Serological Detection of Bean Pod Mottle Virus in Bean Leaf Beetles

S. A. Ghabrial and F. J. Schultz

Department of Plant Pathology, University of Kentucky, Lexington 40546.
The authors are indebted to K. V. Yeargan, Department of Entomology, University of Kentucky, for assistance in the collection and identification of leaf-feeding beetles.
Journal Series Paper 82-11-162, University of Kentucky Agricultural Experiment Station.
Accepted for publication 5 October 1982.

ABSTRACT

Ghabrial, S. A., and Schultz, F. J. 1983. Serological detection of bean pod mottle virus in bean leaf beetles. Phytopathology 73:480-483.

Purified bean pod mottle virus (BPMV) serially diluted with buffered saline or extracts from virus-free bean leaf beetles (*Ceratoma trifurcata*) could be detected by enzyme-linked immunosorbent assay (ELISA) at concentrations as low as 2.0 ng/ml. The virus was readily detected by ELISA in virus-containing beetles homogenized and diluted in buffered saline up to dilutions of 1:10,000–1:40,000 (w/v). The ELISA of beetle extracts was highly reliable in predicting the incidence of BPMV in soybean fields in several locations in Kentucky. BPMV apparently accumulates in the beetles during acquisition feeding since the virus titer in the beetles was higher than that in the infected plants on which they have fed. Monitoring BPMV titer in viruliferous beetles following inoculation feeding, however, indicates a decline in virus titer during this period. Because of its sensitivity, reliability, and suitability for large-scale testing, the ELISA for BPMV in beetle extracts should provide a valuable tool for virus surveys and epidemiological studies on BPMV. In addition to detection of BPMV, the ELISA test with beetle extracts could be extended to include other beetle-vectored as well as stable-nonvectored soybean viruses.

Additional key words: soybean mosaic virus, tobacco ringspot virus.

Bean pod mottle virus (BPMV) is widespread in soybeans (*Glycine max* (L.) Merrill) in many of the soybean-growing areas in the United States, particularly in the southern and eastern states (6,9,12,14,18). Soybean yield losses of 10–40% have been reported as a result of BPMV infection (11,14). Double infections of soybean with BPMV and soybean mosaic virus (SMV) occur in nature and result in synergistic host response with severe yield losses (14).

The epidemiology of BPMV is little understood. The virus is transmitted in nature by several chrysomelid beetles. The bean leaf beetle, *Ceratoma trifurcata* Forst, is considered the most efficient vector (8,12,13). BPMV is not seedborne in soybean. Infected perennial, leguminous weeds, including *Desmodium paniculatum* (L.) DC., and overwintering viruliferous adult beetles have been suggested as possible sources of primary virus inoculum (4,10,21). The importance of these sources in virus spread under field conditions has not been evaluated (4).

The recent adaptation of the enzyme-linked immunosorbent assay (ELISA) to plant virus detection (2) has given an impetus to epidemiological studies on virus diseases (1). Because of its sensitivity, versatility, and efficiency in large scale testing, the ELISA method provides a valuable tool for such studies. ELISA was successfully applied for virus detection in aphid, leafhopper, and plant hopper vectors (1,3,5,7).

The present paper reports on the sensitivity and reliability of the ELISA method in detecting BPMV in bean leaf beetles and describes the application of this test in BPMV surveys and forecasting disease incidence.

MATERIALS AND METHODS

Viruses. A subculture of the G-7 Kentucky isolate of BPMV (6) was used for virus purification and in transmission experiments. The virus was maintained and increased in soybean cultivar York. The purification procedure described by Semancik and Bancroft (17) was followed except that final purification was made by centrifugation on sucrose-density gradients (100–400 mg/ml in 0.1 M phosphate buffer, pH 7.0), and the pooled middle and bottom components constituted the purified preparation. Virus concentration in purified preparations was determined spectrophotometrically using $A^{0.1\%}_{260\ nm} = 8.7$ (16).

An isolate of SMV obtained from infected soybean seeds of a plant introduction (PI 85-663) was used. The virus culture was maintained in soybean cultivar Dare.

Beetles. Bean leaf beetles were collected from a soybean field at the University of Kentucky farm in Fayette County near Lexington, KY. Since previous virus surveys (6) failed to detect BPMV in this area, beetles collected from this location were considered virus-free and were used as controls in the serological assays. The nonviruliferous nature of these beetles was later confirmed by transmission and serological tests. Beetles were maintained on cultivar York soybean seedlings until used.

Preparation of beetle extracts. Individual or groups of five beetles were ground in a mortar and pestle in the presence of 0.02 M phosphate buffered saline (PBS) containing 2% polyvinylpyrrolidone (PVP), 0.05% Tween-20, and 0.02% sodium azide (PBS-PVP-T). Bulk beetle samples (25 beetles) were homogenized in PBS-PVP-T with a Polytron homogenizer using a PT-20 generator (Brinkmann Instruments Inc., Westbury, NY 11590). The homogenates were clarified by passage through two layers of cheesecloth. The average weight of a single bean leaf beetle was determined to be 10 mg. The extraction buffer was used at the rate of 0.4–0.5 ml per beetle.

Procedure for ELISA. An antiserum to isolate G-7 of BPMV, produced in a previous study (6), was used for the ELISA tests. Partially purified γ-globulin was obtained from this antiserum by precipitation with half-saturated ammonium sulfate followed by passage through a DEAE cellulose column (2). The γ-globulin preparation (1 mg/ml) was stored at −80 C in PBS containing 0.02% sodium azide. The ELISA test was performed in polystyrene Microelisa plates (Dynatech Corp., Alexandria, VA 22314). The plates were coated with γ-globulin in carbonate buffer, pH 9.6 (2), at 1.0 μg/ml, 200 μl per well. Following a 2–4 hr incubation at 30 C, the test samples were added at a rate of 200 μl per well and the plates were incubated overnight at 4 C. Alkaline phosphatase-labeled γ-globulin, prepared as described by Clark and Adams (2), was used at a dilution of 1:1,000; this corresponds to 1.0 μg/ml, assuming no change in γ-globulin concentration during the conjugation process. The conjugate was added to the wells, 200 μl per well, and the plates were incubated for 4 hr at 30 C. The wells

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.

were rinsed between each step with PBS containing 0.05% Tween and 0.02% sodium azide. The enzyme substrate, *p*-nitrophenyl phosphate (1.0 mg/ml in 10% diethanolamine buffer, pH 9.8), was added at the rate of 200 μl per well and the enzyme-substrate reaction was measured photometrically at 405 nm by using a Titertek Multiskan photometer (Flow Laboratories, McLean, VA 22102). The instrument was routinely blanked against extraction buffer and the plates were read at 15-min intervals during a period of 1 hr.

The ELISA method was also used for detection of SMV and tobacco ringspot virus (TRSV) in beetle and leaf extracts. Coating and enzyme-labeled γ-globulin from an antiserum to SMV were used in the ELISA test at the rate 2.5 μg/ml; those from an antiserum to TRSV were used at a concentration of 1.0 μg/ml. Other details of the ELISA procedures for detection of SMV or TRSV were the same as described for BPMV.

RESULTS

Detection of BPMV by ELISA. Purified BPMV serially diluted with PBS-PVP-T or with extracts from virus-free bean leaf beetles could be detected by ELISA at concentrations as low as 2 ng/ml (Fig. 1). The relationship between ELISA absorbance values and virus concentration was nearly stoichiometric in the range 2.0–100 ng/ml (Fig. 1, incubation time of 30 min). The results of BPMV detection with ELISA were reproducible with three different virus preparations tested on separate dates. Furthermore, comparable ELISA results were obtained for purified virus when extracts from virus-free beetles were used as diluent instead of PBS-PVP-T. The concentration of BPMV antigen in extracts from virus-containing beetles may, thus, be quantitated by interpolation in a standard curve made with a series of purified virus dilutions. To test the accuracy of ELISA in quantitative assays, known amounts of purified BPMV were mixed with extracts from virus-containing beetles, and the viral antigen concentration in both the beetle extracts and the mixtures were determined by quantitative ELISA. The results showed excellent agreement between expected and test values for antigen concentration in the mixtures (Table 1).

Assay of bean leaf beetles for BPMV. Bean leaf beetles were collected by cooperators from soybean fields in two counties (Fulton and Meade) in Kentucky. Nine samples (five beetles each) from each location were tested for BPMV by ELISA. All beetle samples from Fulton County were positive for BPMV whereas those from Meade County were negative. Subsequent ELISA tests with soybean leaves collected from the same fields sampled for beetles confirmed the incidence of BPMV in the first location and the absence of it in the second. In further tests, we collected bean leaf beetles from several soybean fields in three counties in Kentucky. Soybean leaf samples were also collected from the same rows of plants sampled for beetles. In all tests, the ELISA with beetle extracts was highly reliable in predicting the incidence of BPMV in soybean fields from which the beetles were collected. Higher ELISA values were obtained with bulk beetle samples (25 beetles each) from a soybean field with apparently 100% BPMV-infected plants than with those from fields with lower incidence of BPMV. Although ELISA tests for SMV with leaf samples from two counties indicated the incidence of SMV and/or double infections with SMV and BPMV in some of the surveyed soybean fields, extracts from all corresponding beetle samples were negative for SMV.

The beetle extracts could be stored for several months without deleterious effects on detection of BPMV by ELISA. Furthermore, the ELISA absorbance values were virtually unchanged when subsamples of the extracts were tested after 6 mo of storage at 4 C.

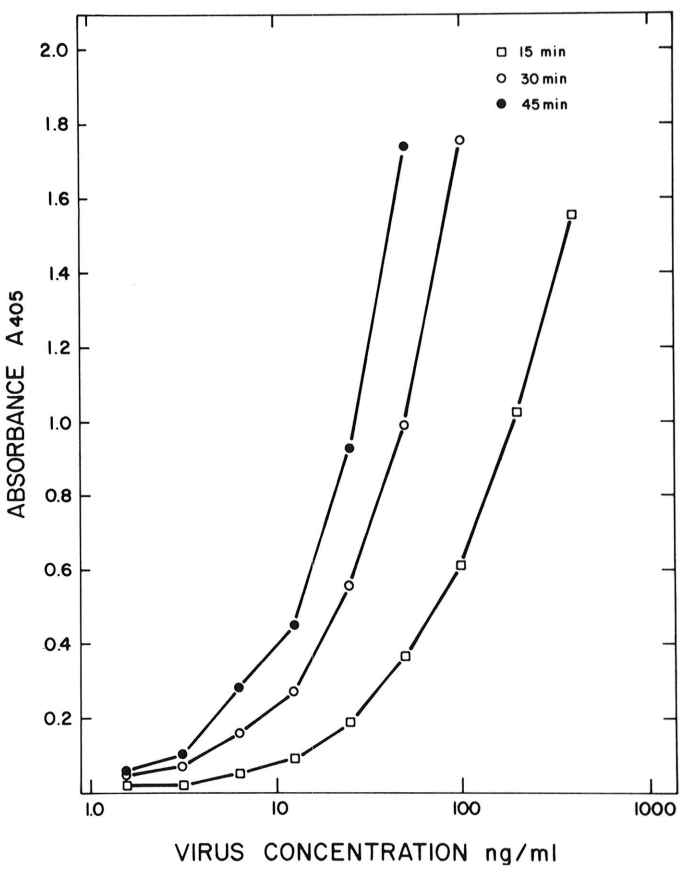

Fig. 1. Relationship between enzyme-linked immunosorbent assay absorbance values at 405 nm and virus concentration in a purified bean pod mottle virus preparation serially diluted with buffered saline (PBS-PVP-T). Absorbance measurements were made at 15-min intervals following substrate addition.

TABLE 1. Reliability of enzyme-linked immunosorbent assay (ELISA) test for quantitative determination of bean pod mottle viral antigen in extracts from bean leaf beetles

	ELISA absorbance value ($A_{405\ nm}$)[a]		Viral antigen concentration (ng/ml)[b]		
				Beetle extract + purified virus[d]	
Replication	Beetle extract alone[c] (1:5,000, w/v)	Beetle extract +[d] purified virus	Beetle extract alone[c] (1:5,000, w/v)	Test	Expected
1	0.881 ± 0.053[e]	1.086 ± 0.071	14.0	17.9	17.6
2	0.764 ± 0.023	0.980 ± 0.063	12.7	16.0	16.4
3	0.925 ± 0.054	1.175 ± 0.028	15.0	19.5	18.5
Means			13.9	17.8	17.5

[a] ELISA readings of 0.009–0.020 were obtained for extracts from virus-free beetles tested in the same plate.
[b] Determined by interpolation in a standard curve made with purified BPMV in the range of 1.5–400 ng/ml.
[c] Original extract (1:50, w/v) was prepared by homogenizing virus-containing beetles in PBS-PVP-T (0.5 ml per beetle) and three samples were withdrawn and individually diluted 100-fold with PBS-PVP-T prior to test.
[d] Samples from the same extract described in footnote "c" were diluted 1:5,000 (w/v) and mixed with purified BPMV (50 ng/ml) at a ratio of 9:1 (v/v).
[e] Values are means for quadruplet wells ± standard deviation; readings made 45 min after addition of substrate.

In addition to bean leaf beetles, spotted cucumber beetles, *Diabrotica undecimpunctata*, were collected from these soybean fields, but in fewer numbers. Extracts from these beetles also contained the BPMV antigen and gave ELISA values comparable to those from bean leaf beetles.

The BPMV antigen concentration in field collected virus-containing bean leaf beetles was determined by quantitative ELISA and was in the range of 500–1,500 ng per beetle. These results were based on tests with three samples of 25 beetles each collected from a soybean field with 100% BPMV infection. Lower ELISA values (corresponding to 50–300 ng BPMV per beetle) were obtained when individual virus-free bean leaf beetles were allowed to feed on caged BPMV-infected soybean plants for 14 days.

TABLE 2. Decline in bean pod mottle virus (BPMV) titer in viruliferous bean leaf beetles during a 6-day inoculation feeding period as determined by enzyme-linked immunosorbent assay (ELISA)

Inoculation feeding (days)	Extract dilution (w/v)	ELISA absorbance ($A_{405\ nm}$)[a] for beetle sample no. 1	2	3
0[a]	1:400	>2.0[b]	>2.0	>2.0
	1:4,000	0.836	1.336	1.084
	1:10,000	0.336	0.546	0.424
	1:40,000	0.077	0.123	0.091
6[c]	1:100	1.724	>2.0	0.196
	1:1,000	0.260	0.267	0.064
	1:10,000	0.016	0.037	0.024
Control[d]	1:400	0.030	···	···

[a]Three samples of 25 beetles, each collected from a soybean field with apparently 100% BPMV infection, were homogenized in PBS-PVP-T (0.4 ml per beetle) and several dilutions of the extracts were tested by ELISA.
[b]Values are means for triplicate wells; readings were made 60 min after addition of substrate.
[c]Three samples of five beetles each, obtained from the same source described in footnote "a," were allowed to feed on three healthy cultivar Essex soybean plants for 6 days, then homogenized and diluted with PBS-PVP-T.
[d]Virus-free beetles homogenized and diluted with PBS-PVP-T.

TABLE 3. Assay of virus source plants, test plants, and bean leaf beetles for soybean mosaic virus (SMV) and bean pod mottle virus (BPMV) by the ELISA method

Experiment	Virus tested	Treatments	Rep	ELISA absorbance values ($A_{405\ nm}$) for extract from[a] Source plants	Beetles	Test plants
1[b]	SMV	Single infection		1.898[c]	0.005	···
		Control[d]		0.018	0	···
	BPMV	Single infection		1.085	>2.0	···
		Control		0.022	0.056	···
2[e]	SMV	Double infection	1	1.949	0.022	0.003
			2	1.705	0.033	0.008
		Control		0.002	0.025	···
	BPMV	Double infection	1	>2.0	1.411	1.840
			2	1.896	1.436	1.859
		Control		0.001	0.021	-

[a]ELISA plates were read at 30 and 15 min following substrate addition in experiment 1 and 2, respectively.
[b]Virus-free bean leaf beetles were allowed to feed on either SMV- or BPMV-infected cultivar Essex soybean plants for 72 hr; five beetles were used per plant. Extracts from beetles and source plants were both prepared in PBS-PVP-T at a ratio of 1:50 (w/v).
[c]Values are averages for duplicate wells.
[d]Virus-free beetles were allowed to feed on noninoculated cultivar Essex soybean plants for periods comparable to the virus treatment.
[e]Virus-free bean leaf beetles were allowed an acquisition feeding period of 90 hr on soybean cultivar Essex plants doubly infected with SMV and BPMV, then transferred to healthy soybean seedling (test plants) and allowed to feed for 72 hr. All extracts were prepared in PBS-PVP-T at a ratio of 1:100 (w/v).

Decline in BPMV titer in bean leaf beetles following acquisition. Bean leaf beetles collected from a soybean field with apparently 100% BPMV-infected plants and presumed to be viruliferous were divided into two groups. Samples of 25 beetles each from one group were homogenized in PBS-PVP-T (0.4 ml per beetle) and the extracts were stored at 4 C until used. Beetles in the second group were allowed to feed on three healthy soybean seedlings for 6 days (five beetles per plant) prior to homogenization. Extracts from the group allowed a 6-day inoculation feeding had a much reduced BPMV titer compared to extracts from the group that was processed immediately after virus acquisition (Table 2). All three test plants on which the viruliferous beetles had fed became infected with BPMV as evidenced by symptom development and ELISA tests.

Assay of beetle extracts for SMV and TRSV. The SMV antigen could not be detected by ELISA in extracts from bean leaf beetles that fed for 72 hr on SMV-infected soybean plants (Table 3). Furthermore, bean leaf beetles that had fed on soybean plants doubly infected with SMV and BPMV transmitted only BPMV to healthy soybean plants. The BPMV antigen, but not the SMV antigen, was detected by ELISA in extracts from such beetles (Table 3). It is of interest that, in the case of single infection with BPMV (Table 3), the beetle extracts had higher virus titers than extracts from the plants on which the beetles had fed. These results were reproduced in a second experiment in which virus-free beetles were allowed 48 hr of acquisition feeding on BPMV-infected soybean plants. The ELISA values for the beetle extracts were two- to threefold higher than those for plant extracts.

Unlike SMV, TRSV was readily detected by ELISA in extracts from bean leaf beetles that fed on soybean plants singly infected with TRSV or plants doubly infected with TRSV and BPMV. The ELISA test was highly sensitive since TRSV was readily detected in beetles homogenized in PBS-PVP-T and diluted 1:10,000 (w/v).

DISCUSSION

BPMV can be readily detected in beetle extracts at dilutions as high as 1:10,000–1:40,000 (w/v). Since the average weight of a single bean leaf beetle is 10 mg and the extraction buffer is used at a ratio of 0.4 ml per beetle, these dilutions (1:10,000 and 1:40,000) simulate extracts of beetle samples with the equivalent of one virus-containing beetle homogenized with 250 and 1,000 virus-free beetles, respectively. A comparable level of sensitivity for ELISA may also be deduced from the results of quantitative ELISA with field-collected virus-containing bean leaf beetles since values for viral antigen concentration of 500–1,500 ng per beetle were determined and since the lower limit of detection of BPMV by ELISA is 2 ng/ml.

ELISA was previously used for virus detection in aphid, leafhopper, and plant hopper vectors (1,3,5,7). This study is the first report of the detection of a beetle-transmitted virus in its vector by ELISA. The ELISA test with beetle extracts was highly reliable in predicting the incidence of BPMV in soybean fields in several locations in Kentucky. It should provide a valuable tool for virus surveys and epidemiological studies on BPMV. Collection of beetles, rather than leaf samples, is much more efficient and less time-consuming since obtaining representative leaf samples requires close examination of plants for disease symptoms.

The presence of inhibitors in bean leaf beetle hemolymph was reported to interfere with virus assay by infectivity tests (15). The components of beetle extracts, however, did not interfere with the specific reaction in the ELISA test for BPMV. Beetle extracts could be stored for several months at 4 C without affecting the ELISA readings. This may be important in handling or transporting a large number of samples. Furthermore, ELISA values for extracts from bulk beetle samples may serve as an indicator to the extent of BPMV incidence in a given location.

BPMV has been reported to survive in overwintering adult bean leaf beetles (22). Alternatively, such beetles may acquire the virus from perennial leguminous weeds, which serve as a reservoir for

BPMV (10). These overwintering beetles become active well before soybeans germinate, and can be found on other leguminous crops (20) and possibly on leguminous weeds (10). Since BPMV has a limited host range and some of the leguminous crops colonized or fed upon by beetles (20) are not hosts for BPMV, testing of plant material for BPMV may not be conclusive. Collection of beetles from leguminous crops and weeds prior to soybean planting and testing for BPMV by ELISA would provide a simple and efficient means for understanding the epidemiology of BPMV in a given soybean-growing area and may aid in forecasting the use of insecticidal sprays for beetle control on soybean earlier in the growing season.

The BPMV titer in extracts from bean leaf beetles was higher than that in extracts from infected tissues on which beetles had fed. Similar observations were reported with the cowpea strain of southern bean mosaic virus (CP-SBMV). The concentration of CP-SBMV could reach higher levels in the regurgitant of bean leaf beetles than that in sap from infected tissues (4). Although these findings suggest virus accumulation in beetles during acquisition feeding, a decline in BPMV titer in viruliferous beetle extracts was detected following inoculation feeding (Table 2). Likewise, a decline in CP-SBMV activity in beetle regurgitant was reported (4).

The objective of testing beetle extracts for the presence of SMV and TRSV, viruses not vectored by beetles, was to expand the ELISA test for beetle extracts to include other economically important soybean viruses. The ability to detect TRSV, but not SMV, by ELISA in extracts from beetles that fed on infected plants probably reflects differences in stability between these two viruses and suggests that SMV was modified or degraded beyond serological recognition in the beetle digestive tract. Successful detection of TRSV by ELISA in beetle extracts may not be surprising since it was possible to detect the virus by infectivity assays in regurgitant from bean leaf beetles that fed on infected plants (19). In addition to the nonvectored TRSV, the beetle-vectored cowpea mosaic and cowpea chlorotic mottle viruses are obvious candidates to be included in ELISA tests with beetle extracts.

LITERATURE CITED

1. Bar-Joseph, M., and Garnsey, S. M. 1981. Enzyme-linked immunosorbent assay (ELISA): Principles and applications for diagnosis of plant viruses. Pages 35–59 in: Plant Diseases and Vectors: Ecology and Epidemiology. K. Maramorosch and K. F. Harris, eds. Academic Press, New York.
2. Clark, M. F., and Adams, A. N. 1976. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
3. Clarke, R. G., Converse, R. H., and Kajma, M. 1980. Enzyme-linked immunosorbent assay to detect potato leafroll virus in potato tubers and viruliferous aphids. Plant Dis. 64:43-45.
4. Fulton, J. P., Scott, H. A., and Gamez, R. 1980. Beetles. Pages 115–132 in: Vectors of Plant Pathogens. K. F. Harris and K. Maramorosch, eds. Academic Press, New York.
5. Gera, A., Loebenstein, G., and Raccah, B. 1978. Detection of cucumber mosaic virus in viruliferous aphids by enzyme-linked immunosorbent assay (ELISA). Virology 86:542-545.
6. Ghabrial, S. A., Pickard, C. M., and Stuckey, R. E. 1977. Identification and distribution of virus diseases of soybean in Kentucky. Plant Dis. Rep. 61:690-694.
7. Hibino, H., and Kimura, I. 1982. Detection of rice ragged stunt virus in insect vectors by enzyme-linked immunosorbent assay. Phytopathology 72:656-659.
8. Horn, N. L., Newsom, L. D., Carver, R. G., and Jensen, R. L. 1970. Effects of virus diseases on soybeans in Louisiana. Louisiana Agric. 13:12.
9. Horn, N. L., Newsom, L. D., and Jensen, R. L. 1973. Economic injury thresholds of bean pod mottle and tobacco ringspot virus infection of soybean. Plant Dis. Rep. 57:811-813.
10. Moore, B. J., Scott, H. A., and Walters, H. J. 1969. *Desmodium paniculatum*, a perennial host of bean pod mottle virus in nature. Plant Dis. Rep. 53:154-155.
11. Myhre, D. L., Pitre, H. N., Haridasan, M., and Hesketh, J. D. 1973. Effect of bean pod mottle virus on yield components and morphology of soybeans in relation to soil water regimes: A preliminary study. Plant Dis. Rep. 57:1050-1054.
12. Patel, V. C., and Pitre, H. N. 1971. Transmission of bean pod mottle virus to soybean by the striped blister beetle, *Epicauta vittata*. Plant Dis. Rep. 55:628-629.
13. Ross, J. P. 1963. Transmission of bean pod mottle virus in soybean by beetles. Plant Dis. Rep. 47:1044-1050.
14. Ross, J. P. 1968. Effect of single and double infections of soybean mosaic and bean pod mottle viruses on soybean yields and seed characters. Plant Dis. Rep. 52:344-348.
15. Scott, H. A., and Fulton, J. P. 1978. Comparison of the relationship of southern bean mosaic virus and the cowpea strain of tobacco mosaic virus with the bean leaf beetle. Virology 84:197-199.
16. Semancik, J. S. 1972. Bean pod mottle virus. No. 108 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
17. Semancik, J. S., and Bancroft, J. B. 1965. Stability differences between the nucleoprotein components of bean pod mottle virus. Virology 27:476-483.
18. Sinclair, J. B. 1982. Compendium of Soybean Diseases. 2nd ed. Am. Phytopathol. Soc., St. Paul, MN.
19. Slack, S. A., and Fulton, J. P. 1971. Some plant virus-beetle vector relations. Virology 43:728-729.
20. Waldbauer, G. P., and Kogan, M. 1976. Bean leaf beetles: Bionomics and economic roles in soybean agroecosystems. Pages 619–628 in: World Soybean Research Proc., World Soybean Res. Conf. 1975. L. D. Hill, ed. Interstate Printers and Publishers, Danville, IL.
21. Walters, H. J., and Lee, F. N. 1969. Transmission of bean pod mottle virus from *Desmodium paniculatum* to soybean by the bean leaf beetle. Plant Dis. Rep. 53:411.
22. Walters, H. J., Lee, F. N., and Jackson, K. E. 1972. Overwintering of bean pod mottle virus in bean leaf beetles. (Abstr.) Phytopathology 62:808.