Enzyme-Linked Immunosorbent Assay of Viruses Infecting Forage Legumes

M. R. McLaughlin, O. W. Barnett, P. B. Gibson and P. M. Burrows

Former visiting assistant professor, Department of Plant Pathology and Physiology; professor, Department of Plant Pathology and Physiology; research agronomist, U. S. Department of Agriculture, Agricultural Research Service, Department of Agronomy; and professor, Experimental Statistics Unit; Clemson University, Clemson, SC 29631. Present address of senior author: research plant pathologist, U. S. Department of Agriculture, Agricultural Research Service, Crop Science Research Laboratory, Department of Plant Pathology and Weed Science, Mississippi State University, P. O. Drawer PG, Mississippi State 39762.

We thank the cooperators mentioned in the text and are grateful for the technical assistance of Flora King, Tracy Zimmerman, Dick Baker, and Louis Jackson. Our research was sponsored by the South Carolina Agricultural Experiment Station and the USDA Agricultural Research Service, under Cooperative Agreement 12-14-7001-1134, and contributed to Regional Research Project S-127, Forage Legume Viruses. Technical Contribution 2063 of the South Carolina Agricultural Experiment Station, Clemson University.

Mention of a trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture and does not imply approval to the exclusion of other products or vendors that may also be suitable.

Accepted for publication 23 March 1984.

ABSTRACT

McLaughlin, M. R., Barnett, O. W., Gibson, P. B., and Burrows, P. M. 1984. Enzyme-linked immunosorbent assay of viruses infecting forage legumes. Phytopathology 74:965-969.

Enzyme-linked immunosorbent assay (ELISA) was adapted for detection of alfalfa mosaic, bean yellow mosaic (BYMV), clover yellow mosaic, clover yellow vein (CYVV), peanut stunt, red clover vein mosaic and white clover mosaic viruses. ELISA was versatile, practical and reliable in indexing forage legumes for viruses, and also differentiated between BYMV and CYVV. ELISA was well suited for large-scale screening programs, and its potential was further extended by mailing sensitized ELISA plates between cooperating laboratories.

Symptom expression in virus-infected forage legumes may vary from no visible symptoms to severe mosaics with stunting, leaf deformation, and necrosis (3,4,25). Symptoms caused by different viruses may be similar and symptoms caused by multiple infections may be indistinguishable from those caused by single infections. Indexing methods using indicator hosts (2,10,18), immunodiffusion tests (14), and serologically specific electron microscopy (11) are reliable; however, the need for a simpler and faster means of detecting and identifying several viruses and indexing large numbers of plants led us to examine the enzyme-linked immunosorbent assay (ELISA) (7,28,29).

In this paper, we describe use of several sample buffer additives to minimize nonspecific positive ELISA reactions. We also describe experiments to discriminate between the closely related viruses (bean yellow mosaic virus [BYMV] and clover yellow vein virus [CYVV]) that infect many of the same forage legume species, are serologically closely related, and are difficult to differentiate (14,16). The use of ELISA as a survey tool involving the mailing of sensitized test plates between researchers in different states is also described. Abstracts of this research have been published (5,21–23).

MATERIALS AND METHODS

Antisera sources. Antisera to alfalfa mosaic virus (AMV), clover yellow mosaic virus (CYMV), CYVV, and white clover mosaic virus (WCMV) were produced by O. W. Barnett (2). Antiserum to BYMV-Ky 204-1 (14) was kindly provided by T. P. Pirone (University of Kentucky, Lexington), that to peanut stunt virus (PSV, B-2) by S. A. Tolin (Virginia Polytechnic Institute and State University, Blacksburg), and that to red clover vein mosaic virus (RCVMV) by R. O. Hampton (USDA, ARS at Oregon State University, Corvallis). Conjugated antibody preparations of RCVMV were cross-absorbed with sap from healthy white clover plants extracted (5%, w/v) in PBS-Tween buffer (0.02 M phosphate, 0.15 M NaCl, 0.003 M KCl, pH 7.3, containing 0.05% Tween-20). Absorbed conjugate was incubated 1 hr at 5 C and centrifuged at 6,500 *g* for 15 min before use in ELISA.

ELISA. The double antibody sandwich ELISA (7) was used with modifications (20,24). Optimum concentrations of coating and conjugate were determined experimentally for each antiserum. Quantitative assays were as described (6,24). Tests with optical density values (400 nm wavelength) more than double the negative controls were scored positive. Qualitative tests were judged by visual comparison with appropriate positive and negative controls.

Comparative tests. Plant tissue samples were ground with mortar and pestle in PBS-Tween, squeezed through cheesecloth, and aliquots were tested by ELISA and on indicator hosts. *Phaseolus vulgaris* L. 'Bountiful' was used for detection of AMV (necrotic local lesions) and WCMV (chlorotic local lesions and systemic mosaic), *Chenopodium amaranticolor* Coste & Reyn. for BYMV and CYVV (necrotic local lesions), *C. quinoa* Willd. for CYMV (chlorotic local lesions and systemic mosaic), and *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Blackeye' for PSV (chlorotic local lesions, systemic vein clearing, and mosaic). Indicator hosts were inoculated and maintained in the greenhouse or in plant growth chambers (16-hr day, 11,000 lux, 30 C; 20 C night), and symptom development was observed for 1–3 wk.

In comparisons of ELISA with latex agglutination tests (LAT) (1), we used infected plant tissue or purified preparations of CYMV, CYVV, or WCMV in PBS-Tween buffer containing 2% (w/v) polyvinylpyrrolidone (PVP, 40,000 MW).

Sample buffer additives. Purified preparations of CYVV and WCMV were mixed with buffer and with buffer homogenates of healthy red or white clover. Leaf tissue was ground 1:4 (w/v) in PBS-Tween with or without various combinations of 2.0% (w/v) PVP, 0.02 M sodium diethyldithiocarbamate (NaDIECA), and 0.02 M 2-mercaptoethanol (2-ME). Effects of additives were determined by using a 2^4 factorial set of treatments arranged in balanced 4 × 4 lattice square designs with five replications. Treatment means were adjusted for row and column effects in squares (6,24). Concurrent experiments were conducted for both viruses with buffer alone, buffer containing white clover sap, and buffer containing red clover sap.

Mailing ELISA plates. Polystyrene plates were sensitized with

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.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1984.

antibody and rinsed with PBS-Tween. A lid (spent plate) was placed on each. Plates were sealed inside plastic bags containing moist paper towels and shipped via first class mail to cooperators. Healthy and infected plant tissues, either fresh or freeze-dried, were included with each shipment along with test reagents and instructions. Cooperators prepared leaf samples and incubated the charged plates (with lids, inside plastic bag moist chambers) overnight at 5 C. Plates were rinsed with cold tap water, covered, resealed in plastic bags and returned by mail for completion of ELISA. Initial tests of this procedure were made in cooperation with T. P. Pirone, Lexington, KY. Subsequent tests involved the cooperation of E. M. Clark and W. C. Johnson, Auburn, AL; B. G. Harville, Baton Rouge, LA; J. P. Meiners, Beltsville, MD; R. G. Pratt and W. E. Knight, Mississippi State, MS; W. A. Cope and C. L. Campbell, Raleigh, NC; S. A. Tolin and S. Boatman, Blacksburg, VA; D. P. Maxwell, Madison, WI; and K. T. Leath, University Park, PA.

Detection of BYMV and CYVV in mixtures. Purified BYMV (Ky 204-1) and CYVV (Pratt) were tested against homologous and heterologous antisera. Viruses at 0.1, 1, 5, 10, 25, 50, 75, 100, 125, 250, 500, and 1,000 ng/ml in PBS-Tween were tested in parallel experiments with each antiserum by using 5 × 5 balanced lattice square designs with 25 treatments (twelve concentrations of each virus and one buffer control) and six replications. Corresponding replicate squares of the parallel experiments were done on opposite halves of the same polystyrene plates, ie, BYMV antiserum on one side and CYVV antiserum on the other. Treatment means were adjusted for row and column effects in squares and plotted as a function of virus concentration (Fig. 1).

Purified preparations of BYMV (Ky 204-1) and CYVV (Pratt) were tested in all combinations of concentrations 0, 250, 500, 1,000, and 2,000 ng/ml against BYMV and CYVV antisera in a 7 × 7 lattice square design with 49 treatments and four replications. Antisera to BYMV and CYVV were combined for tests of 0 ng/ml of both BYMV and CYVV, thus reducing the number of treatment combinations from 50 to 49.

TABLE 1. Comparison of symptom expression, indicator host plant assay, and enzyme-linked immunosorbent assay (ELISA) for detection of alfalfa mosaic virus (AMV) in *Trifolium repens*

Source plants		AMV detected			
		ELISA		Host assay[a]	
Symptoms	No.	+	−	+	−
Mosaic	69	63	6	63	6
Indistinct	18	9	9	8	10
None	43	17	26	17	26
Total	130	89	41	88	42

[a] Mechanical inoculation of local lesion host, *Phaseolus vulgaris* 'Bountiful.' All AMV infections detected by this method were also detected by ELISA.

TABLE 2. Comparison of indicator host plant assay with enzyme-linked immunosorbent assay (ELISA) for detection of alfalfa mosaic virus (AMV), clover yellow vein virus (CYVV), peanut stunt virus (PSV), and white clover mosaic virus (WCMV) in 200 plants of *Trifolium repens*

	Infected plants detected (no.)			
Virus	Total	ELISA	Host[a]	Discrepancies
AMV	13	13	11	2[b]
CYVV	54	50	52	6[c]
PSV	112	97	110	17[d]
WCMV	28	20	18	18[e]

[a] Mechanical inoculation of *Phaseolus vulgaris* 'Bountiful,' *Chenopodium armaranticolor*, and *Vigna unguiculata* subsp. *unguiculata* 'California Blackeye.'
[b] Two infections detected by ELISA only.
[c] Two infections detected by ELISA only, four by host only.
[d] Two infections detected by ELISA only, 15 by host only.
[e] Ten infections detected by ELISA only, eight by host only.

Motorized sap extractor. A motorized "leaf squeezer" similar to machines developed in North Dakota (12) and Europe (8) was constructed. Leaves were passed between motorized counter-rotating stainless steel rollers wetted with sample buffer from a wash bottle, and sap was collected by rinsing with additional sample buffer. To prevent virus contamination between samples, rollers were rinsed 7 sec with cold tap water and blotted dry with paper towelling. This standard method was used for all qualitative ELISA. Subsequent to this research, similar machines became commercially available (Piedmont Machine and Tool, Inc., Box 109, Six Mile, SC 29682).

RESULTS

Comparative tests. Sap from virus-infected greenhouse plants tested by ELISA and indicator hosts, respectively, had dilution end points as follows: AMV (white clover), 10^{-5} and 10^{-2}; BYMV (alsike clover), 10^{-3} and 10^{-3}; CYMV (white clover), at least 10^{-6} and 10^{-5}; CYVV (white clover), 10^{-2} and 10^{-2}; WCMV (Alaska pea), 10^{-4} and at least 10^{-7}; and WCMV (Bountiful bean), 10^{-3} and 10^{-5}. Results of indicator host assays and ELISA for AMV, CYVV, PSV, and WCMV in *T. repens* are shown in Tables 1 and 2.

In ELISA and LAT with purified virus, we detected CYVV at 100 ng/ml with ELISA and 10 μg/ml with LAT. The lower limits of virus detection under optimum routine ELISA conditions were 120 ng/ml for BYMV and 42 ng/ml for CYVV (Fig. 1).

The reliability of ELISA compared with indicator host assays and LAT in detecting viruses from field-grown clovers, was also tested. Host assays and ELISA results agreed for 23 of 27 plants infected with BYMV, CYVV, WCMV, or combinations of these viruses. In two plants BYMV was detected by ELISA only, and in two others WCMV was detected by host assay only. We detected 15 BYMV/CYVV-type infections by host assays but could not distinguish single and mixed infections, whereas we detected 17 such infections by ELISA and differentiated seven BYMV, one CYVV, and nine mixed. Results of LAT, done for CYVV and WCMV only, agreed with ELISA results for all but one plant, in which we detected WCMV by LAT only.

Sample buffer additives. Results are shown in Tables 3 and 4. Generally, plant sap in the sample buffer decreased the sensitivity of ELISA. Red clover sap generally had a greater effect than white clover sap. Strong nonspecific reactions sometimes observed in ELISA of WCMV in field-grown red clover did not occur, perhaps because we used fresh tissue from greenhouse-grown plants. The presence of NaDIECA in the sample buffer generally improved the

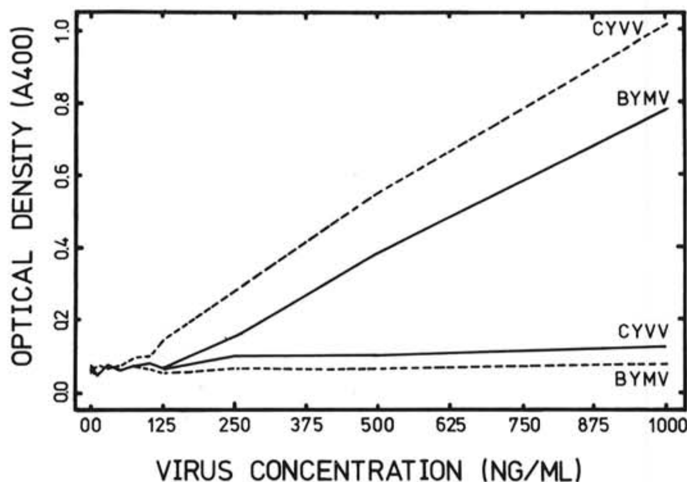

Fig. 1. Reactivity of bean yellow mosaic virus (BYMV) and clover yellow vein virus (CYVV) with BYMV antiserum (solid lines, standard error per adjusted mean = 0.02) and with CYVV antiserum (dashed line, standard error per adjusted mean = 0.01) in ELISA. Test amounts were 100 μl of reactants per well; coating and conjugated antibody concentrations were 2.5 μg/ml and 1.25 μg/ml, respectively. The experimental design is described in the text.

ELISA results, whereas, other additives and combinations gave erratic results.

Mailing ELISA plates. In an initial test with BYMV, CYVV, and WCMV, no adverse effects due to mailing were observed. Samples containing virus were accurately identified in all but one instance in blind tests. In one instance, soybean mosaic virus (SMV) reacted so strongly with CYVV antiserum that the test was interpreted as positive for CYVV. During the following 14 mo, 142 plates were sent in 25 mailings to cooperators in nine states (Table 5). A total of 2,748 plant samples were indexed for three to seven viruses for a total of 13,649 virus tests; 1,142 of these were positive.

Infected fresh plant material withstood mailing well enough for use as virus control samples, but some deterioration occurred when the material remained in the mail longer than 2 or 3 days, especially during the warmer months. Freeze-dried sap used to avert this problem was prepared in PBS-Tween-NaDIECA buffer and reconstituted in distilled water. If reconstituted in PBS-Tween buffer, the antigenicity of some viruses, especially AMV, was severely reduced.

Detection of BYMV and CYVV in mixtures. Low optical density

TABLE 3. Effect of additives in sample-grinding buffer on detection of clover yellow vein virus (CYVV) by enzyme-linked immunosorbent assay (ELISA)

	Adjusted mean optical density (400 nm)					
	Experiment I[a] (buffer only)		Experiment II[a] (buffer + white clover sap)		Experiment III[a] (buffer + red clover sap)	
Buffer + additives[b]	Virus[c]	No virus	Virus	No virus	Virus	No virus
PBS+Tw	1.34	0.03	1.04	0.04	0.09	0.10
PBS+Tw+M	1.45	0.05	1.14	0.04	0.45	0.05
PBS+Tw+D	1.38	0.09	1.23	0.02	0.40	0.08
PBS+Tw+P	0.66	0.00	0.68	0.06	0.20	0.06
PBS+Tw+M+D	1.44	0.06	1.29	0.02	0.19	0.04
PBS+Tw+M+P	0.98	0.02	0.52	0.00	0.43	0.03
PBS+Tw+D+P	0.94	0.04	0.84	0.08	0.41	0.04
PBS+Tw+M+D+P	1.09	0.05	0.89	0.05	0.28	0.03
S.E.	±0.04		±0.03		±0.02	

[a] Balanced 4 × 4 lattice square design with five replications for each 2^4 factorial experiment.
[b] Phosphate-buffered saline (0.02 M phosphate, 0.15 M NaCl, 0.003 M KCl, pH 7.3) containing 0.05% Tween-20 (PBS-Tw); 0.02 M 2-mercaptoethanol (M); 0.02 M sodium diethyldithiocarbamate (D); and 2.0% polyvinylpyrrolidone, 40,000 MW (P).
[c] Purified CYVV, 50 μg/ml, 200-μl sample per test well.

TABLE 4. Effect of additives in sample-grinding buffer on detection of white clover mosaic virus (WCMV) by enzyme-linked immunosorbent assay (ELISA)

	Adjusted mean optical density (400 nm)					
	Experiment I[a] (buffer only)		Experiment II[a] (buffer + white clover sap)		Experiment III[a] (buffer + red clover sap)	
Buffer + additives[b]	Virus[c]	No virus	Virus	No virus	Virus	No virus
PBS+Tw	0.15	0.05	0.18	0.04	0.17	0.05
PBS+Tw+M	0.21	0.06	0.14	0.06	0.10	0.05
PBS+Tw+D	0.15	0.05	0.28	0.06	0.27	0.06
PBS+Tw+P	0.16	0.05	0.18	0.06	0.20	0.05
PBS+Tw+M+D	0.21	0.06	0.12	0.05	0.15	0.05
PBS+Tw+M+P	0.21	0.06	0.10	0.05	0.08	0.05
PBS+Tw+D+P	0.15	0.06	0.19	0.05	0.14	0.04
PBS+Tw+M+D+P	0.21	0.06	0.13	0.05	0.07	0.05
S.E.	±0.006		±0.009		±0.008	

[a] Balanced 4 × 4 lattice square design with five replications for each 2^4 factorial experiment.
[b] Phosphate-buffered saline (0.02 M phosphate, 0.15 M NaCl, 0.003 M KCl, pH 7.3) containing 0.05% Tween-20 (PBS-Tw); 0.02 M 2-mercaptoethanol (M); 0.02 M sodium diethyldithiocarbamate (D); and 2.0% polyvinylpyrrolidone, 40,000 MW (P).
[c] Purified WCMV, 50 μg/ml, 200-μl sample per test well.

TABLE 5. Summary of 'ELISA by mail' from June, 1978 through August, 1979

			Virus infections detected (no.)/no. tests[a]							
State	Mailings (no.)	Plates (no.)	AMV	BYMV	CYMV	CYVV	PSV	RCVMV	WCMV	Total
AL	2	8	0/70	0/70	0/70	4/70	nt	nt	3/274	7/554
KY	2	8	0/772	12/84	0/72	0/84	3/72	0/72	0/84	15/540
LA	6	18	2/130	3/130	0/60	159/456	94/456	30/196	148/386	436/1,814
MD	1	7	0/77	30/77	0/77	8/77	11/77	0/77	0/77	49/539
MS	5	31	0/651	259/867	0/111	16/867	13/797	0/104	0/111	288/3,508
NC	3	20	10/378	6/378	0/238	59/378	43/378	0/238	17/378	135/2,366
PA	2	3	5/31	4/31	0/17	3/31	0/31	2/31	0/31	14/203
VA	2	18	7/202	18/202	0/202	50/202	24/202	0/202	14/202	113/1,414
WI	2	29	16/449	21/449	0/449	14/449	10/449	24/449	0/17	85/2,711
Total	25	142	40/2,060	353/2,288	0/1,296	313/2,614	198/2,462	56/1,369	182/1,560	1,142/13,649

[a] Samples included various species of forage and other legumes collected in nature, from experimentally inoculated and naturally infected plants in the field and from greenhouse collections; "nt" means "not tested."

values were observed in heterologous ELISA of BYMV and CYVV (Fig. 1), thus allowing the determination of heterologous reaction limits. When mixtures of BYMV and CYVV at known concentrations were tested and the results interpreted based on the heterologous reaction limits, accurate interpretations were possible in 88 of 98 tests. Inaccurate interpretations were associated with relatively high concentrations of BYMV in the absence of CYVV (when reactions were mistakenly interpreted as due to virus mixtures) and with relatively high concentrations of CYVV in the presence of relatively low concentrations of BYMV (when reactions were mistakenly interpreted as due to CYVV only).

We also observed occasional nonspecific reactions with some greenhouse-grown white clover plants in ELISA of BYMV and CYVV (*unpublished*). No virus could be isolated by sap inoculation even after concentration of inoculum. Periodic ELISA of the same plants gave erratic results. Addition of sugars (9) or NaDIECA to the grinding buffer did not prevent these occasional nonspecific reactions.

Motorized sap extractor. For qualitative tests, two people could process 250–500 samples in about 6 hr depending on the number of viruses to be tested and, therefore, the number of wells to be charged with each sample. Double rinsing and drying of rollers was required to prevent carryover of AMV. Silicone coating on the rollers made roller washing more rapid, but drying was still required to prevent carryover.

DISCUSSION

The sensitivity of ELISA relative to infectivity tests varied among the forage legume viruses tested and with experimental conditions such as differences in IgG preparations, dilution of coating antibody, and timing of assay steps.

The reliability of ELISA was generally as good or better than LAT. Koenig (15) also observed greater sensitivity of ELISA over LAT. Symptom expression of white clover plants inoculated with AMV was not a reliable criterion for determining infection. Testing by ELISA enabled detection of AMV in symptomless plants (Table 1). Agreement between ELISA and indicator host assays (Tables 1 and 2) was variable, but the ease and speed of ELISA made it the preferable method for large-scale testing.

Sample buffer additives had marked effects on ELISA (Tables 3 and 4). Inclusion of 2% PVP in PBS-Tween sample buffer, had little or no effect on detection of WCMV (Table 4) but was detrimental in some CYVV tests (Table 3). Although our tests were done by adding purified virus to extracts of healthy plants, similar observations of the effects of PVP were made by Mowat (26) for plants infected with narcissus tip necrosis and narcissus mosaic viruses. Hill et al (13) tested purified soybean mosaic virus (SMV) and reported severe aggregation of SMV in PBS-Tween-PVP as responsible for the apparent decrease in ELISA sensitivity, but also observed aggregation in PBS-Tween without PVP and suggested high salt concentrations may be responsible. In our tests, no single buffer concoction was optimum for all systems. Whether examined by analysis of interactions within the factorial arrangement of treatments, or by comparison of binding ratios as suggested by Hill et al (13), addition of NaDIECA gave best results overall. Although buffer containing 2-ME alone or with PVP, or containing NaDIECA with PVP, gave better results in some tests, the overriding requirement for simplicity in testing for several viruses in several plant species caused us to adopt PBS-Tween-NaDIECA for routine testing of clovers.

High background optical density readings may result from several unrelated causes, including antibodies to host components. This problem may be overcome by absorption with healthy host material (17) or by use of short substrate incubation times. Ghabrial et al (9) used sugars in the antigen buffer to prevent high nonspecific background due to lectins. Hill et al (13) used a secondary ovalbumin coat to lower background, supposedly by preventing nonspecific adsorption to the plate. We observed that 10% diethanolamine can also cause high backgrounds if stored for 3–4 mo before use.

The use of ELISA by mail offers new dimensions in research potential to plant breeders and pathologists not otherwise equipped for virus detection and identification work (16,19,27).

Distinguishing closely related viruses by ELISA proved especially useful with BYMV and CYVV. The procedure we described to distinguish heterologous reactions from reactions of virus mixtures according to experimentally determined criteria for heterologous reactions was about 90% accurate within the range of virus concentrations tested. Although this margin may not be acceptable for some studies, it is a significant improvement over use of indicator hosts for separation of BYMV and CYVV. Complications may arise however, if more than two closely related viruses need to be distinguished. We found reliable serodiagnosis of BYMV and CYVV in mixed infections was possible only from original plants, and that mechanical or aphid inoculations to transfer natural or experimentally produced mixed virus cultures often resulted in loss of one or the other virus.

LITERATURE CITED

1. Abu Salih, H. S., Murant, A. F., and Daft, M. J. 1968. The use of antibody-sensitized latex particles to detect plant viruses. J. Gen. Virol. 3:299-300.
2. Barnett, O. W., and Gibson, P. B. 1975. Identification and prevalence of white clover viruses and the resistance of *Trifolium* species to these viruses. Crop Sci. 15:32-37.
3. Bercks, R. 1971. White clover mosaic virus. No. 41 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
4. Bos, L., and Jaspars, E. M. J. 1971. Alfalfa mosaic virus. No. 46 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
5. Burrows, P. M., McLaughlin, M. R., Barnett, O. W., and Baum, R. H. 1981. Quantitative ELISA technique: uniformity analysis and experimental design. (Abstr.) Phytopathology 71:206-207.
6. Burrows, P. M., Scott, S. W., Barnett, O. W., and McLaughlin, M. R. 1984. Use of experimental designs with quantitative ELISA. J. Virol. Meth. 8:207-216.
7. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. J. Gen. Virol. 34:475-483.
8. DeBokx, J. A. 1972. Test plants. Pages 102-110 in: Viruses of Potatoes and Seed-Potato Production. J. A. DeBokx, ed. PUDOC, Wageningen, The Netherlands.
9. Ghabrial, S. A., Li, D., and Shepherd, R. J. 1982. Radioimmunosorbent assay for detection of lettuce mosaic virus in lettuce seed. Plant Dis. 66:1037-1040.
10. Hampton, R., Beczner, L., Hagedorn, D., Bos, L., Inouye, T., Barnett, O., Musil, M., and Meiners, J. 1978. Host reactions of mechanically transmissible legume viruses of the north temperate zone. Phytopathology 68:989-997.
11. Harville, B. G., and Derrick, K. S. 1978. Identification and prevalence of white clover viruses in Louisiana. Plant Dis. Rep. 62:290-292.
12. Hastings, R. C., Kucera, H. L., and Tool, E. 1971. North Dakota State leaf "squeezer." Am. Potato J. 48:33-34.
13. Hill, J. H., Bryant, G. R., and Durand, D. P. 1981. Detection of plant virus by using purified IgG in ELISA. J. Virol. Meth. 3:27-35.
14. Jones, R. T., and Diachun, S. 1977. Serologically and biologically distinct bean yellow mosaic virus strains. Phytopathology 67:831-838.
15. Koenig, R. 1978. ELISA in the study of homologous and heterologous reactions of plant viruses. J. Gen. Virol. 40:309-318.
16. Leath, K. T., and Barnett, O. W. 1981. Viruses infecting red clover in Pennsylvania. Plant Dis. 65:1016-1017.
17. Lister, R. M. 1978. Application of the enzyme-linked immunosorbent assay for detecting viruses in soybean seed and plants. Phytopathology 68:1393-1400.
18. Lucas, L. T., and Harper, C. R. 1972. Mechanically transmissible viruses from ladino clover in North Carolina. Plant Dis. Rep. 56:774-776.
19. McLaughlin, M. R. 1979. Recent progress of regional research project S-127 on forage legume viruses. Pages 76-80 in: Proc. 36th Southern Pasture and Forage Crop Improvement Conf., 1-3 May, Beltsville, MD.
20. McLaughlin, M. R., and Barnett, O. W. 1978. Enzyme-linked immunosorbent assay (ELISA) for detection and identification of forage legume viruses. Pages 138-145 in: Proc. 35th Southern Pasture and Forage Crop Improvement Conf., 13-14 June, Sarasota, FL.
21. McLaughlin, M. R., Barnett, O. W., and Gibson, P. B. 1978. Detection

and differentiation of bean yellow mosaic virus and clover yellow vein virus by ELISA. (Abstr.) Phytopathology News 12(9):198.

22. McLaughlin, M. R., and Barnett, O. W. 1979. The influence of plant sap and antigen buffer additives on the enzyme-immunoassay of two plant viruses. (Abstr.) Phytopathology 69:1038.
23. McLaughlin, M. R., Barnett, O. W., and Gibson, P. B. 1979. Mailing ELISA plates extends virus indexing potential. (Abstr.) Phytopathology 69:530.
24. McLaughlin, M. R., Barnett, O. W., Burrows, P. M., and Baum, R. H. 1981. Improved ELISA conditions for detection of plant viruses. J. Virol. Meth. 3:13-25.
25. Mink, G. I. 1972. Peanut stunt virus. No. 46 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
26. Mowat, W. P. 1978. Methods of detecting viruses in narcissus. 24th Annual Report for the year 1977. Scottish Horticultural Research Institute. Invergowrie, Dundee, Scotland. 96 pp.
27. Velde, M., Weissman, A., Maxwell, D. P., and Smith, R. R. 1979. Survey of red clover for virus in Wisconsin with the ELISA technique. Clovers and Special Purpose Legumes Research Prog. Rep. 12:55-57.
28. Voller, A., Bartlett, A., Bidwell, D. E., Clark, M. F., and Adams, A. N. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 33:165-167.
29. Voller, A., Bidwell, D. E., and Bartlett, A. 1976. Enzyme immunoassays in diagnostic medicine. Theory and practice. Bull. W.H.O. 53:55-65.