Sensitive Serologic Detection of Barley Stripe Mosaic Virus in Barley Seed

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ABSTRACT

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Enzyme-linked immunosorbent assay readily detected barley stripe mosaic virus in extracts from individual barley seeds, endosperms, and embryos. Test sensitivity depended on the concentrations of immunoglobulins used, but these tests and those of bulk seed extracts indicated that it was possible to detect very low proportions of infected seed in seed samples and to discriminate between samples with different proportions of infected seed. Although serologically specific electron microscopy was also a sensitive test procedure, it required expensive equipment and expert personnel.

Barley stripe mosaic virus (BSMV) is controlled in Montana by the planting of virusfree seed that has been certified by seed testing (5). At present, all certification is based on "SDS disk" tests of embryos (4), from a method originated by Hamilton (8) that uses sodium dodecyl sulfate (SDS) detergent to degrade virus particles and permit diffusion of viral antigen through agar.

In the SDS disk test, embryo extracts are absorbed on filter paper disks placed in a circular arrangement on buffered agar containing SDS. These disks surround a central disk containing antibody. Diffusion of viral antigen is detected by antisera elicited to SDS-treated preparations of BSMV. The test can handle large numbers of individual embryos, and the percentage of infected embryos is an adequate estimate of the percentage of infected plants a seed lot is likely to produce (8).

Unfortunately, however, both antibody titers and the concentration of appropriate antigens in embryo extracts are low. Nonspecific precipitates from other seed constituents also reduce the visualization of immunoprecipitates. The technique is therefore relatively insensitive and suitable only for tests of individual embryo extracts at low dilutions.

We report here on investigations of the potential of more sensitive serologic assays in testing for BSMV. Most work was done with the enzyme-linked immunosorbent assay (ELISA) (6), but some experiments were also conducted with serologically specific electron

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microscopy (SSEM) (1,7). Both methods showed promise for use in barley seed certification. The tests were conducted at Purdue University and at Montana State College at Bozeman. Preliminary work with ELISA was reported earlier (12).

MATERIALS AND METHODS

Seed and seed preparation. Seed infected with BSMV was produced at Bozeman, MT, from barley (*Hordeum vulgare* L.) plants grown in the 1976–1978 seasons. Seed lots comprised about 1 kg of well-mixed seeds from plants singly infected with one of four strains of BSMV. We assumed that infected seeds were scattered randomly throughout each lot. The combinations of virus strain and barley cultivar tested were MI-1 (3) in Atlas, MI-2 (isolate originally from Compana barley) in Atlas, MI-3 (4) in Vantage, and type strain (2) in Atlas. Virusfree seeds of Atlas and Vantage barleys were collected from healthy barley plants also grown at Bozeman.

We prepared seeds for testing by separating embryos and endosperms or by milling. In the former procedure, seeds were soaked in water overnight, dehulled by hand, and the embryos carefully and cleanly eased away from the endosperms with a small instrument or thumbnail. Milled seed was prepared by milling dry seed complete with hulls, as finely as possible, in a Buhler-Miag mill (Type MLI-204, Buhler-Miag Inc., Minneapolis, MN) at Purdue or in a Cyclone sample mill (Udy-Analyzer Co., Boulder, CO) at Montana. To avoid cross-contamination, we carefully cleaned the mills and passed batches of healthy barley seeds through them between samples.

ELISA tests. All ELISA tests were conducted at Purdue by slightly modified standard procedures (6). Seeds or seed parts for testing were hand ground with a pestle and mortar in 1:2 to 20 (w/v) ratios of 0.2 M sodium borate, pH 9.0 (9), and then ground further after adding an equal volume of phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 2% polyvinylpyrrolidone (PVP) (mol wt 40,000) (PBS/PVP/Tween). Each test sample was duplicated at least once in paired wells in microELISA plates (1-223-29, Dynatech Laboratories Inc., Alexandria, VA), and test reproducibility was excellent.

Extracts from a Purdue stock of healthy Moore barley seed were used as controls. In preliminary tests, these extracts gave the same ELISA values as those from the healthy Atlas and Vantage seeds. Because they originated at Bozeman, the latter seeds may have had occasional viral contamination in endosperms even when SDS disk tests of embryos were negative.

Immunoglobulin was purified from antiserum to the type strain of BSMV (precipitin titer 1:2,048) by ammonium sulfate precipitation and chromatography on diethylaminoethyl cellulose. Coating immunoglobulin was stored at 1 mg/ml with 0.02% azide in PBS; conjugate immunoglobulin at 1 mg/ml in PBS was labelled, after thorough dialysis against PBS, with twice its weight of pelleted alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO), by incubation with 0.06% glutaraldehyde for 4 hr at 25 C. After further thorough dialysis against PBS, the conjugate was stored with an equal volume of bovine serum albumin at 10 mg/ml containing 0.04% sodium azide. Both coat and conjugate were used in tests at 2.5–10 μg/ml. Coat was diluted in carbonate buffer, pH 9.6; however, to reduce nonspecific background reactions we cross-absorbed the conjugate by dilution with healthy barley leaf extracts (11). These were made by grinding Moore barley leaf (1:2, w/v) in a 1:1 mixture of borate buffer and PBS/PVP/Tween containing 0.2% ovalbumin. Similarly, we interposed a step involving the addition of healthy leaf extracts between the coating and antigen addition steps (see below). These precautions would probably be unnecessary with more highly specific antibody.

The substrate used was *p*-nitrophenyl phosphate at 1 mg/ml in 10% diethanolamine at pH 9.8. For assays, 250-μl samples of solutions per well were used according to the following procedure: coating for 2 hr at 37 C, healthy extract added for 2 hr at 37 C, antigen added overnight at 5 C, conjugate added for 4–6 hr at 37 C, and substrate added and allowed to react for 30 min before adding 50 μl of 3 *N* sodium hydroxide to stop hydrolysis. Between each step, wells were rinsed with PBS/Tween. Absorbances (A_{405nm}) of the reacted substrate samples were measured after five fold dilution in water, and means (ELISA values) were recorded for each test sample.

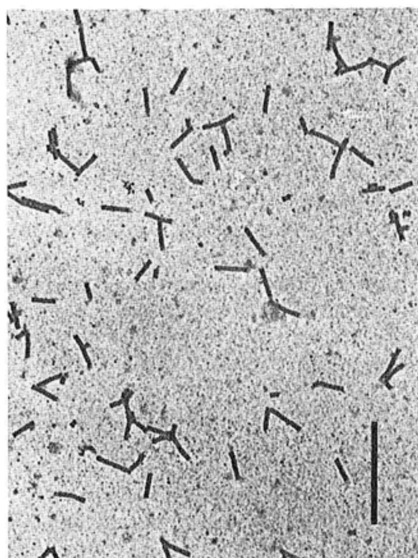


Fig. 1. Particles of barley stripe mosaic virus captured on a grid coated with antiserum in a typical serologically specific electron microscopy test. Magnification ×27,000. Bar = 0.5 μm.

SDS disk and SSEM tests. All SDS disk and SSEM tests were conducted at Bozeman. For SDS disk tests, filter paper disks (6 mm diam) containing embryo extracts were arranged in five-place circular patterns on Noble agar containing SDS (4) 4 mm away from a central disk containing antiserum raised in rabbits by injection of SDS-treated preparations of partially purified BSMV (MI-3 strain). The antiserum titer was 1:4 in SDS disk tests with 200 μg/ml of partially purified BSMV as antigen, regardless of whether the MI-1, MI-2, MI-3, or type strain was used.

Similarly, when used undiluted in SDS disk tests the antiserum routinely detected all four strains of BSMV at concentrations as low as 25 μg/ml. However, with infected embryo extracts, clear immunospecific precipitin lines were observed only to antigen dilutions of 1:2 or 1:4. Visibility was sometimes inhibited by nonspecific cloudiness.

For SSEM, previous procedures (1,7) were modified. Parlodion-filmed electron microscopy grids (300-mesh copper, 74 μm) were coated with antibody by being floated for 30 min on drops of the antiserum diluted to 1:1,000 with 0.05 M tris-HCl buffer at pH 7.2. They were then rinsed on three successive drops of buffer followed by three successive drops of distilled water. Test extracts were prepared by hand-grinding 2-g samples of millings from 50-seed bulk samples of barley seed lots with a pestle and mortar with 20 ml of tris-HCl buffer (1:10, w/v). The extracts were allowed to settle 10–15 min before a drop of the supernatant fluid was transferred to a plastic petri dish for testing. Antibody-coated grids were then floated on the drops of extract for 45 min, rinsed in tris-HCl, and then in water as before.

The grids were stained by being floated on 5% uranyl acetate in 50% ethanol for 1–1½ min, then rinsed in 95% ethanol, blotted dry with filter paper, and stored until examined in a Zeiss EM9S-2 electron microscope. Three fields of each grid were photographed at ×5,000 or 9,000, and the resulting negatives were enlarged to a final total magnification of ×27,000 on a 127 × 178 mm (5 × 7 in.) positive. The number of particles on each photograph (Fig. 1) was hand tallied.

RESULTS

Characteristics of immunoglobulins. Some characteristics in ELISA tests of the immunoglobulins from BSMV-type strain antiserum as determined in various preliminary experiments are summarized in Figure 2. Immunoglobulins from an antiserum to BSMV-ND18 (precipitation titer in tube tests 1:1,024) behaved similarly but less sensitively (12). However, immunoglobulins to BSMV-

type strain detected BSMV-ND18 as sensitively as homologous virus, and there was no indication of differential sensitivity to the other BSMV strains investigated.

Test sensitivity was considerably influenced by conjugate dilution but less so by coat dilution (Fig. 2). Coat and conjugate at 10 and 5 μg/ml (1:100), respectively, detected as little virus as 50 ng/ml and gave much higher ELISA values at low antigen dilutions than did coat and conjugate at 5 and 2.5 μg/ml (1:200), respectively (Fig. 2). The sensitivity of the reaction can thus be modified within wide limits, and the combination used in most tests of 5 μg/ml each of coat and conjugate was a compromise between sensitivity and economy.

Virus in individual seeds. We compared the proportions of virus-containing seeds and seed parts in two series of seed lots as assessed by SDS disk test and ELISA (Table 1). The seed lots tested contained seed from the 1976 harvest (series 1) or mixed seed from the 1976–1978 harvests (series 2) at Bozeman.

The number of seeds included in most tests was reduced to 50, and limited replicate tests were performed because limited numbers of seeds were available. This small sample size may have contributed to the variability observed between replicate estimates of the percentage of embryo infection in some tests (Table 1). However, plots of the ELISA values (Fig. 3) indicated that the viral content of individual embryo extracts sometimes fell into a more or less continuous range in which it was difficult to discriminate between positive and negative assays. This variation may also have affected discrimination in SDS disk tests. It was less obvious in the results for endosperm extracts and contrasted with those for leaf extracts from progeny plants, where ELISA values scored as positive were typically uniform and clearly distinguishable from values scored as negative (Fig. 3). Discontinuity in the range of ELISA values was more apparent for each type of extract at ×3 than at ×2 background. Use of the ×3 background, a common level for distinguishing between positive and negative reactions (14), was also more consistent with the linearity of plots of ELISA values against BSMV dilution in other experiments (Figs. 2, 4, and 5).

All estimates by ELISA of percentage of progeny infection were somewhat lower than corresponding estimates of embryo infection by ELISA or by SDS disk tests (Table 1). Overall, the results suggested that some virus associated with embryos failed to infect progeny plants. This may represent a residue of virus inactivated during seed ripening, as occurs in some associations of virus and seed (13). The virus may also sometimes have been carried superficially in small fragments of endosperm adhering to the separated embryos, because ELISA estimates of the percentage of endosperm infection were generally high (Table 1). However, if this occurred it was erratic, for the results showed no direct relationship between the viral content of endosperm and corresponding embryo tissue; they also confirmed that virusfree embryos can be associated with virus-containing endosperm (Fig. 3).

By comparing the ELISA values for endosperm and embryo extracts with those for virus standards, we found that the extracts contained up to 0.5 mg of viral antigen per milliliter. Because

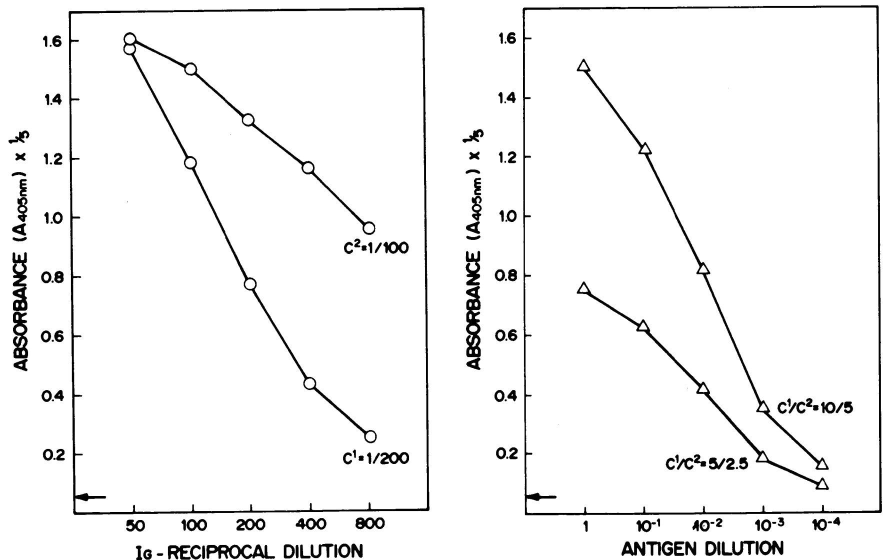


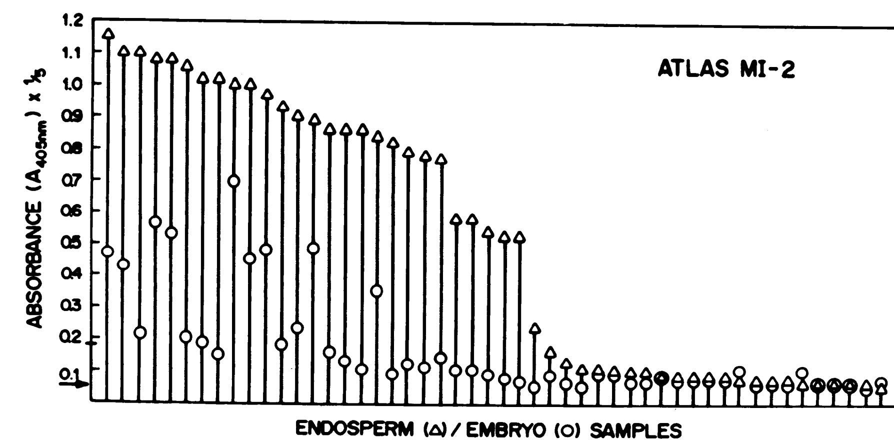
Fig. 2. Plots of enzyme-linked immunosorbent assay values for a barley stripe mosaic virus preparation at 0.5 mg/ml: **(left)** coating immunoglobulin (C^1) at 5 μg/ml (1:200) or conjugate immunoglobulin (C^2) at 5 μg/ml (1:100) used in conjunction with conjugate or coat, respectively, at the dilutions indicated; **(right)** coat and conjugate combinations (C^1/C^2) used at 5/2.5 μg/ml (1:200) and 10/5 μg/ml (1:100). Arrows on vertical axes indicate control values.

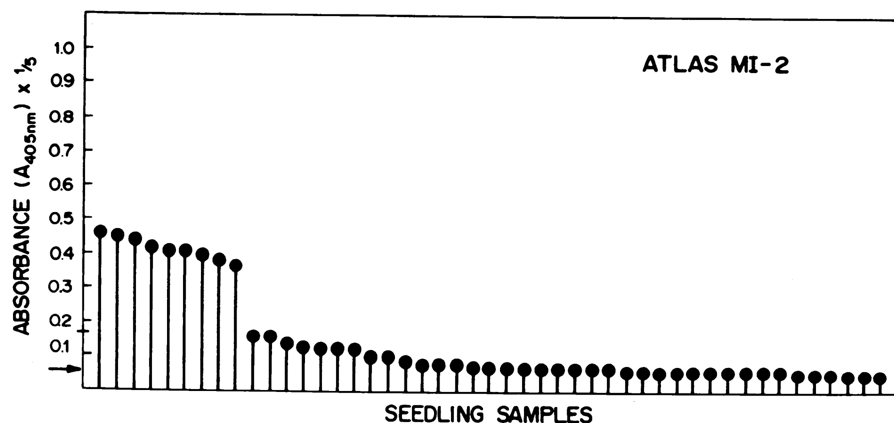
Table 1. Estimates of infection of seed samples with barley stripe mosaic virus as measured by sodium dodecyl sulfate (SDS) disk tests and enzyme-linked immunosorbent asssay (ELISA)

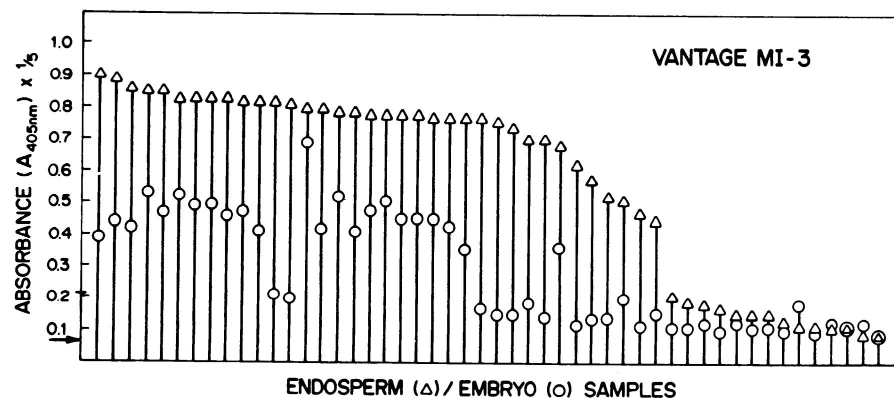
Cultivar/virus strain	No. of seeds per sample (samples)	Estimate of infection (range), %			
		SDS disk	ELISA[a]		
		Embryos	Endosperms	Embryos	Progenies
	Series 1 (1976 seed)				
Atlas/MI-1	200(1)	60	...[b]	...	...
	50(2)	...	82 (74–90)	60 (56–63)	...
	50(2)	...	...	...	33 (30–35)
Atlas/MI-2	200(1)	50	...	...	...
	50(2)	...	81 (78–84)	54 (53–56)	...
Vantage/MI-3	200(1)	70	...	...	...
	50(2)	...	99 (98–100)	81 (71–88)	...
	50(2)	...	...	...	60 (58–62)
	Series 2 (1976-1978 seed mix)				
Atlas/MI-1	50(4)	50 (48–54)	...	...	...
	50(2)	...	79 (78–80)	44	...
	50(2)	...	...	...	43(42–44)
Atlas/MI-2	50(4)	25 (20–32)	...	...	...
	50(2)	...	55 (54–56)	29 (26–32)	...
	50(2)	...	...	...	16(14–18)
Atlas/type	50(4)	23 (12–38)	...	...	...
	50(2)	...	85 (84–86)	12	...
	50(2)	...	...	...	6
Vantage/MI-3	50(4)	59 (46–66)	...	...	...
	50(2)	...	73 (72–74)	51 (46–56)	...
	50(2)	...	...	...	49 (40–58)
Atlas/control	50(4)	0	...	...	...
Vantage/control	50(4)	0	...	...	...

[a] Mean values from tests of extracts made by grinding individual embryos, endosperms, or leaf samples (about 0.05 g) in 250 μg of 0.2 M borate buffer, pH 9.0, followed by 2 ml of phosphate-buffered saline/polyvinylpyrrolidone/Tween buffer. Controls were similar extracts from whole, bulk, healthy Moore barley seed. ELISA values (A_{405nm}) exceeding three times the value for healthy seed extracts were scored as positive.

[b] ... = not tested.







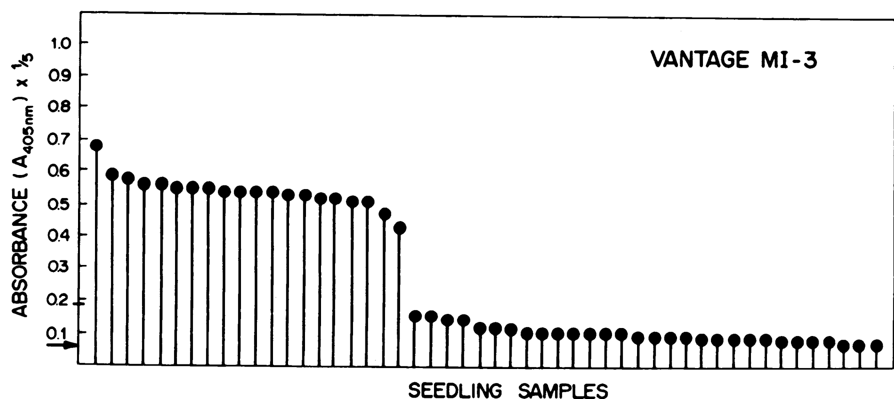


Fig. 3. Enzyme-linked immunosorbent assay values for extracts from barley infected with barley stripe mosaic virus strains Atlas MI-2 and Vantage MI-3, as indicated. Embryo and endosperm values are arranged in descending order of endosperm value; those on the same vertical line correspond to the same seed. Progeny seedlings are random samples of plants grown from seed batches of the same lots, with almost 100% germination in each case. Each presoaked embryo, endosperm, or 0.05-g fresh leaf sample was extracted in 250 μl of 0.2 M borate buffer, pH 9.0, and 2.0 ml of phosphate-buffered saline/polyvinylpyrrolidone/Tween. Coat immunoglobulin was used at 5 μg/ml, and conjugate was used at 5 μg/ml with embryo and endosperm extracts and 2.5 μg/ml with leaf extracts. Control and ×3 control values are indicated on the vertical axes by arrows and crosslines, respectively.

embryos from presoaked seed weighed about one-tenth as much as endosperms but were extracted in the same volumes of buffer, the results also suggested that embryos can contain higher concentrations of virus than endosperm tissue. The ratio, however, was variable.

Virus in bulk seed lots. Embryo or progeny plant samples must be tested to estimate virus transmission through seed, but the sensitivity of ELISA suggested its use in tests of whole seed to detect infected seed lots. If seeds likely to produce infected progeny must be stringently excluded, this information could aid in a decision on seed lot acceptability.

Figure 4 summarizes ELISA values obtained for bulk seed extracts in a series of experiments. Duplicate 3-g samples (about 60 seeds) of each of the seed lots in series 1 (Table 1) were presoaked and ground in borate buffer at 1:2 (w/v), followed by further grinding in PBS/PVP/Tween at 1:2 (w/v). ELISA values for undiluted samples were anomalous and those for low dilutions of the extracts covered a wide range, but all were closely similar at higher dilutions (Fig. 4). The results indicated that extracts had dilution end points similar to that of a standard preparation containing BSMV at 0.5 mg/ml, giving ELISA values clearly distinguishable from background at dilutions of 10^{-4}.

These results suggested the possibility of identifying infected seed in mixtures containing one infected seed per 1,000 or even one per 10,000, assuming that the effect of "dilution" of virus-containing seed by the presence of virusfree seed was

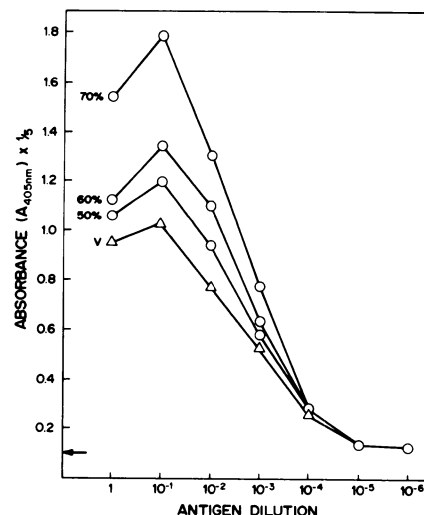


Fig. 4. Plots of enzyme-linked immunosorbent assay values for dilutions of barley seed extracts (O) and barley stripe mosaic virus (BSMV) preparation at 0.5 mg/ml (Δ). Coat and conjugate immunoglobulins were used at 5 μg/ml. Buffered extracts (1:4, w/v) were from seeds of Atlas barley 50% infected with BSMV strain MI-1, Atlas barley 60% infected with strain MI-2, and Vantage barley 70% infected with strain MI-3. Arrow on vertical axis indicates control value.

similar to dilution of extracts with buffer. Further tests validated this assumption. We used ELISA tests to compare extracts from dry-milled seed from the nominally 50%-infected Atlas barley seed lot (Atlas MI-2, Table 1, series 2) when diluted with dry-milled, healthy Moore barley seed, with buffer extracts from healthy seed, or with PBS/PVP/Tween. The results confirmed that each way of diluting the virus-containing seed sample gave similar ELISA values (Table 2).

In further experiments, virus was easier to detect in bulk seed samples from seed lots of series 2 (Table 1) if seeds were presoaked rather than dry milled before being ground in a mortar. Dry-milled seed samples were soaked overnight in enough water to compensate for the amount taken up by presoaked seed, and extracts were then compared by ELISA. The results (Fig. 5) indicated that presoaking improved the sensitivity of virus detection up to tenfold. Dry milling probably reduces the efficiency of viral antigen solubilization and of hydration in virus-containing seed tissue.

Detection of BSMV in bulk seed mixtures. ELISA and SSEM were used to detect BSMV in various seed mixtures prepared at Bozeman by blending infected seed samples with virusfree seed of Vantage barley. We mixed the seeds to preselected levels of embryo infection based on the data in Table 1. SDS disk tests confirmed the levels of infection (Table 3).

Extracts from 12 of the mixtures were tested by ELISA at Purdue as unknowns, and four were also checked for virus particle content by SSEM at Bozeman. The results confirmed the usefulness of both ELISA and SSEM tests in determining whether seed lots contained even small amounts of seed derived from infected plants (Table 3). When compared with those of SDS disk tests, the results of ELISA and SSEM were presumably both enhanced and modified by the presence of virus in endosperms. However, we found surprising agreement among the three test procedures in their ranking of seed mixtures according to level of infection.

DISCUSSION

ELISA was more precise in comparing the viral content of individual seeds and seed plants, but it had no special advantage over the SDS disk test in testing individual seeds in a seed certification program except that of convenience in a laboratory already equipped for ELISA tests. Both ELISA and SSEM, however, showed considerable potential for usefulness in the routine testing of bulk seed. Such testing could be done when seeds originating from infected plants, and thus likely to produce a proportion of infected progeny, must be excluded. It could also be used where a specific level of acceptability is to be applied based on the expected performance of seeds containing some virus.

In these situations, ELISA offers the advantages of simplicity and economy in detecting low levels of virus-containing seed and of discriminating among the amounts of virus in different seed lots. Where facilities are available, however, SSEM is a valuable method for analyzing a limited number of bulk seed lots in certification programs. It is presently used in this way to monitor SDS disk test results in seed certification in Montana.

We found no indications that ELISA has greater specificity than other serologic tests with the strains of BSMV in our study, but the other evidence of enhanced specificity with this test (10) requires that the possibility be investigated

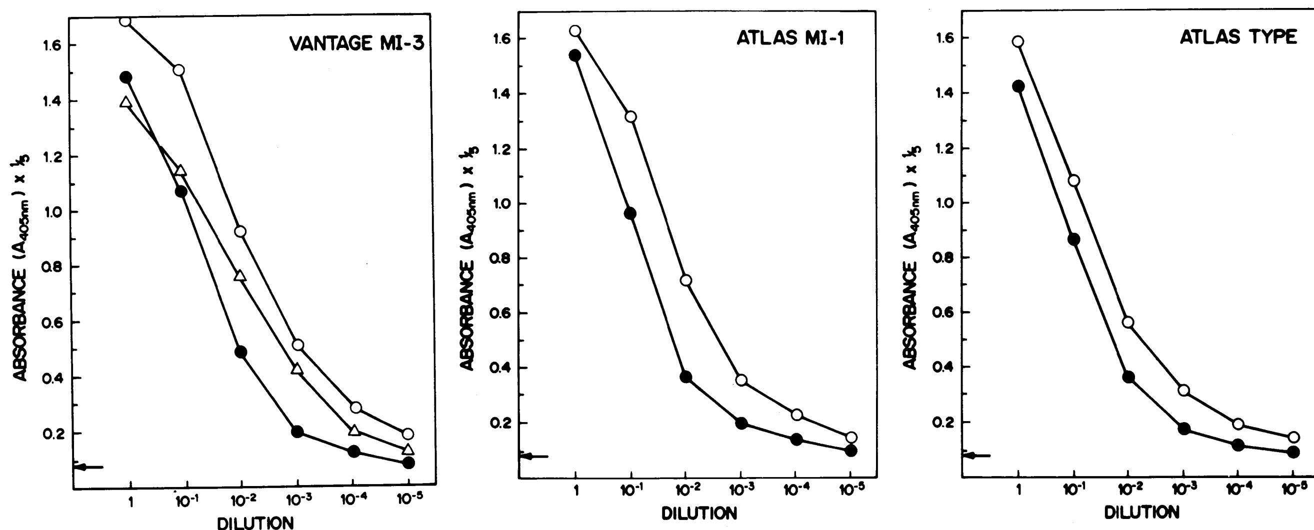


Fig. 5. Plots of enzyme-linked immunosorbent assay values for dilutions of barley stripe mosaic virus (BSMV) preparation at 0.5 mg/ml (Δ) and various barley seed extracts: **(left)** Vantage barley infected with BSMV strain MI-3; **(center)** Atlas barley infected with strain MI-1; and **(right)** Atlas barley infected with type strain. Open circles = buffered extracts (1:12, w/v) from presoaked seed; solid circles = extracts from dry-milled seed. Coat and conjugate immunoglobulins were used at 5 μg/ml. Arrows on vertical axes indicate control values.

Table 2. Absorbance values for dilutions of barley stripe mosaic virus (BSMV) and extracts from milled seed as measured by enzyme-linked immunosorbent assay

Extract	Diluent	Absorbance[a] at dilution of					
		1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
BSMV (0.5 mg/ml)	PBS/PVP/Tween[b]	···	0.96	0.72	0.48	0.23	0.16
Milled, infected seed[c]	Dry-milled, healthy seed[d]	1.08	0.82	0.65	0.38	···	···
Milled, infected seed	Extract from healthy seed[d]	1.09	0.82	0.56	0.42	0.33	0.30
Milled, infected seed	PBS/PVP/Tween	···	0.88	0.69	0.54	0.35	0.23
Milled, healthy seed	None	0.16	···	···	···	···	···

[a] A_{405nm} values for reacted substrates diluted fivefold. All absorbances are means of duplicate values from each of two separate experiments.
[b] Phosphate-buffered saline/polyvinylpyrrolidone/Tween.
[c] From a nominally 50% BSMV-infected Atlas barley seed batch. Each seed extract was from 2 g of milled seed ground in 8 ml of 0.2 M borate buffer plus PBS/PVP/Tween buffer at 8 ml/g. All further dilutions were in the latter buffer.
[d] From Moore barley.

Table 3. Detection of barley stripe mosaic virus (BSMV) in seed mixtures[a] by sodium dodecyl sulfate (SDS) disk tests, enzyme-linked immunosorbent assay (ELISA), and serologically specific electron microscopy (SSEM)

Cultivar/virus strain[b]	Embryo infection		Whole seed infection	
	Calculated, %[c]	SDS disk evaluation, %[d]	Mean ELISA value (range)[e]	Mean number of particles observed by SSEM (range)[f]
Vantage/MI-3	50	54	0.78 (0.75–0.81)	430 (110–802)
Atlas/MI-1	30	31	0.71 (0.66–0.76)	219 (147–342)
Vantage/MI-3	25	22	0.69 (0.65–0.73)	...[g]
Atlas/MI-1	20	22	0.71 (0.67–0.75)	...
Atlas/type	10	16	0.68 (0.58–0.77)	...
Atlas/MI-2	15	14	0.74 (0.68–0.80)	...
Atlas/MI-2	10	8	0.70 (0.55–0.86)	95 (54–161)
Atlas/MI-2	5	6	0.60 (0.53–0.66)	...
Atlas/type	5	4	0.57 (0.56–0.58)	12 (1–30)
Atlas/MI-1	5	4	0.56 (0.53–0.59)	...
Atlas/type	1	2	0.37 (0.32–0.41)	...
Vantage/MI-3	1	2	0.26 (0.18–0.34)	...
Atlas/control[h]	0	0	...	0
Vantage/control[h]	0	0	...	0
Healthy barley control[i]	...	...	0.09 (0.08–0.10)	...
BSMV (500 μg/ml)	...	...	1.27 (1.2–1.34)	...
BSMV (50 μg/ml)	...	...	0.67 (0.63–0.71)	...
BSMV (5 μg/ml)	...	...	0.41 (0.39–0.42)	...
BSMV (0.5 μg/ml)	...	...	0.24 (0.22–0.25)	...

[a] Blends of selected barley cultivar seeds and virus isolate combinations with virusfree seed of the Vantage barley control (Table 1).
[b] Listed in order of SDS disk evaluation of embryos.
[c] Preselected levels of embryo infection were calculated for each seed mixture based on predetermined values observed for each virus-barley combination (Table 1).
[d] Based on a test of 50 embryos.
[e] ELISA values are means (×1/5) of duplicate values for undiluted, buffered extracts (1:12, w/v) from milled, whole seed of each of two 50-seed samples or for a purified preparation of BSMV (type isolate). Coat and conjugate were used at 5 and 2.5 μg/ml, respectively.
[f] SSEM values are numbers of particles of buffered extracts from milled, 50-seed samples. Means are based on counts from nine electron micrographs (three per seed sample, three samples per source). Each micrograph (×27,000) measured 127 × 178 mm.
[g] ... = not tested.
[h] Free from BSMV as determined by previous SDS disk tests of embryos.
[i] Buffered extract from healthy Moore barley seed.

further. We suggest a program, especially with ELISA, of estimating the viral status of bulk seed lots by comparing preparations from them with those from standard seed lots of known viral content or with standard viral preparations. The seed lot preparations would most simply be made from milled dry seed, but test sensitivity could be improved by using presoaked seed and by other modifications in extraction. Sensitivity could also be improved by adjusting the concentration of immunoglobulins.

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