Comparative Immunoassays of Bean Common Mosaic Virus in Individual Bean (*Phaseolus vulgaris*) Seed and Bulked Bean Seed Samples

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ABSTRACT

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Indirect enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (MAb) or polyclonal antiserum (PC) and dot immunoassay using a MAb were compared for the detection of bean common mosaic virus (BCMV) in seed of *Phaseolus* from the U.S. Department of Agriculture germ plasm collection. A small portion of seed coat, testa, and cotyledon was removed from each of 270 seeds and tested for BCMV in each of the three assays. The seeds were then planted and the resultant seedlings were tested for BCMV by ELISA:MAb. ELISA:MAb proved to be the most accurate technique for BCMV detection in individual seed. Further tests by ELISA:MAb on 1,350 seeds indicated that an effective ELISA threshold could not be established because of erratic virus distribution in the individual seed parts. An indirect ELISA using six samples of flour prepared from eight seeds each per seed lot was developed to predictively estimate the incidence of BCMV-infected seedlings in germ plasm accessions.

Germ plasm accessions have been recognized (3,7,8) as potential sources for new virus diseases. Consequently, elimination of seedborne viruses from collections of germ plasm is widely desired. Accessions in the United States Department of Agriculture (USDA) germ plasm collection of *Phaseolus* have been shown to be contaminated with bean common mosaic virus (BCMV) (11). As a component of a BCMV elimination program, we evaluated methods for determining BCMV infection in *Phaseolus* accessions.

Although serological methods have been used to detect BCMV-infected bean (*P. vulgaris* L.) seed (4,6,9,14,15,22), the relationship between detectable BCMV antigen in seeds and infection of the resulting seedling has not been fully determined (22). The germ plasm collection of bean is diverse and the accessions are contaminated with many different isolates of BCMV (10), making it likely that there will be considerable diversity in seed transmission among accessions (16). Therefore, the parameters of BCMV detection in individual germinable seeds were investigated using enzyme-linked immunosorbent assay (ELISA) and dot immunoassay (DIA) (14,19). A preliminary report has been published (12).

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MATERIALS AND METHODS

Antisera. Antisera used in these assays included a rabbit polyclonal antiserum (PC) prepared against the NY-15 strain of BCMV (21) and an ascites fluid containing a monoclonal antibody (MAb) that reacts with all known strains of BCMV (23).

ELISA and DIA comparison. Thirty seeds from each of nine Plant Inventory (PI) accessions of *P. vulgaris* were tested individually by ELISA and DIA using the MAb as well as by ELISA using the PC. A small piece of each seed was removed with an electric saw from the seed end distal to the primary axis. The small seed piece, consisting of testa and cotyledon tissue, was ground to a fine flour with a mortar and pestle and stored until use. The flour was then suspended approximately 1:50 (w/v) in 0.05 M carbonate buffer, pH 9.6, to which was added 2% polyvinylpyrrolidone and 0.2% ovalbumin. Aliquots of the flour were pipetted into microtiter plates (Immulon 2, Dynatech Laboratories, Inc., Alexandria, VA) or adsorbed to nitrocellulose membrane (Bio-Rad, Inc., Richmond, CA); 200 µl of sample was used for each of two microtiter wells, whereas 20 µl was adsorbed to the membrane. Indirect ELISA (13) with both the MAb (ELISA:MAb) and the PC (ELISA:PC) was done as described previously (11).

For DIA, membranes were washed vigorously in phosphate-buffered saline solution (0.01 M phosphate, 0.15 M NaCl, pH 7.4) with 0.05% Tween 20 (PBS-T); all washes were repeated three times for 5 min. After the wash steps, membranes were incubated for 30 min in 1% bovine serum albumin, washed as described earlier, incubated for 1 hr at 37 C in a 1:10,000 dilution of MAb in PBS-T, washed as described earlier, incubated for 1 hr at 37 C in a 1:100 dilution of antimouse IgG/horseradish peroxidase conjugate (Sigma) in PBS-T, washed as above, and incubated in a substrate solution prepared as described by Powell (19). Both ELISA tests and the DIA were done concurrently.

Positive and negative controls were prepared from bean seed harvested from BCMV-infected PI 474518 and healthy Bountiful plants, respectively. Positive and negative controls were prepared identically to the sample seed, except the controls were prepared in adequate quantities such that the same controls were used in all assays. This facilitated direct comparisons of tests run in different microtiter plates on different days. ELISA plates were read with a Dynatech Minireader II at 410 nm when the absorbance of the positive control first exceeded 2.0 at A_{410} (about 2–3 hr). Samples with an average absorbance two times the mean of the negative control (ELISA:PC) or 0.2 at A_{410} (ELISA:MAb) were considered positive for BCMV. The results of the DIA tests were determined visually. The remaining seed piece was planted in the greenhouse, and the resultant seedling was tested for BCMV infection in ELISA:MAb 2–3 wk after emergence. The relative accuracy of the respective assays was measured by determining the percent agreement between assays on seed pieces and the corresponding definitive seedling assay. Not uncommonly, seed piece assays were positive for BCMV antigen, but the seedling arising from the seed was uninfected; the converse was also observed. These were designated as false positive and false negative assays, respectively, whereas seed piece assays in agreement with the seedling test were considered positive or negative assays.

Additional tests were conducted using ELISA:MAb only. Thirty bean seeds from each of 45 PI accessions were tested individually as described earlier. Stan-

dardized positive and negative controls were used in each ELISA plate to facilitate comparisons among ELISA tests done on different dates with different accessions. Seedlings from each tested seed were tested as described. Seedlings testing negatively for BCMV infection were allowed to grow to maturity and were retested at least once during this time.

Seed dissection tests. Thirty seeds from each of nine bean accessions were dissected, while dry, into seed coat, cotyledon, and primary axis. In addition, 95 seeds from plants of a single accession were dissected into their component parts; these 95 seeds were harvested from plants produced from infected seed. Each part from each seed was tested individually with ELISA:MAb.

Bulk seed tests. Six samples of eight seeds each from each of 29 PI bean accessions were ground to a fine flour in a small coffee mill, and a small portion of the flour from each sample was tested as described earlier with both ELISA:MAb and ELISA:PC. Standardized positive and negative controls were included in each ELISA plate; plates were read as described earlier. Sample absorbance readings in each plate were adjusted to a uniform positive control reading of 2.0 at A_{410}; this was done by dividing 2 by the actual positive control absorbance in each plate and then multiplying all sample absorbances by this ratio. The six sample absorbances from each accession were then averaged. An additional sample of 30–50 seeds from each accession was planted and tested for BCMV infection using ELISA:MAb. The relationship between average seed sample ELISA absorbance and percent seedling infection was determined by correlation analysis.

RESULTS

Reliable prediction of seedling infection from immunoassays on individual seedling proved difficult. Discrepancies among results obtained were common when results with flour of individual seeds were compared with those obtained from plants grown from each sampled seed (Table 1). Both false positive and false negative reactions were observed, with false negative results being more common than false positives. However, there were relatively fewer false negatives when ELISA:MAb was used, probably because ELISA:MAb had much lower background absorbances. Some seed samples gave weakly positive reactions in DIA or gave ELISA values slightly less than the positive/negative threshold; these sample results sometimes proved to be false negatives. However, lowering the threshold retrospectively to evaluate all these seed tests as positive served only to increase the incidence of false positives while slightly decreasing the incidence of false negatives. Eleven false negative ELISA:MAb and ELISA:PC results occurred in tests of a single accession, whereas discrepancies in DIA results were randomly distributed among accessions. Of the three immunoassays tested, ELISA:MAb proved to be the most reliable, although 8% of the test results were either false negative or false positive. Consequently, additional seed tests were conducted with ELISA:MAb to determine the source of these discrepancies.

During these additional seed tests, it was not possible to establish a threshold absorbance value that would eliminate or meaningfully reduce the discrepancy between individual seed and seedling tests. However, a relationship exists between the seed ELISA absorbances and the incidence of seedling infection by BCMV (Table 2).

ELISA:MAb on seed parts of 363 seeds from 10 accessions indicates that ELISA-detectable BCMV antigen is erratically distributed within bean seed. Only 43% of the seed either contained antigen in the three seed parts or contained no antigen in any part. More than 53% had detectable antigen only in the seed coat. In one accession, PI 474518, 96% of the seed coats of seed harvested from BCMV-infected plants were ELISA positive, whereas 51% of the cotyledons were ELISA positive; BCMV was seed transmitted to 70% of the seedlings derived from a matching seed sample. In one instance, BCMV was detected only in the primary axis.

The relationship between individual seed ELISA absorbances and seedling infection by BCMV suggested a bulked seed test, based on relatively small seed numbers, was a feasible method of estimating seedling BCMV incidence. When this was tested with seed from 29 accessions, the average ELISA absorbance of six flour samples derived from eight seeds seed each was correlated with the percent infection of seedlings from the same seed source. Although the correlation was stronger when the seed tests were done with ELISA:MAb ($r = 0.92$), a significant correlation ($r = 0.82$) was also observed in ELISA:PC.

Table 1. Comparison of three immunoassays for the detection of bean common mosaic virus (BCMV) in individual bean seeds and their resulting seedlings

Result	Immunoassay[a]			Seedling infection[b]
	ELISA:MAb	ELISA:PC	DIA	
Verified positives	37	26	29	51
Verified negatives	183	187	182	188
Total (%)	220 (92.1)	213 (89.1)	211 (88.3)	239
False positives[c]	4	1	5	···
False negatives[d]	15	25	23	···
Total (%)	19 (7.9)	26 (10.9)	28 (11.7)	···

[a] ELISA:MAb = indirect enzyme-linked immunosorbent assay with a monoclonal antibody; ELISA:PC = indirect enzyme-linked immunosorbent assay with a polyclonal antiserum; and DIA = dot immunoassay with a monoclonal antibody.
[b] Detected with ELISA:MAb 2–3 wk after emergence. A total of 270 seeds were tested, 239 of which gave rise to seedlings (89% germination). Of the 31 nongerminating seeds, seven tested positive for BCMV in ELISA:MAb.
[c] False positive = positive immunoassay result with seeds, negative ELISA result with seedling (healthy plant).
[d] False negative = negative immunoassay result with seed, positive ELISA result with seedling (BCMV-infected plant).

Table 2. Relationship between substrate absorbance in enzyme-linked immunosorbent assay (ELISA) for bean common mosaic virus (BCMV) in individual seeds from 45 Plant Introduction *Phaseolus vulgaris* accessions and seedling infection by BCMV in the same seeds

ELISA A_{410}	Seedling infection		
	No. tested	No. infected	Percent infection
0–0.12	928	6	0.6
0.13–0.25	101	7	6.9
0.26–0.50	55	9	16.4
0.51–1.00	23	16	69.6
1.01–2.00	17	16	94.1
≥2.01	34	32	94.1
Total	1,158[a]	86	$x = 7.4$

[a] A total of 1,350 seeds were tested, 1,158 of which gave rise to seedlings (86% germination).

DISCUSSION

DIA has been reported to have sensitivity and accuracy comparable or superior to that of ELISA in several pathogen-host testing systems (14,23). ELISA, however, proved to be the more accurate technique for testing individual seeds on a wide range of bean genotypes and BCMV strains. The greater accuracy of ELISA is likely attributable to the easier differentiation of reaction by quantitative readings in ELISA with a spectrophotometer. Regardless of the

causes of the decreased accuracy, there seems little reason to use DIA for detection of BCMV in *Phaseolus* spp. seed because DIA tests use greater quantities of immunological reagents, do not yield easily quantifiable results, and require more operator time.

Sutula et al (20) reviewed the interpretation of ELISA results and noted that the effectiveness of positive/negative threshold values can be judged by the frequency of erroneous results. In individual seed tests for BCMV with ELISA:MAb, it was not possible to establish a threshold value without incurring an unacceptably high incidence of discrepancies between seed and seedling results. The erratic distribution of BCMV or ELISA-reactive antigen within bean seed appears to be the primary cause of these discrepancies. False positive seed tests are likely attributable to virus or viral antigen present only in the cotyledon or other seed parts, which is not transmitted to the seedling, whereas false negative tests are presumably attributable to BCMV infection of the primary axis without infection or contamination of the testa or cotyledons. The latter could also be attributable to virus being present but below the concentration that this assay can detect. Infection solely of the primary axis was not observed in other virus-host combinations (2,17,18), but this may be a function of the high rate of BCMV seedling infection and the wide range of host genotypes and BCMV strains associated with accessions in the bean germ plasm collection.

Unrestricted elimination of diseased plants from a germ plasm collection can result in the loss of genetic diversity (1), but up to 10% of a bean germ plasm accession can be discarded before or during seed propagation without significantly reducing genetic diversity, provided this is not done in successive generations. Individual seed tests were contemplated as a means of eliminating BCMV-infected seeds from planting material before propagation, but elimination of seeds testing positively in ELISA would frequently exceed 10% of an accession, even when seedling infection would have been less than 10% because of the prevalence of false positive seed tests. In addition, the presence of false negative seed tests would necessitate seedling tests; this second round of testing is financially and temporally prohibitive.

To prioritize accessions for effective use of facilities in a virus elimination program, it is essential to identify accessions with less than 10% incidence of seedling infection by BCMV. Thus, a testing procedure was devised that used a small number of samples composed of relatively few seeds to identify accessions within the desired limits of seedborne BCMV incidence. Although the relationship between average ELISA absorbance of bulk seed samples and seedling infection was analyzed as linear, ELISA-dose response curves are usually logarithmic and deviations from linearity may be encountered as more of the 10,000 *Phaseolus* accessions are tested. These deviations, however, would most likely occur at incidences much greater or much less than the 10% infection critical value. The testing of small bulk seed samples is not limited to ELISA systems using MAbs, as evidenced by the high degree of correlation obtained in tests with the PC. It may, however, be limited to virus tests using antisera of equal affinity to all virus strains likely to be encountered so that virus titer and antibody affinity effects are not confounded. This procedure may be of special interest to others considering the elimination of a seedborne virus from a germ plasm collection, especially when the number of seed available for testing is not suitable for group testing (5).

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