Serological Assays for Oat Blue Dwarf Virus

E. E. Banttari

Professor, Department of Plant Pathology, University of Minnesota, St. Paul 55108.
Scientific Journal Series Paper 10,799, Minnesota Agricultural Experiment Station, St. Paul.
Accepted for publication 18 March 1981.

ABSTRACT

Banttari, E. E. 1981. Serological assays for oat blue dwarf virus. Phytopathology 71:1242-1244.

Agar double diffusion (DD), latex flocculation (LF), and enzyme-linked immunosorbent assay (EIA) were effective for detecting oat blue dwarf virus (OBDV) in barley, oat, and flax plants. In DD plates, visible precipitin bands were obtained by using a constant concentration of clarified sap extract from oat plants as the antigen and an optimum antiserum dilution between 1:8 and 1:32. At a constant 1:16 dilution of antiserum, antigen dilution end points of partially clarified concentrated sap extracts of OBDV-infected barley, oat, and flax plants were 1:4, 1:8, and 1:16, respectively. In LF assays, antigen dilution end points of nonconcentrated sap extracts of barley, oats, and flax were 1:16, 1:16, and 1:32, respectively, determined by using latex-sensitized OBDV γ-globulin diluted 1:800 to 1:1,400. Antigen dilution end points of nonconcentrated plant sap from OBDV-infected barley, oats, and flax were 1:28, 1:128, and 1:512, respectively, when assayed by EIA. All serological assays indicated that the OBDV titer in flax was at least twofold higher than in oat or barley plants.

Oat blue dwarf virus (OBDV) is a 28–30 nm RNA-containing isometric virus transmitted by the aster leafhopper, *Macrosteles fascifrons* Stål, and has a broad monocotyledonous and dicotyledonous host range (2). Detection of infected plants has been either by visual examination in those species that develop symptoms or by transmission of the virus via leafhoppers to assay plants. Leafhopper transmission assays are laborious as well as time consuming. Approximately 2 wk of incubation of the virus in the vector and 2–3 wk for symptoms to develop in test plants are necessary. Moreover, stocks of vectors must be continuously maintained and transmission may be adversely affected by various factors including elevated summer temperatures in the greenhouse. Latex flocculation (LF) and enzyme-linked immunosorbent assay (EIA, ELISA) serological methods have been used successfully for assays of several plant viruses including the leafhopper-transmitted sugar beet curly top virus (1,4,5,7). Purification of OBDV enabled production of a specific antiserum and development of serological assays for the virus.

MATERIALS AND METHODS

Preparation of the antiserum. The virus was purified from infected oat plants by using column chromatography and density gradient centrifugation (3). Rabbits were immunized over a 5-wk period by a combination of weekly intramuscular (IM) and intravenous (IV) injections of purified virus (1 ml, $OD_{260}=5.9$). For IM immunizations, the virus was thoroughly mixed with an equal volume of Freund's incomplete adjuvant. The rabbits were bled 1 wk after the fifth weekly immunization and biweekly thereafter following booster injections. The serum fractions were separated and stored at −10 C. The γ-globulin fraction was precipitated from whole antiserum by using saturated ammonium sulfate. After centrifugation (2,800 *g*, 30 min), the precipitate was resuspended in 0.1 M Tris-HCl buffer pH 7.4.

Whole antiserum or the γ-globulin fraction diluted with 0.1 M Tris-HCl buffer pH 7.4, was used in agar double-diffusion (DD) tests.

Plates for DD assays contained 0.5% agarose and 0.85% sodium chloride in distilled water and sodium azide (0.02%) as a preservative. Clarified and partially concentrated plant sap extracts were used for DD assays. Equal weights of plant tissues were ground in 25 ml of 0.02 M phosphate buffer pH 7.2. The ground pulp was squeezed through cheesecloth and the expressed sap was centrifuged (6,000 *g*, 15 min). The supernatant fluid was centrifuged (79,000 *g*, 2.5 hr) and the pellets were resuspended and further dilutions were made by adding 0.02 M phosphate buffer, pH 7.2.

To produce latex-sensitized antiserum, latex beads (0.794 μm, LB-8, Sigma Chemical Company, St. Louis, MO 63178) were combined with the γ-globulin fraction of OBDV antiserum according to techniques described by Bercks et al (4), with some modifications described in Results. The latex-sensitized γ-globulin was preserved with 0.02% sodium azide, filtered through Whatman No. 2 filter paper, and stored at 4–5 C until used. These antisera were effective for more than 9 mo.

For routine LF tests for virus in plants, two or three drops of plant sap (approximately 40–60 μl) from a Pasteur pipet were mixed with 0.8 ml of 0.1 M Tris-HCl buffered saline, pH 7.4, and one drop of the diluted sap was added to one drop of latex-sensitized antiserum in each well of a Micro Test II culture plate (Falcon. Div., Becton, Dickinson and Co., Oxnard, CA 93030. The plates were covered and shaken for 60 min on a TekTator V shaker (American Hospital Supply Corp., Evanston, IL 60201) at 200 oscillations per minute and results were observed using a dissecting microscope at ×30.

The double antibody sandwich method with alkaline phosphatase enzyme and *p*-nitrophenyl phosphate substrate as described by Clark and Adams (6) was used for EIA. Coating γ-globulin dilutions of 0.1, 1.0 and 10.0 μg/ml of OBDV-antiserum and enzyme conjugate dilutions of 1:200, 1:400, 1:800, and 1:3,200 were tested.

Coating γ-globulin was incubated in cuvettes for 3–5 hr; plant extracts were incubated overnight at 3–5 C and enzyme-globulin conjugates were incubated at room temperature (approximately 22 C) for 4–6 hr. Substrate (*p*-nitrophenyl phosphate, Sigma 104) at 1 μg/ml in 2,2′-iminodiethanol-HCl, pH 9.8, was added to the cuvettes, incubated at approximately 22 C and absorbance measurements made immediately and at 30 and 60 min. A Gilford PR-50 EIA Processor Reader (Gilford Instrument Laboratories, Oberlin, OH 44074) was used to wash cuvettes between each step and to obtain quantitative measurements (A_{405nm}) of hydrolyzed enzyme substrate. Readings were considered positive when they exceeded the mean absorbancies plus two standard deviations of the mean of extracts from healthy plants.

RESULTS

Agar double diffusion assays. Precipitin bands were formed using whole antiserum dilutions as high as 1:128 and partially clarified and concentrated sap extracts from OBDV-infected oat plants. However, the most distinct bands occurred when antiserum

0031-949X/81/12124203/$03.00/0

dilutions between 1:8 to 1:32 were used (Fig. 1). No precipitin bands occurred between sap of healthy oats and the virus antiserum nor between sap of infected plants and serum from nonimmunized rabbits (normal serum).

The relation of age of plants after inoculation to virus titer and reaction in DD assays was tested. Oat plants were harvested 13 days, 42 days, and 4 mo after being exposed to viruliferous leafhoppers. The 4-mo-old plants had been cut back once and younger tillers were used. One, 2, and 4 g of plant leaves and stems of each age class were processed for double-diffusion assay. Precipitin bands developed for extracts of each age group and quantity of plant tissue except for 1 g of plants with 13-day-old infections.

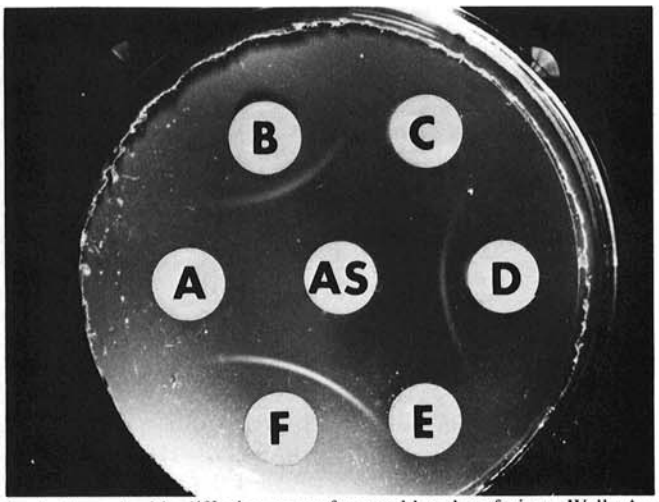

Fig. 1. Agar double-diffusion assay for oat blue dwarf virus. Wells A = healthy barley, B = infected barley, C = healthy oats, D = infected oats, E = healthy flax, F = infected flax, and AS = 1:16 dilution of antiserum.

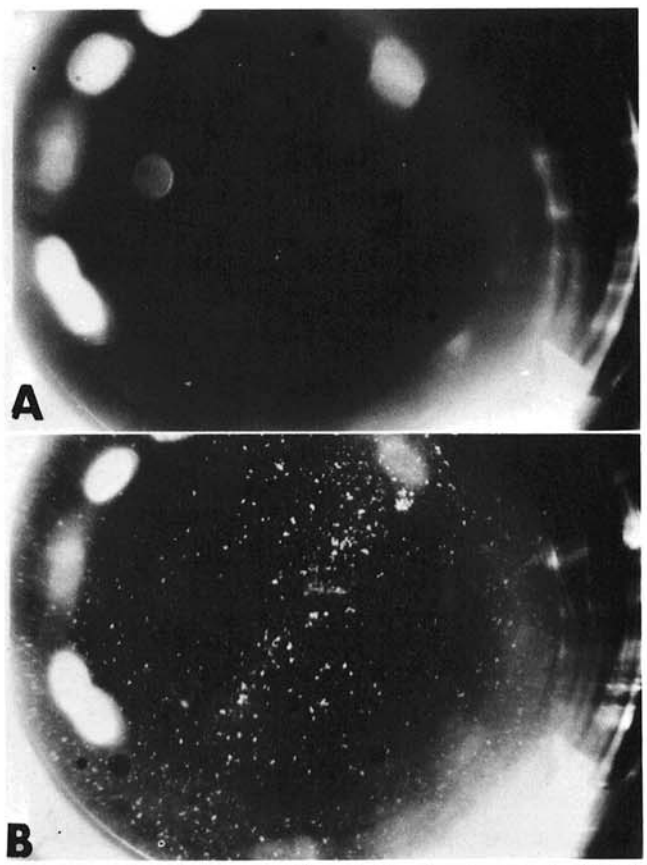

Fig. 2. Latex flocculation assay for oat blue dwarf virus: **A,** Negative reaction; and **B,** Positive flocculation for infected oats in well of Microtiter II plate (×30).

Leaves and stems of oats were compared as sources of virus in double diffusion assays. Twofold dilutions of concentrated extracts were made in 0.02 M phosphate buffer, pH 7.2, and placed in wells of double diffusion plates. A constant 1:16 dilution of antiserum was used. No consistent differences were detected between stems or leaves as sources of the virus.

Latex flocculation serology. More distinct flocculation occurred when antiserum was sensitized with latex at ratios of 5.0–7.5 μl of latex beads per milliliter of antiserum than when 3.25 μl/ml was used (Fig. 2). Maximum sensitivity occurred at dilutions of γ-globulin between 1:800–1:1,400. Approximately 60 μl of plant sap was mixed into 0.8 ml of 0.1 M Tris-HCl buffer, pH 7.4, then one drop of this dilution delivered from a Pasteur pipet was mixed with one drop of latex conjugate in a well of a Micro Test II culture plate. Flocculation occurred in samples containing virus shaken for 30 min at 180–200 oscillations per minute at 24–26 C; however, flocculation was more intense if shaking was extended to 1 hr.

No flocculation was observed in any of the following controls used in these experiments: healthy plant sap mixed with latex sensitized OBDV γ-globulin; healthy or OBDV-infected plant sap mixed with latex-sensitized normal serum γ-globulin; and healthy or OBDV-infected sap mixed with latex in 0.1 M Tris-HCl buffer, pH 7.4.

Enzyme-linked immunosorbent assay. When infected oats were the source of OBDV, maximum abosrbance ($A_{405\ nm}$) was obtained by using the coating γ-globulin at 10 μg/ml sap (diluted 1:1 in PBS-Tween + 0.01 M sodium diethyldithiocarbamate [NaDIECA]) and enzyme γ-globulin dilution of 1:400. However, positive absorbance values could be observed with samples of infected oats diluted 1:128 in healthy oat sap by using a coating γ-globulin dilution of 1.0 μg/ml and globulin-enzyme conjugate dilutions as high as 1:800.

Virus concentration in barley, oats, and flax assayed by agar double diffusion, latex flocculation, and enzyme-linked immunosorbent assays. Oat blue dwarf virus titers in barley, oats, and flax were assayed by LF, DD, and EIA. Plants of each species were inoculated and approximately 8 wk later healthy and inoculated plants were harvested. For DD assay the plants were ground in a blender and concentrated as described above. Twofold dilutions of these sap concentrates up to 1:4,096 in 0.02 M phosphate buffer, pH 7.2, and a constant 1:16 dilution of antiserum were used. For LF and EIA assays, twofold dilutions to 1:4,096 of nonconcentrated infected sap of each species were made by using healthy sap of each species as the diluent. For LF 1:800 and 1:1,200 dilutions of γ-globulin coated latex were used and two assays were made for each sap dilution. For EIA, wells were coated with γ-globulin (10.0 μg/ml) each antigen was placed in triplicate randomized wells containing 0.1 ml of PBS-T + 0.01 M NaDIECA, and a 1:400 dilution of enzyme conjugate was used.

Visible precipitin bands were produced at maximum dilutions of 1:4, 1:8, and 1:16 of barley, oat, and flax extracts, respectively, in DD assays (Table 1). In LF assays, distinct flocculation was obtained at maximum dilutions of 1:16, 1:16, and 1:32 of barley, oat, and flax extracts, respectively. In EIA, positive absorbance values (A_{405nm}) were obtained using maximum dilutions of 1:128, 1:128, and 1:512 of barley, oat, and flax extracts, respectively.

Comparison of LF and leafhopper transmission assays for detection of OBDV in barley, flax, and oat plants. To compare LF and leafhopper transmission assays for detection of OBDV in barley, oat, and flax cultivars Manker, Lodi, and Marine 62, respectively, were inoculated in the greenhouse by caging viruliferous leafhoppers on the seedlings for 1 wk, after which the leafhoppers were killed and the plants grown in the greenhouse until used for these assays. The ages of the plants varied from 3 to 8 wk and intensity of symptoms of virus infection varied from distinct to none. Checks consisted of healthy plants of each cultivar. Approximately 10 virus-free aster leafhoppers were placed in each plastic cylindrical cage on each of 180 inoculated and healthy plants in two experiments. Leafhoppers were given acquisition access on the test plants for 1 wk, then transferred to

TABLE 1. Comparison of latex flocculation, agar double-diffusion, and enzyme-linked immunosorbent assays for detection of oat blue dwarf virus in three hosts

Assay[a]	Host	Flocculation, precipitin bands, or positive absorbance values in assays at reciprocal dilutions of:[b]											
		2	4	8	16	32	64	128	256	512	1,024	2,048	4,096
DD	Barley	+	+	−	−	−	−	−	−	−	−	−	−
	Oats	+	+	+	−	−	−	−	−	−	−	−	−
	Flax	+	+	+	+	−	−	−	−	−	−	−	−
LF	Barley	+	+	+	+	−	−	−	−	−	−	−	−
	Oats	+	+	+	+	−	−	−	−	−	−	−	−
	Flax	+	+	+	+	+	−	−	−	−	−	−	−
EIA	Barley	+	+	+	+	+	+	+	−	−	−	−	−
	Oats	+	+	+	+	+	+	+	−	−	−	−	−
	Flax	+	+	+	+	+	+	+	+	+	−	−	−

[a] DD = agar double diffusion, LF = latex flocculation, and EIA = enzyme-linked immunosorbent assay.

[b] Maximum reciprocal dilutions at which precipitin bands occurred in agar double diffusion assays, flocculation occurred in latex flocculation assays, or absorbance (A_{405nm}) exceeded the mean absorbance plus two standard deviations of the mean or extracts from healthy plants. Three experiments were done.

healthy oat seedlings for 12 days, and to a second set of oat seedlings for another 12 days. The leafhoppers were killed and the seedling plants were placed in the greenhouse and scored for symptoms after 6 wk.

The same test and control plants on which the leafhoppers were given acquisition feeding were harvested, sap was extracted, and the LF assay was made as described for the experiments above. A 1:1,200 dilution of latex-sensitized antiserum was used, the plates were rotated for 1 hr and results were recorded. In two experiments, in which a total of 180 assays of barley and oat plants were made, the results of 162 LF assays agreed with results obtained by transmission assays (LT). In 18 assays that did not agree, 10 resulted in negative LF tests and positive transmission tests; the other eight resulted in positive LF and negative transmission assays.

DISCUSSION

Latex flocculation tests and EIA were convenient, quick, and sensitive methods for assaying OBDV. The reasons for disagreement between LF and LT assays were not apparent; however, transmission from plants which were negative in LF tests may be because leafhoppers acquire OBDV from some plants that do not contain sufficient virus titers to give positive LF assays. Although EIA was the most sensitive serological assay, LF assay is probably sufficiently accurate for field surveys for OBDV. Latex flocculation assays were most convenient and results were available within 1.5 hr, whereas the results for LT were obtained only after 40 days. For certain assays, the increased sensitivity of EIA over LF would justify its use.

Assays by DD were less sensitive than LF or EIA, and, in addition, clarification, and partial concentration of sap extracts were necessary. However, DD assays may be useful in making serological comparisons between OBDV and other similar viruses. All three serological assays revealed that virus titer was at least 2–3 times higher in flax than in barley and oats. This suggests that flax may be preferable to the grasses as a propagation species for virus purification.

Several factors affected sensitivity of the LF assay for OBDV. Flocculation was more distinct if 5.0–7.5 μl of latex beads per milliliter of γ-globulin were used to sensitize the γ-globulin than smaller amounts of latex beads. The concentration of γ-globulin was important; most flocculation occurred with this particular antiserum at γ-globulin dilutions between 1:800 and 1:1,400. Therefore, it will be necessary to titrate each batch of antiserum to determine the optimum γ-globulin concentration. Finally, shaking for 1 hr resulted in more flocculation than shaking for 30 min.

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