Enzyme-Linked Immunosorbent Assay for Barley Yellow Dwarf Virus Using Antiserum Produced to Virus from Field-Infected Plants

B. DOUPNIK, JR., Visiting Professor, R. E. STUCKEY, Associate Extension Professor, G. R. BRYANT, Postdoctoral Research Associate, and T. P. PIRONE, Professor, Department of Plant Pathology, University of Kentucky, Lexington 40546

ABSTRACT

Doupnik, B., Jr., Stuckey, R. E., Bryant, G. R., and Pirone, T. P. 1982. Enzyme-linked immunosorbent assay for barley yellow dwarf virus using antiserum produced to virus from field-infected plants. Plant Disease 66:812-815.

The use of naturally infected oat leaves as a source of barley yellow dwarf virus (BYDV) for purification eliminated the need for rearing vectors and yielded amounts of virus comparable to those reported from greenhouse-grown tissue. An antiserum was produced that gave positive reactions in enzyme-linked immunosorbent (ELISA) tests with homologous virus and with the RPV and PAV isolates but not with the MAV or RMV isolate of BYDV. Samples of small grains suspected of infection with BYDV were tested by ELISA. Freezing and thawing of extracts increased ELISA values for positive samples and decreased values for negative samples. Samples from throughout Kentucky reacted positively with our BYDV antiserum. Negative samples did not react with any of four isolate-specific antisera.

Rapid and accurate diagnosis of barley yellow dwarf virus (BYDV) infection is of particular importance in areas such as Kentucky where small grains are double-cropped with soybeans. Plowing under a BYDV-infected, small-grain crop with low potential yield allows earlier planting of soybeans, improving potential yield of this crop. We have not felt confident in our ability to identify BYDV in small grains other than oats, based on visual symptoms, and assay by means of aphid transmission has not been practical in our facilities. Availability of an antiserum produced to BYDV isolates that occur naturally in Kentucky and the demonstrated suitability of the enzyme-linked immunosorbent assay (ELISA) technique for BYDV diagnosis (6,7,9) should make routine diagnosis possible.

Purification of BYDV for use in serological and other procedures has thus far been done from plants grown and infected under greenhouse or laboratory conditions (5,8). Objectives of our research were to prepare an antiserum that could be used for qualitative and quantitative assay of BYDV in a small-grain breeding program and that could also be used for diagnosis of BYDV in commercial cereal crops. Purification of BYDV from plants that were naturally infected in the field offered the following advantages: a) It is unnecessary to rear or inoculate with the different and specific aphid vectors of the various BYDV isolates, and b) the purified virus isolates would represent those of importance in the geographic area from which they were obtained.

This paper reports the feasibility of using field-infected material for virus purification and antiserum production, development of procedures for handling and preparing samples for large-scale testing by ELISA, comparison of visual and ELISA diagnoses of BYDV, and the testing of our antiserum against suspected BYDV samples from various areas of the state.

Permanent address of first author: University of Nebraska, South Central Station, Clay Center, NE 68933.

Kentucky Agricultural Experiment Station Journal Series Paper 81-11-154 and Nebraska Agricultural Experiment Station Journal Series Paper 6690.

Accepted for publication 5 December 1981.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0191-2917/82/09081204/$03.00/0

MATERIALS AND METHODS

Tissue for virus purification. Various cultivars and breeding lines of oats (*Avena sativa* L.) in the uniform winter oat hardiness nursery at Lexington, planted in September 1979, were used as the virus (BYDV-Ky) source. Naturally infected plants in the prejointing stage, with red-leaf symptoms typical of BYDV infection and free of symptoms of other viruses, were harvested in May 1980 by clipping aboveground parts with scissors. Immediately after harvesting, subsamples from each of the lots to be used for virus purification were sent to W. F. Rochow to verify that they were infected with BYDV. In his ELISA tests, the samples reacted strongly with antiserum to the *Rhopalosiphum padi* specific virus (RPV) isolate of BYDV and less strongly to the *R. padi* and *Sitobion* (= *Macrosiphum*) *avenae* nonspecific virus (PAV) isolate antiserum, suggesting the presence of a mixture of isolates.

Stems and very large leaf veins were removed, and leaves were cut into 1–2 cm pieces and frozen at −20 C until use 6–10 mo later. Just prior to use, the tissue was transferred to a freezer at −80 C for 12–24 hr and then freeze-dried for 48 hr. In a typical experiment, 250 g (fresh weight) of tissue yielded about 60 g of dried material. The freeze-dried tissue was ground in a Wiley mill to pass a 60-mesh screen prior to virus purification. Healthy oat leaves from greenhouse-grown plants were processed in the same manner and used as controls.

Virus purification. The procedure used for purification was basically that described by Paliwal (5). Sixty grams of freeze-dried, ground tissue was soaked overnight in 300–350 ml of 0.1 M potassium phosphate buffer, pH 7.0. The next day, an additional 50–100 ml of buffer was added just prior to homogenization in a Waring Blendor for 1 min. Subsequent steps were according to Paliwal's (5) method until the step just prior to density gradient centrifugation. At this point, the pooled, low-speed supernatants were layered over a 4- to 5-ml cushion of 30% sucrose in buffer in a tube measuring 1 × 3.5 in. and centrifuged for 2 hr at 45,000 rpm in a Spinco 50.2 Ti rotor.

The pellet was covered with 1 ml of buffer and allowed to resuspend overnight with the aid of a small stirring bar. An additional 1 ml of buffer, used to rinse the tube after removal of the resuspended virus, was pooled with the first milliliter and centrifuged at 5,000 *g* for 10 min. The supernatant was then layered over one or two sucrose gradients (100–400 mg/ml of buffer) and centrifuged for 4 hr at 25,000 rpm in an SW-27 rotor. The distinct virus band about 3 cm from the top of the gradient was removed, diluted with 20–25 ml of buffer, and centrifuged for 2 hr at 46,000 rpm in a 50.2 Ti rotor. The final pellet was dissolved in 0.5 ml of buffer. Virus concentration was estimated using an $E^{0.1\%}_{259}$ value of 6.0 (5).

Antiserum preparation. A rabbit was immunized using the following schedule: Day 1, 130 μg BYDV intramuscularly (i.m.); day 23, 50 μg intravenously (i.v.); day 45, 85 μg i.m.; day 59, 45 μg i.v. and 45 μg i.m. Serum collected 55 days after the initial injection had a titer (ring

interface test) of 1:512; sera collected after 68 and 81 days had titers of 1:1,024. The 68-day serum and the methods of Clark and Adams (2) were used for immunoglobulin (Ig) preparation and for ELISA tests. The BYDV-Ky antiserum was not cross-absorbed with healthy oat extracts. ELISA absorbances at 405 nm were measured directly from the plate with a Titertek Multiskan photometer (Flow Laboratories, McLean, VA 21102).

Sample sources and handling. Thirty-two samples (25 wheat, 5 barley, 2 rye) were received in March and April 1981 in response to a request sent to extension agents in each county for samples of suspected BYDV. Upon arrival, most samples were evaluated for condition (good, fair, or poor), and all were rated as very likely, likely, possibly, unlikely, or very unlikely to be infected with BYDV on the basis of symptoms. Samples were refrigerated at 5 C and within 2 days were chopped into 1- to 2-cm pieces, mixed well, and frozen at −20 C until processed for ELISA.

Extraction procedures for ELISA. Four methods of BYDV extraction were compared. The first was by grinding tissue with buffer (1:1, w/v) in a mortar, with liquid nitrogen, followed by clarification by treatment with chloroform and centrifugation as described by Rochow (7). The second method was identical to the first but without clarification or centrifugation. The third utilized freeze-dried tissue ground to pass a 60-mesh screen as described above, soaked in buffer (1:1, w/v; fresh weight basis) for 3 hr, and expressed through cheesecloth. In the fourth procedure, tissue was homogenized in buffer (1:3, w/v) in a Brinkmann PT 45 homogenizer (Polytron) with a PT 20 probe generator (Brinkmann Instruments, Westbury, NY 11590). Homogenization was for 10 sec, and the liquid was expressed through cheesecloth 0.5 hr after homogenization. The starting material in these experiments was 3 g of naturally infected oat leaves from one of the lots used for virus purification (BYDV-Ky) and frozen for 10–11 mo or from healthy greenhouse-grown frozen oat leaves (control). The extraction buffer was 0.02 M potassium phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 and 2% polyvinylpyrrolidone. Each method was evaluated on two separate occasions, and each homogenate was divided into duplicate samples for ELISA.

RESULTS

Virus purification and serology. In seven separate purifications, yields ranged from 25 to 60 μg ($\bar{x}$ = 40 μg) of virus per 100 g of fresh weight. $A_{260/280}$ values ranged from 1.67–1.87. Typical BYDV particles were observed in the electron microscope. Exhaustive examination revealed the occasional presence of rod-shaped particles morphologically similar to but shorter (<100 nm) than those of soilborne wheat mosaic virus (1). These were present at not more than 0.02% of the concentration of the BYDV particles in any preparation and were not present in all preparations.

Losses of virus at various steps in the purification process were monitored by ELISA, using an antiserum to RPV provided by W. F. Rochow (9). Some loss occurred at most steps in the procedure, particularly during resuspension of pellets. Careful and twice repeated extraction of pellets reduced these losses; however, because this increased the time required for purification and because yields of virus were judged adequate without repeated extraction, this was not done routinely.

Of the other modifications tested, the addition of the sucrose cushion step was judged most useful, as the resulting material was visibly cleaner. Concentration of virus by precipitation in the presence of 6 or 8% polyethylene glycol (PEG; mol wt of 6,000) and 4% potassium chloride, in place of high-speed centrifugation, was tested in a number of experiments. This procedure reduced the overall time required and allowed processing of larger initial volumes than was practical using ultracentrifugation. Final virus yield from the PEG procedure, however, was never more than half that of controls prepared using the standard procedure, and in some cases no virus was recovered. Similar results with PEG have been reported by others (8).

Ig from the antiserum prepared to BYDV-Ky was tested against tissue infected with the homologous virus and with tissue infected with the RPV, PAV, *Macrosiphum avenae* specific virus (MAV), and *R. maidis* specific virus (RMV) isolates of BYDV provided by W. F. Rochow. Ig from Rochow's antiserum to RPV (cross-absorbed with healthy oat leaf extract) was used for comparison. As expected from the ELISA results with the source plants, positive readings with the BYDV-Ky Ig were obtained with the BYDV-Ky, RPV, and, to a lesser extent, the PAV isolate, but not with the MAV or RMV isolates (Table 1). RPV Ig reacted only with the RPV and BYDV-Ky isolates.

Extraction procedures for ELISA. The liquid nitrogen-chloroform extraction method produced the highest ELISA readings for BYDV-infected tissue (1.391; >2.000) in both experiments. The three other methods produced similar but somewhat lower readings, averaging in the range of 0.630 to 0.750. When adjusted for differences in dilution factors (1:3 [w/v] for Polytron; 1:1 for others), extraction by the Polytron procedure was similar to that of the liquid nitrogen-chloroform procedure and was more efficient than the other methods. Because Polytron extraction was simpler, faster, and less messy than the others, it was used in subsequent experiments.

Effect of freezing and thawing. When Polytron-extracted BYDV-Ky sap was tested immediately (day 0) and after being frozen for 4 and 28 days at −20 C, progressively higher ELISA values were obtained (0.622, 1.378, and >2.000, respectively). BYDV-Ky extracts freshly prepared on the same day that the extracts frozen for 4 and 28 days were tested had values similar (0.584, 0.578) to that of material freshly prepared on day 0. Healthy oat extracts, on the other hand, gave readings of 0.055, 0.043, and 0.024 for 0, 4, and 28 days frozen, respectively. Healthy oat extracts freshly prepared on the same day that the extracts frozen for 4 and 28 days were tested had values of 0.039 and 0.058,

Table 1. Reaction of two barley yellow dwarf virus (BYDV) antisera in enzyme-linked immunosorbent assay tests against various BYDV isolates

	Absorbance at 405 nm[b]			
	BYDV-RPV[c]	BYDV-Ky		
Tissue[a]	1/400, 1/400[d]	1/200, 1/500	1/400, 1/500	1/800, 1/500
Healthy	0.024	0.027	0.021	0.050
BYDV-RMV	0.010	0.000	0.015	0.033
BYDV-MAV	0.006	0.022	0.012	0.006
BYDV-PAV	0.006	0.170	0.121	0.111
BYDV-RPV	>2.000	1.344	1.016	1.041
BYDV-Ky	0.369	0.395	0.302	0.277
Purified virus[e]	>2.000	1.225	0.881	0.877

[a] Tissues infected with specific BYDV isolates were provided by W. F. Rochow; RMV = *Rhopalosiphum maidis* specific virus isolate, MAV = *Macrosiphum avenae* specific virus isolate, PAV = *R. padi* and *M. avenae* nonspecific virus isolate, RPV = *R. padi* specific virus isolate, and BYDV-Ky = subsample of material used for virus purification. All tissues were freeze-dried and ground to pass a 60-mesh screen. Samples of 0.2 g were soaked in 3 ml of buffer overnight and filtered through cheesecloth; 200 μl was placed in each well.

[b] Means of duplicate wells.

[c] BYDV-RPV antiserum was absorbed with healthy oat extract prior to immunoglobulin (Ig) preparation; BYDV-Ky was not.

[d] Ig coating dilution, Ig conjugate dilution. Initial concentration of Ig for coating and conjugate preparation was 1 mg/ml.

[e] Purified BYDV-Ky; 1 μg/well.

respectively. Thus, freezing seemed to increase ELISA values of BYDV containing sap and to lower values of controls.

The effect of freezing and thawing was subsequently tested on a large scale with the small-grain samples submitted for diagnosis. Following extraction, ELISA tests were run prior to and after freezing for 21 days at −20 C. Results showed that freezing and thawing tended to increase positive values and to decrease negative ones (Table 2). The usefulness of this procedure can be seen in the case of the one sample that was first considered questionable (initial reading only slightly more than twice the control) but that after freezing and thawing was clearly negative.

BYDV distribution. The geographic distribution of samples that reacted positively and negatively in ELISA tests is shown in Figure 1. The antiserum prepared to the isolates present in Fayette County (BYDV-Ky) reacted with BYDV from throughout the state.

Because our antiserum did not react in ELISA with the MAV and RMV isolates of BYDV, negative readings would be expected with samples infected with these isolates. For this reason, seven samples that reacted negatively with our antiserum, but that were judged most likely (of the 17 negative samples) to be infected with BYDV based on symptomology, were sent to W. F. Rochow for testing against MAV and RMV, as well as RPV and PAV, antisera (9). Three samples that gave positive ELISA readings with our antiserum (one strongly positive, two intermediate) were sent as controls. None of the samples that gave negative reactions in our tests reacted with any of the isolate-specific antisera. Of the three positive samples from our tests, two reacted with Rochow's RPV antiserum and one with PAV antiserum.

Symptomologic vs. ELISA diagnosis. Of the four samples rated "very likely" to be infected with BYDV on the basis of visual observation, three were positive in the ELISA tests. Three of 10 rated "likely," 3 of 9 "possibly," 4 of 8 "not likely," and the sole "very unlikely" samples were positive for BYDV by ELISA. Eight of the samples that arrived in "good," three in "fair," and three in "poor" condition gave positive ELISA readings. Of the samples that did not react, three were in "good," six in "fair," and five in "poor" condition on arrival. There was no correlation between the condition of the plants and our inability to diagnose BYDV visually.

DISCUSSION

The use of naturally infected plants as the source of BYDV resulted in virus yields comparable to those reported for greenhouse-grown plants (5,8). The purified virus reacted with RPV and PAV antisera, indicating the presence of these isolates; the possibility that the preparations contained other, morphologically indistinguishable virions cannot be excluded, however. The BYDV-Ky antiserum reacted with BYDV-infected plants collected from various areas of Kentucky, indicating that the isolates used for immunization occur throughout the state.

Use of the Polytron for homogenization and the fact that BYDV antigen remains reactive in ELISA after at least two cycles of freezing and thawing make diagnostic surveys feasible on a fairly large scale. We plan to offer this service in our Plant Diagnostic Laboratory on a routine basis with ELISA tests to be run at periodic, stated intervals. The tendency of positive samples to become more positive and for negative samples to become more negative in ELISA tests following freezing and thawing is of evident value and might be applicable for viruses other than BYDV.

Our lackluster record in diagnosing BYDV on the basis of symptoms confirms the need for diagnosis by more reliable means, at least in Kentucky. Because samples that did not react in ELISA tests with our antiserum to BYDV-Ky also did not react in tests with MAV and RMV antiserum, it would appear that—of the four isolates (RPV, PAV, MAV, RMV) to which antisera are currently available—the RPV and PAV isolates were most common here, at least in 1981. The possibility that some of the visually positive samples were infected with other BYDV isolates, such as *Schizaphis graminum* specific virus isolate, or with luteoviruses other than BYDV cannot be excluded, however; hence, antisera that could detect as many luteoviruses as possible would unquestionably be more desirable. This might be accomplished by exchange of our antiserum for those reactive with other luteoviruses or by use of equally sensitive, but less strain-specific, methods such as indirect ELISA (3) or radioimmunoassay (4).

Table 2. Enzyme-linked immunosorbent assay (ELISA) values of extracts of barley yellow dwarf virus survey samples before and after freezing

Initial diagnosis[a]	No. of samples	Range of readings[b]		No. of readings that	
		Before freezing	After freezing	Increased >10%	Decreased >10%
Negative	17	0.017–0.055	0.000–0.037	2	13
Positive	14	0.132–1.569	0.260–1.964	9	2
Questionable	1	0.081	0.028	...	...
	Control	0.039	0.054[c]	...	...

[a] Sample considered positive if ELISA reading was more than two times that of the control, negative if not.
[b] Absorbance at 405 nm. Values are ranges of means calculated for duplicate wells.
[c] Freshly prepared, unfrozen control.

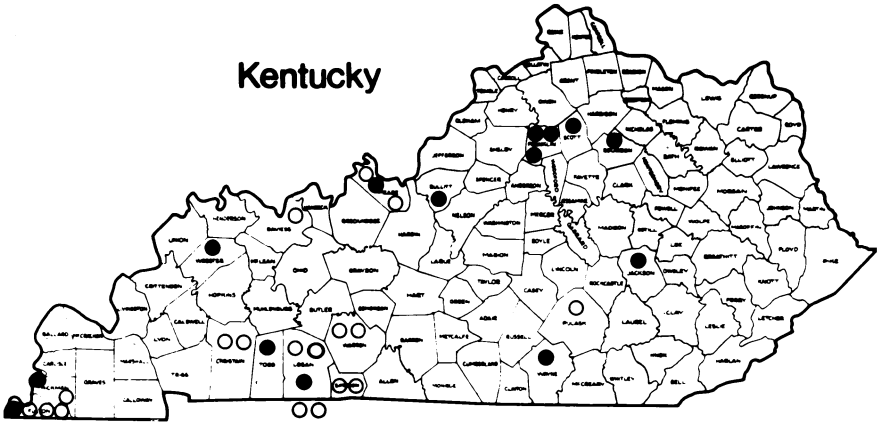

Fig. 1. Distribution of small-grain samples that gave positive (closed circles) or negative (open circles) readings in enzyme-linked immunosorbent assay tests for barley yellow dwarf virus.

ACKNOWLEDGMENTS

We thank W. F. Rochow for his invaluable assistance in diagnosing the BYDV isolates and for providing RPV antiserum and tissue infected with the specific BYDV isolates, and we thank R. L. Mernaugh for electron microscopy.

LITERATURE CITED

1. Brakke, M. 1971. Soil-borne wheat mosaic virus. Descriptions of Plant Viruses. No. 77. Assoc. Appl. Biol./Commonw. Mycol. Inst. Kew, Surrey, England. 4 pp.
2. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
3. Crook, N. E., and Payne, C. C. 1980. Comparison of three methods of ELISA for baculoviruses. J. Gen. Virol. 46:29-37.
4. Ghabrial, S. A., and Shepherd, R. J. 1980. A sensitive radio-immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 48:311-317.
5. Paliwal, Y. C. 1978. Purification and some properties of barley yellow dwarf virus. Phytopathol. Z. 92:240-246.
6. Rochow, W. F. 1979. Comparative diagnosis of barley yellow dwarf by serological and aphid

transmission tests. Plant Dis. Rep. 63:426-430.
7. Rochow, W. F. 1982. Identification of barley yellow dwarf viruses: Comparison of biological and serological methods. Plant Dis. 66:381-384.
8. Rochow, W. F., Aapola, A. I. E., Brakke, M. K., and Carmichael, L. E. 1971. Purification and antigenicity of three isolates of barley yellow dwarf virus. Virology 46:117-126.
9. Rochow, W. F., and Carmichael, L. E. 1979. Specificity among barley yellow dwarf viruses in enzyme immunosorbent assays. Virology 95:415-420.