Serologic Detection of Bean Common Mosaic and Lettuce Mosaic Viruses in Seed

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ABSTRACT

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Serologic methods for detecting seedborne bean common mosaic (BCMV) and lettuce mosaic (LMV) viruses were investigated because preliminary tests showed that enzyme-linked immunosorbent assays (EIA) could be used to detect the purified viruses at concentrations as low as 10 ng per milliliter. With artificially contrived levels of infection, the equivalent of one BCMV-infected embryo homogenized with 2,000 healthy embryos and one LMV-infected seed homogenized with 1,400 healthy seed could be detected. EIA was more sensitive and convenient in routine screening of lettuce seed lots for LMV than the *Chenopodium quinoa* assay or "seedling grow-out" indexing methods currently used by the California lettuce industry. EIA detected BCMV in all flower and seed parts tested in infected bean. In mature bean seed, the virus was mainly in the embryo, with only a small amount in the seed coat. In contrast, considerable virus was detected in the coat of immature seed as well as in the embryo after surface decontamination.

Transmission of viruses by seed is important in the epidemiology of some diseases. Randomly distributed infected seed are sources of disease when the plants emerge from the soil, and the timing and distribution are optimum for virus spread. Such seedborne sources of virus inoculum can be extremely important in an epidemic of a virus that also is spread efficiently by insects. Both bean common mosaic (BCMV) and lettuce mosaic (LMV) viruses are transmitted in seed and by aphids in a nonpersistent manner (7,9,16) and have the potential for rapid spread (15).

Because BCMV and LMV cause damaging diseases wherever susceptible crops are grown and because both are transmitted primarily through seed, virusfree seed is especially important for disease control (7,9,11,16). Methods of monitoring seed lots for the viruses should be convenient and effective. Current clean-seed programs for control of BCMV and LMV rely on visual surveys of seed fields and tests of seed lots in the greenhouse. These methods are laborious and time-consuming and, furthermore, fail to detect masked infections. We felt that enzyme-linked immunosorbent assay (EIA), the recently developed sensitive immunologic method for detecting viruses (4–6,18,19), might be more convenient and reliable for evaluating seed lots for virus content. This is the report of our investigation of EIA for detecting seedborne LMV and BCMV.

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MATERIALS AND METHODS

Source of virus isolates. Three BCMV isolates that induced typical symptoms (2) were used: (i) American Type Culture Collection (ATCC) PV-25-LN, (ii) A. W. 15B isolated from Pinto

bean and received from A. W. Saettler, Michigan State University, East Lansing, and (iii) Pinto 111 seeds infected with strain NY15 and received from M. J. Sibernagel, Washington State University, Pullman.

LMV isolates were obtained from seedlings grown from LMV-infected lettuce seed in the greenhouse. The identity of the virus was determined by sap inoculation to lettuce (*Lactuca sativa* L.), *Chenopodium quinoa* Willd, *C. amaranticolor* Coste and Reyn, and *Gomphrena globosa* L., all of which developed characteristic symptoms of LMV (17). Antiserum to LMV, provided by K. A. Kimble (University of California, Davis) was used to confirm identity of the virus.

Virus purification. The procedure for virus purification was similar to that used previously (8) for tobacco etch and other members of the potyvirus group. Leaf tissue with distinct symptoms, collected about 15–18 days after mechanical inoculation, was homogenized with a Waring blender in cold (4 C) 0.5 M potassium phosphate buffer, pH 7.2, containing 0.5 M urea, 1% Na_2SO_3, 0.5% 2-mercaptoethanol, and 0.05 M disodium ethylenediaminetetraacetate (EDTA) (1 g of fresh tissue per 1.2 ml of buffer). Crude extracts were filtered through two layers of cheesecloth and clarified by shaking for 5 min with an equal volume of chloroform. The emulsion was centrifuged at 8,000 rpm for 25 min. BCMV was precipitated from the collected supernatant by adding solid polyethylene glycol 6,000 (PEG) to an 8% (w/v) level, plus 0.5% (w/v) NaCl. Stirring was continued for 0.5 hr at 4 C, after which the extract was incubated overnight at the same temperature. The precipitate was collected by centrifugation for 20–30 min at 8,000 rpm, and the pellets were resuspended for 4 hr in about 1/10 of the original volume of 0.025 M phosphate buffer, pH 7.2, containing 1 M urea and 0.1% 2-mercaptoethanol. The virus then was subjected to two cycles of differential centrifugation. After high-speed centrifugation for about 2 hr at 27,000 rpm in a No. 30 rotor of a Spinco Model L ultracentrifuge, the virus was further purified by equilibrium density-gradient centrifugation in saturated CsCl (about 23 C) in glass-distilled water at 32,000 rpm for 19 hr in an SW 50.1 rotor. The virus band was collected and dialyzed overnight against sodium borate buffer 0.025 M, pH 8.2.

LMV was purified by a modification of the procedure used for BCMV. *C. quinoa* was used as the host for virus propagation. For clarification, *n*-butanol (thoroughly washed with sodium bisulfite) was added and the mixture was stirred for 30 min and allowed to stand at 4 C for 2.5 hr. Then, 60% (v/v) cold chloroform was added and the mixture was shaken for 5 min, followed by low-speed centrifugation at 8,500 rpm for 20 min in a Sorvall GSA rotor. The aqueous phase was withdrawn by aspiration. After two cycles of differential centrifugation, the virus was stabilized by adding formaldehyde to a final concentration of 1.8% and incubating at 4 C for 3 hr, followed by high-speed centrifugation and resuspension in buffer. Further purification was achieved by two cycles of equilibrium density-gradient centrifugation with saturated CsCl.

The concentration of both viruses was determined in a Beckman DK-2A spectrophotometer using an extinction coefficient of 2.4/mg/ml at 261 nm, as reported for tobacco etch virus by Purcifull (13).

Antiserum production. Antiserum for BCMV was prepared by injecting rabbits with purified whole virus. Initially, about 1 mg of virus was injected into the marginal ear vein and 2 mg of virus emulsified 1:1 (v/v) with Freund's complete adjuvant was injected into a leg muscle. Four more intramuscular injections of virus in Freund's incomplete adjuvant were made at 1-wk intervals. The animals were bled 10 days after the last injection and at 10-day intervals thereafter. After the third bleeding, the animal was rested about 2 mo. Booster injections of about 9 mg of purified virus in adjuvant then were given, and the rabbit was bled at 1-wk intervals. The microprecipitin titer of serum from the first bleeding was about 1/2,000.

Since the antiserum reacted with healthy bean sap in double-diffusion tests, it was cross-absorbed with host protein.

Highly specific antiserum against LMV was produced. The antiserum reacted strongly with purified virus in both microprecipitin and tube tests up to a dilution of 1/512. Sap from healthy *C. quinoa* did not react with the LMV antiserum in double-diffusion in agar tests.

Isolation of bean embryos. Two methods were used to prepare bean embryos. In one, the seeds were soaked overnight in water and the embryos were separated manually from the rest of the seed. When a large number of embryos was needed, the procedure used by G. Bruening and M. L. Russel (*personal communication*), Department of Biochemistry, University of California, Davis, was followed. Bean seeds, ground in a blender until broken, were stirred in a mixture of cyclohexane and carbon tetrachloride (1:2.5). Cell debris was allowed to settle for several minutes, and the embryos from the surface of the solution were collected and drained for 20–30 min. At this stage, embryos were mixed with some seed coats, but the coats floated and the embryos settled after stirring in water. The embryos were collected and dried for 3–4 hr at room temperature and stored under refrigeration. For EIA, embryos were weighed and homogenized in 6 volumes (w/v) of phosphate-buffered saline and Tween 20 containing 2% polyvinylpyrrolidone.

Enzyme-linked immunosorbent assays. EIA was done as described for detecting plant viruses (4,18). The γ-globulin fractions of both antisera were precipitated with half-saturated ammonium sulfate and were further purified by passage through a DEAE-cellulose column. The concentration of γ-globulin used for optimum coating of the wells of the polystyrene plate was 0.5 μg/ml for BCMV and 0.3 μg/ml for LMV. Globulin fractions of each antiserum were conjugated with alkaline phosphatase (E.C. 3.1.3.1, Sigma Chemical Co., St. Louis, MO 63178), with 0.06% glutaraldehyde as the coupling agent (1). Test samples and conjugates of enzyme-labeled γ-globulin were diluted in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20, 2% polyvinylpyrrolidone (PVP, mol wt 40,000), and 0.2% ovalbumin. Results were considered positive for test samples if the average absorbance at 405 nm was twice that of healthy control samples in the same plate (20).

RESULTS

Sensitivity and specificity of EIA in detecting BCMV and LMV. Serial dilutions of infected sap and purified preparations of the viruses were tested. BCMV was detected by EIA in infected Red Kidney bean leaf extract diluted to about 1:25,600 (Fig. 1). Purified preparations of BCMV were detected at a concentration as low as about 8 ng per milliliter (Fig. 1). LMV was detected by EIA in infected *C. quinoa* leaf extract diluted to about 1:12,800 and in a purified virus preparation at a concentration as low as about 9 ng per milliliter (Fig. 2). When compared with infectivity tests of the same extracts (Table 1), virus detection with the EIA procedure was about eight times more sensitive than infectivity for BCMV and about 16 times more sensitive for LMV.

Comparison of infectivity tests and EIA for seedborne BCMV. To determine the reliability of EIA for detecting seedborne virus in bean seedlings, a small amount of tissue was taken from germinating seed without destroying the seedling. About 5-mm^2 sections of primary leaves of Pinto 111 infected with BCMV (strain NY15) were taken shortly after plant emergence from the soil and placed individually in depressions in a Plexiglas board. These were ground with glass rods in the presence of PBS-Tween 20 and 2% PVP. The extracts were transferred to the wells of a microtiter plate previously coated with antiserum and were tested by EIA. The remainder of the seedlings were kept in the greenhouse and disease symptoms were recorded after about 2 wk. Symptoms and serology correlated perfectly. Of 67 seedlings tested, 33 reacted positively to EIA and later developed virus symptoms.

Distribution of BCMV in seed. Fully mature Pinto bean seeds infected with BCMV (strain NY15, exhibiting approximately 50% seed transmission) were soaked for 18 hr in distilled water, then dissected into seed coat, cotyledons, and remainder of the embryos. The dissected parts were washed in running water for 20–25 min, then homogenized individually with a pestle and mortar in PBS-Tween 20 and 2% PVP in a ratio of 1:10 (g tissue/ml). Testing the thoroughly triturated portions by EIA showed that the virus was

distributed mainly in embryos and cotyledons; very little virus was detected in mature dry seed coats (Table 2).

The same test was conducted with immature Pinto bean seeds infected with BCMV (NY 15, approximately 50% seed transmission). Fifteen green pods (before striation) were chosen at random, and two or three immature seeds were removed and mixed. Four samples of six seeds each were tested. The samples were dissected into seed coats, cotyledon, and remainder of the embryo and were decontaminated by washing for 20–25 min in running water, ground in buffer, and tested by EIA. The concentration of virus was much higher in immature seed coats than in mature seed coats but higher in mature cotyledons and embryos than in immature ones (Table 2).

Detection of BCMV in infected bean embryos. Artificially contrived mixtures of infected bean embryos homogenized with a large number of healthy embryos were used to evaluate the sensitivity of EIA for detecting BCMV in bean seed. Extracted bean embryos were divided in half longitudinally with a sterilized razor blade, and single halves were tested by EIA to identify the infected embryos. Selected infected halves were mixed with 500, 800, or 1,000 healthy embryos to simulate one infected embryo in a sample of 1,000, 1,600, or 2,000 embryos. The grinding buffer (PBS-Tween 20, 2% PVP, and 0.2% ovalbumin) was in a ratio of about 6:1 (ml/g tissue). One infected embryo could be detected consistently in a mixture of up to 2,000 healthy embryos (Table 3).

Detection of LMV in lettuce seed. In a similar experiment, EIA was done on extracts from halves of lettuce seed to identify infected seed. Infected seed halves then were mixed with healthy seed (300, 500, or 700) to simulate samples with low rates of seed transmission. Seed were ground in buffer at a ratio of 7 ml per gram of tissue. A single LMV-infected seed could be detected in about 1,400 healthy seed (Table 4).

Because dry lettuce seed are difficult to grind, germinated seed were used. Seed were collected randomly from a seed lot with approximately 10% seed transmission. Eight replications of about

TABLE 1. Infectivity of bean common mosaic virus from Red Kidney bean and lettuce mosaic virus from *Chenopodium quinoa* in serial twofold dilutions of infected leaf extracts

Sap dilution	BCMV-inoculated Red Kidney bean[a] (%)	LMV-inoculated *Chenopodium quinoa*[b] (%)
1:50	100	100
1:100	100	100
1:200	100	66
1:400	100	33
1:800	57	17
1:1,600	28	0
1:3,200	14	0
1:6,400	0	0

[a] Expanded primary leavess of seven Red Kidney beans were inoculated with each dilution.
[b] Six healthy *C. quinoa* at the fully developed four-leaf stage were inoculated with each dilution.

TABLE 2. Comparison of EIA with bean common mosaic virus in various parts of mature and immature bean (*Phaseolus vulgaris* L.) seed

Seed parts	Fully mature dry seed[a]	Immature seed from green pod[a]	Healthy seed[b]	Infected leaf[b]
Coat	0.188 ± 0.08	1.3 ± 0.53	0.02 ± 0.01	6.4 ± 0.5
Cotyledon	2.35 ± 0.14	1.8 ± 0.31	0.02 ± 0.01	···
Embryo	2.31 ± 0.24	1.79 ± 0.45	0 02 ± 0.01	···

[a] Average $A_{405\ nm}$ values of four samples of six seeds each (about 50% seed transmission), with standard errors.
[b] Average $A_{405\ nm}$ of three samples, with standard errors.

TABLE 3. Sensitivity of EIA for detecting a single infected bean embryo homogenized with a large number of healthy embryos[a]

Sample size	$A_{405\ nm}$	Positive samples/ samples tested
1 infected embryo + 1,000 healthy embryos	0.16 ± 0.034	10/10
1 infected embryo + 1,600 healthy embryos	0.134 ± 0.02	6/6
1 infected embryo + 2,000 healthy embryos	0.096 ± 0.034	6/6
Healthy embryos	0.02 ± 0.01	0/5

[a] Embryos were weighed and homogenized in six volumes (w/v) of buffer.

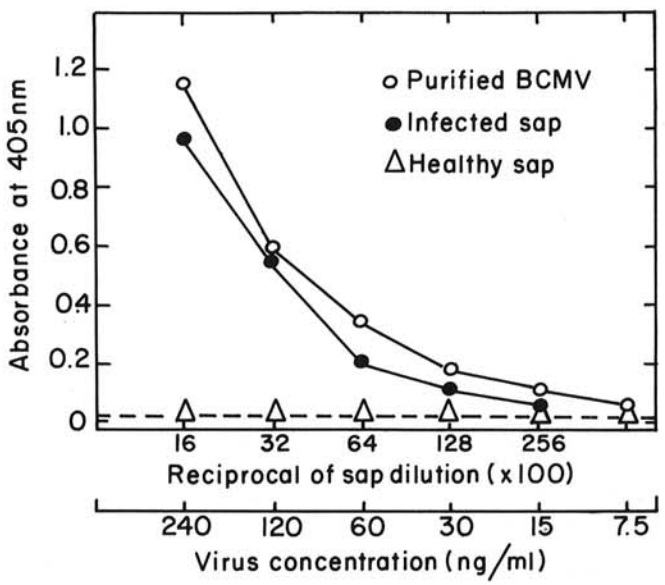

Fig. 1. Results of enzyme-linked immunosorbent assays with bean common mosaic virus in diluted extracts of infected Red Kidney bean (upper scale) and purified virus (lower scale).

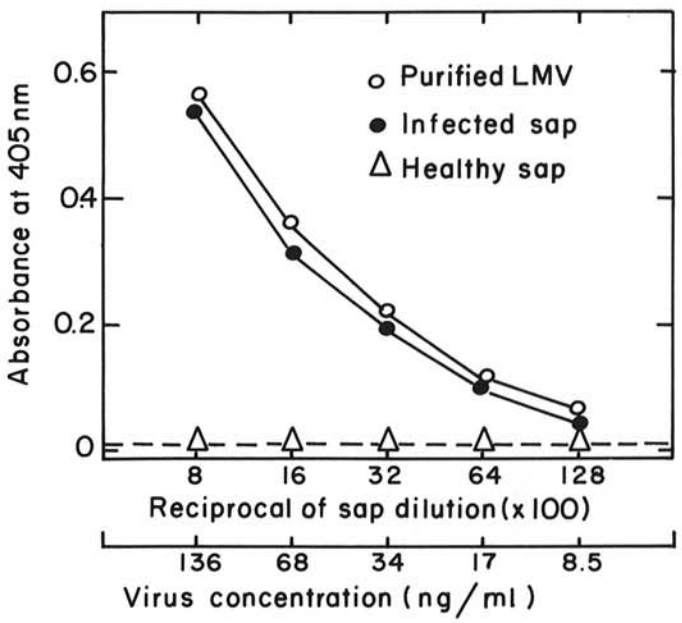

Fig. 2. Results of enzyme-linked immunosorbent assays with lettuce mosaic virus in diluted extracts of infected *Chenopodium quinoa* (upper scale) and purified virus (lower scale).

100 lettuce seed each were placed on filter papers in plastic petri dishes each day over a 4-day period. The papers were moistened with distilled water to induce germination, and moisture was replenished as needed each day. The dishes were kept under continuous fluorescent light at room temperature (approximately 25 C). On the fourth day, each sample was ground thoroughly with mortar and pestle in the presence of PBS-Tween 20 and 2% PVP (10 ml/g of tissue) and tested by EIA. For comparison, ungerminated seed samples were moistened with buffer to allow the seed coat to soften before grinding. The amount of virus was about the same in germinated and ungerminated seed. The relative virus concentration in germinated seedlings also was about the same for the first three days of germination (Table 5).

A series of tests were done with lettuce seed lots with different amounts of seed transmission to relate EIA absorbance values to predetermined levels of virus seed transmission. The results are shown in Table 6.

DISCUSSION

The EIA technique detected small amounts of BCMV and LMV in leaf extracts, infected seed, and purified virus preparations. The method is sensitive and inexpensive and appears to be a practical alternative to the methods now used for screening seed lots for these viruses. In fact, our results with BCMV (consistent detection of one infected embryo in 2,000 healthy embryos) and LMV (detection of one infected seed in 1,400 healthy seeds) indicate that EIA is superior to any other method.

TABLE 4. Sensitivity of EIA for detecting a single infected lettuce seed homogenized with a large number of healthy seeds

Sample size	$A_{405\ nm}$	Positive samples/ samples tested
1 infected seed + 600 healthy seeds	0.27±0.04	3/3
1 infected seed + 1,000 healthy seeds	0.15±0.03	9/9
1 infected seed + 1,400 healthy seeds	0.09±0.03	9/9
Healthy seed	0.01±0.005	0/2

TABLE 5. EIA with ungerminated and germinated lettuce seed

	Ungerminated seed[a] ($A_{405\ nm}$)	Germinated seed after:[b] 1 day	2 days ($A_{405\ nm}$)	3 days
Infected seed	0.53±0.16	0.65±0.2	0.62±0.22	0.68±0.2
Healthy seed	...[c]	0.02±0.01	...	...

[a] Average of eight samples with 1 g of seed homogenized in 10 ml of buffer.
[b] Average of two samples with 1 g of seed homogenized in 10 ml of buffer.
[c] No test was done.

TABLE 6. EIA absorbance of lettuce seed lots with a high rate of seed transmission of lettuce mosaic virus

Seed lot	Seed transmission (%)	$A_{405\ nm}$[a]
1	1.7	0.42 ±0.5
2	5.0	0.90 ±0.2
3	6.0	0.92 ±0.16
4	10.0	1.27 ±0.12
Healthy seed	0	0.015±0.007

[a] Average absorbance value of four samples of 200 seeds each with 1 g of seed homogenized in 10 ml of buffer.

EIA has several advantages over the seedling grow-out and *C. quinoa* inoculation methods used routinely for screening lettuce seed lots for LMV (11). The EIA method saves much time, space, and effort and may be as reliable as other techniques.

Other investigators (3,10,12) have found EIA with viruses in soybean and pea seed to be less sensitive than our results with BCMV. Pea seed infected with seedborne pea mosaic virus at less than 1% (10) or soybean infected with tobacco ringspot or soybean mosaic viruses at less than 1% (12) have not been detected with EIA by other investigators. This difference probably is not due to the use of whole seed in their tests vs. the use of extracted embryos in our tests, since cotyledons, which make up the bulk of all these seed, have about the same virus content as embryos in beans (Table 2). Perhaps this is to be expected because the cotyledon is part of the embryo. We used extracted embryos because they are easier to grind and the homogenates are less bulky.

Our study of BCMV in bean seed showed considerable virus in immature seed coats even after surface decontamination for 20–25 min in running water (EIA value $A_{405nm} = 1.35 \pm 0.53$). During seed maturation and drying, however, most of the virus was inactivated or had become difficult to extract from the seed coat (mature seed coat extract in EIA tests was $A_{405nm} = 0.188 \pm 0.08$). In contrast, the relative virus concentration was higher in mature embryos and cotyledons than in immature ones (Table 2). This indicates that virus is not inactivated in embryos and cotyledons during maturation and drying. A similar result was noted by Schippers (14), who used plant inoculation assays.

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