# Evaluation of Virus Contents in Soybean by Enzyme-Linked Immunosorbent Assay

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**ABSTRACT**

Moore, D. L., Lister, R. M., Abney, T. S., and Athow, K. L. 1982. Evaluation of virus contents in soybean by enzyme-linked immunosorbent assay. Plant Disease 66:790-793.

Enzyme-linked immunosorbent assay was used to compare virus contents of soybean cultivars and breeding selections inoculated with soybean mosaic virus or tobacco ringspot virus. Differences in virus content detected between cultivars did not always correspond to symptom severity. Results indicated that timing of assays, in relation to environment and plant development, and choice of leaves sampled can be critical factors in making such comparisons.

Breeding for "resistance" to plant virus diseases usually amounts to a search for resistance to pathogenesis, not virus production. Selections are compared by agronomic features such as symptom severity and relative growth and yield rather than by their virus contents. However, information on virus content is a prerequisite for distinguishing between resistance, defined as the ability to reduce virus multiplication; tolerance, defined as the ability to grow and yield normally even though sustaining virus multiplication; and immunity, defined as complete resistance to virus infection. Moreover, the precise comparison of virus contents could provide a basis for understanding the genetics of resistance. It has been suggested that enzyme-linked immunosorbent assay (ELISA) would be appropriate for such comparisons (4,6). Published results (6) show consistent relationships between symptom severity and ELISA values in pepper plants infected with cucumber mosaic virus or potato virus Y.

We report here on work done to determine the feasibility of applying ELISA to quantitative comparisons of the virus contents of soybean entries (cultivars, plant introductions, and breeding selections) inoculated with soybean mosaic virus (SMV) or tobacco ringspot virus (TRSV). These studies were incorporated into continuing studies of the susceptibility of many soybean entries to these viruses. Overall, the results indicated that ELISA is a potentially useful adjunct to visual observation and measurement of plant characteristics in breeding for resistance to these viruses in soybean, but that the timing of assays in relation to environment and plant development, and the choice of leaves sampled, may be critical factors.

Portion of a thesis submitted by the senior author in partial fulfillment of the requirements for the M.S. degree, Purdue University, West Lafayette, IN.

Journal Series Paper 8741 of the Purdue Agricultural Experiment Station.

Supported in part by USDA-ARS Special Grant 9011542 and by the Indiana Crop Improvement Association.

Accepted for publication 1 February 1982.

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0191-2917/82/09079004/$03.00/0


## MATERIALS AND METHODS

**SMV field studies.** *Plant selection and inoculation.* Sampling was conducted in plots on the Purdue Agronomy Farm in which various soybean entries were exposed to infection with SMV. The entries examined in most detail were grown in replicated 3-ft rows (24–30 plants each) alternating with infector rows of Midwest soybean. They were progeny from plants infected with an isolate maintained at Purdue as SMV-MI (B. W. Hagood, *unpublished*; 4), which is probably the same as that referred to by Ross (7) as SMV-6. About 30% of these progeny contained seed-transmitted SMV (4). In this design, used routinely in such trials at Purdue, the infected progeny plants provide a source of SMV to ensure exposure of adjacent soybean selections to infection.

Twelve rows of Midwest soybeans and the entry rows planted between them were available for this study. These included 14 and 18 entry rows of the cultivars Amsoy 71 and Bansei, respectively, but only single rows each of Adelphia, Hawkeye 63, Merit, and PI 80837. The latter four entries, and randomly selected rows of Amsoy 71 and Bansei, were hand-inoculated with SMV on 22 and 23 June, before any virus spread was apparent and when plants were at the V1–V3 stage (first or second trifoliolate leaves fully expanded [3]). At the same time, 140 Midwest plants with no symptoms of SMV infection were selected at random for inoculation from the 12 rows and labelled "Midwest inoculated" (MIN) for later sampling. Similarly, 140 Midwest plants showing SMV symptoms were labelled "Midwest infected progeny" (MIP) for later sampling. ELISA confirmed the visual diagnosis of all of the MIN and MIP plants. Soybean entries that were sampled only once were grown in a similarly designed plot but as single, unreplicated, 3-ft rows, except that replicate rows of Bansei were included as a standard for scoring symptoms. All of these entries were also hand-inoculated at the V1–V3 stage.

*Sampling procedure.* All plants were scored for symptom severity throughout the season (Table 1). The entries tested once were sampled between 8 and 14 August, when most were in the R3 stage (3). Three plants chosen at random from each row were sampled by removing the central leaflet from each of the 10 uppermost, fully expanded trifoliolates. The tissue was stored in plastic bags on ice, transferred to the laboratory, and stored at 4 C overnight. The 10 leaflets in each bag were stacked and punched (no. 3 cork borer, 8 mm diameter) to yield a single set of 10 leaf disks. Each of the three sets of 10 disks thus obtained represented a single SMV-inoculated plant.

For the entries examined in detail, plant growth stages were recorded (3) and leaf samples were obtained weekly during the period 26 July to 30 August. At each sampling, three replicate lots of 10 representative, randomly selected MIP, MIN, Amsoy 71, and Bansei plants were uprooted. All completely expanded

**Table 1.** Symptom ratings and enzyme-linked immunosorbent assay (ELISA) values for extracts from 59 soybean entries inoculated with soybean mosaic virus isolate MI

| Symptom rating[y] | Entries (no.)[z] | Mean ELISA value |
|---|---|---|
| 5 | 10 (2) | 0.48 |
| 4 | 12 (12) | 0.40 |
| 3 | 20 (20) | 0.23 |
| 2 | 15 (15) | 0.16 |
| 1 | 12 (10) | 0.06 |

[y] On a scale of 1–5 (1 = no symptoms, 5 = severe symptoms).

[z] Numbers in parenthesis indicate the numbers of different selections included in the total of entries.

trifoliolate leaves were removed and arranged in stacks according to node. Each stack thus contained 10 leaves from the same node and represented the plants from one replicate lot. Three sets of leaf disks were punched from each stack, one from each of the three leaflets of the superimposed trifoliolate leaves. Combining each set thus generated three replicate lots of 30 leaf disks representing each nodal position.

Leaf disks were obtained in the field from Adelphia, Hawkeye 63, Merit, and PI 80837 plants because too few were available to uproot samples. To conserve tissues also, leaves at the lowest four nodes of one to three plants were sampled, and only 10 leaf disks were included in each replicate set representing a particular nodal position. No samples were collected from these plants on 26 July (Table 2).

**TRSV field study.** Plants sampled were from a plot on the Purdue Farm designed for continuing observations on the infectivity and gross effects of TRSV in a range of soybean selections. Each entry was grown in duplicate 3-ft rows, one of which was hand-inoculated at the V1 stage with TRSV derived from an infected stock of Harosoy (4), while the other was left uninoculated. The entries sampled included widely grown commercial cultivars and numbered breeding selections showing resistance to the bud blight disease caused by TRSV (Table 2). They were sampled once only, on 8 August, when plants were in growth stages R3 or R4. The central leaflet from each fully expanded trifoliolate leaf of three plants chosen at random from each 3-ft row sampled was removed, and those from the same node were bulked. Each bulk sample thus consisted of three leaflets representing a specific nodal position. The plants were rated for symptom severity twice during the season (Table 2).

**TRSV greenhouse study.** Entries in the TRSV field study were also assayed for virus content in a greenhouse experiment. Sixty plants of each entry were grown in 6-in. pots (12 pots, five plants per pot) from seeds sown in September, with supplementary fluorescent and tungsten lighting to extend day length to 14 hr. Primary leaves were inoculated when plants were at the V1 stage. Sampling was as for the field plants except that 10 plants per cultivar were sampled instead of three. Thus, each sample consisted of 10 central leaflets from fully expanded trifoliolate leaves, representing a specific nodal position. Cultivars were sampled at the same growth stage as in the field study.

**ELISA of tissue extracts.** Procedures for ELISA (2) were essentially as outlined by Lister (4), using flat-bottomed, polystyrene microELISA plates (Cook/Dynatech, 1-223-29, Dynatech, Alexandria, VA 22314). Coating immunoglobulins (Ig) were used at 5 $\mu$g/ml and conjugated immunoglobulins at 5 $\mu$g/ml for SMV and 20 $\mu$g/ml for TRSV tests, amounts that were determined as adequate and economical in preliminary experiments. To reduce background reactions, conjugated immunoglobulins were diluted in an extract from healthy Wayne soybean leaves made 1:5 (w/v) in phosphate-buffered saline containing 0.05% Tween 20 and 2% polyvinylpyrrolidone (PBS-Tween-PVP) (4). For this purpose also, it was found advantageous with the SMV Ig, but not with TRSV Ig, to preabsorb the antiserum used to prepare coating Ig (5) by incubating it with healthy soybean extract before preparing the Ig by fractionation by ammonium sulfate precipitation and chromatography in diethylaminoethyl cellulose. Immunoglobulin used for conjugate was not preabsorbed.

Coating, antigen addition, conjugate addition, and substrate hydrolysis steps were, respectively, for 3–4 hr at 37 C, 18 hr at 4 C, 4–5 hr at 37 C, and 30 min at room temperature. At each step, 250-$\mu$l aliquots of reactants were used. Substrate hydrolysis was stopped by adding 50 $\mu$l of 3 M sodium hydroxide, and well contents were diluted fivefold with water prior to reading absorbances ($A_{405}$ nm $\times$ 1/5 = ELISA value). Triplicate wells were run for each sample, and mean values were used for comparisons.

Extracts were prepared from SMV-inoculated plants by grinding leaves in 0.2 M sodium phosphate buffer (pH 7.5) at 1:2 (w/v) (B. W. Hagood, *unpublished*), then adding two volumes of PBS-Tween-PVP and regrinding. Extracts from TRSV-inoculated plants were extracted at 1:5 (w/v) in 0.05 M sodium phosphate buffer (pH 7.0). After preparation, 1-ml aliquots of the samples were frozen and stored at −20 C in glass vials. Extracts used as standards of comparison were prepared similarly from SMV-infected Midwest leaves and TRSV-infected Harosoy leaves (4) and stored at −20 C as 1-ml aliquots for use as required. Prior to ELISA, SMV test samples and samples of the SMV standard were thawed and diluted 1:5 with PBS-Tween-PVP. Such dilutions of the standard contained approximately 0.8 $\mu$g/ml of virus as determined by comparison with dilutions of purified virus, and gave ELISA values averaging about 1.2. The TRSV samples and standard were thawed and diluted 1:8, at which the standard contained virus at about 10 $\mu$g/ml and gave ELISA values of about 1.2.

Tests of the standard extracts and healthy tissue control extracts were included in each ELISA plate. Control values were subtracted from sample readings, which were then adjusted in relation to the average values obtained for all tests done with a particular standard aliquot. This was especially important for the SMV-infected entries examined in detail, for which separate conversion factors were developed for each batch of tests. These tests were conducted in batches during 6 January to 15 February. Batch-to-batch fluctuation of ELISA values for the standard extracts in these tests ranged between about 0.8 and 1.5; mean values for the first and last batches almost coincided at 1.48 and 1.50, respectively. Variation was presumably caused by procedural variations and possibly by variations between test plates. Values for the standard extracts included in the other studies, completed over shorter periods, fluctuated less. Where possible, adjusted data were analyzed by Duncan's new multiple range test.

## RESULTS AND DISCUSSION

**SMV field studies.** Data for the group of 59 different entries sampled only once are summarized in Table 1. Although overall, mean ELISA values were remarkably consistent with symptom ratings, the results indicated that valid comparisons would require more detailed investigation. For example, of the 12

**Table 2.** Enzyme-linked immunosorbent assay (ELISA) values and symptom ratings of entries inoculated with tobacco ringspot virus in the field and greenhouse

| Field samples | | | | Greenhouse samples | |
|---|---|---|---|---|---|
| | Mean ELISA value[y] | Symptom rating[z] | | | Mean ELISA value[y] |
| Cultivar | | 22 July | 27 Oct. | Cultivar | |
| Harosoy | 0.70 a | 3 | 4 | Cutler 71 | 0.81 a |
| CX 652-28-2-2 | 0.64 ab | 5 | 4 | CX 655-3-2-2 | 0.80 abc |
| CX 655-3-3-3 | 0.60 ab | 1 | 1 | Amsoy 71 | 0.79 abcd |
| Beeson | 0.55 abc | 3 | 3 | Williams | 0.76 abcd |
| Amsoy 71 | 0.54 abc | 5 | 5 | Harosoy | 0.76 bcd |
| Wells | 0.54 abc | 4 | 3 | Wells | 0.74 cd |
| CX 665-3-2-2 | 0.54 abc | 4 | 4 | CX 655-3-3-3 | 0.74 cd |
| CX 654-19-3-3 | 0.52 bc | 4 | 4 | CX 654-19-3-3 | 0.70 de |
| Williams | 0.52 bc | 3 | 3 | CX 652-28-2-2 | 0.64 ef |
| CX 652-33-2-1 | 0.43 cd | 1 | 2 | CX 652-33-2-1 | 0.61 f |
| Cutler 71 | 0.40 cd | 3 | 4 | BX 652-33-2-2 | 0.60 f |
| CX 652-33-2-2 | 0.36 d | 1 | 1 | Beeson | 0.60 f |

[y] Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.
[z] On a scale of 1–5 (1 = no symptoms, 5 = severe symptoms).

entries rated 1 for symptoms, all but one had mean ELISA values of zero; for the exception (Amsoy), the mean ELISA value was 0.66, which exceeded the mean for all entries. Similarly, the mean ELISA value for Dare, rated as 5 for symptoms, was 0.14, which was less than that for most entries rated 3 or 4. Some of these anomalies were probably the result of inadequate sampling: nine of the entries rated as 5 were replicate rows of Bansei, and ELISA values for these ranged from 0.16 to 0.83, indicating wide variation in this cultivar.

For the detailed study, ELISA values for samples from the MIN, MIP, Amsoy 71, and Bansei plants indicated that, with some exceptions, extractable virus was fairly uniformly distributed between nodes (Fig. 1). Some of the exceptions suggested erratic virus distribution (eg, MIN samples of 9 August), whereas others appeared to reflect rapid growth in relation to the rate of virus spread from older leaves (eg, MIN and MIP samples of 26 July). Data for Adelphia, Hawkeye 63, and Merit were insufficient to draw conclusions because of the small number of plants available. None of the samples collected from plants of PI 80837 reacted positively in ELISA.

A feature of the data for all entries was the occurrence of week-to-week differences in mean ELISA values. Some samplings from Adelphia, Hawkeye 63, and Merit contained no detectable virus. Significant differences in ELISA values between samplings occurred with Amsoy 71, MIN, and MIP plants (Fig. 1); apparent differences with Bansei samples (Fig. 1) were not statistically significant because of the relatively high variability between ELISA values for samples from this cultivar.

We cannot explain these indications that virus content or extractability varied markedly over the season. Possibly some of the variability was caused by difficulties in obtaining uniform extraction efficiency and preventing particle aggregation—a common problem with viruses with long, flexuous particles. Changes in the virus content of plants might be involved, but inspection of meteorologic data indicated no relationship to field temperature, a factor that influences SMV symptoms (1). Also, although relatively low ELISA values were obtained for samples from MIN, MIP, Amsoy 71, and Bansei plants collected at the onset of blooming (R1, Fig. 1), variations in ELISA values were not clearly associated with stages in plant development and physiologic status. ELISA values for samples from MIN and MIP plants followed generally the same trends, except that the values for samples collected from MIN plants on 9 August were relatively high and those for 16 August were relatively low (Fig. 1). Values for some of the Merit samples collected on 30 August seemed anomalously high and probably represented a plant or plants not true to type (Fig. 2).

Because of the sources of error apparent from the above, the overall rankings of entries by ELISA values for samples over the season (Fig. 2) must be viewed with caution, especially for the cultivars Adelphia, Hawkeye 63, and Merit. However, samplings from the cultivar Midwest consistently indicated higher virus contents than those from the other cultivars. Similarly, the results suggested a difference between samples from Bansei and Amsoy 71, although this was not statistically significant, again because of high variability of ELISA values from Bansei samples.

Ranking of entries by ELISA values was compared with the overall ranking by symptom severity, which was Bansei > MIP > MIN > Amsoy > Adelphia = Hawkeye 63 = Merit = PI 80837. Symptoms in Bansei (rated 5) were obvious mosaic, leaf distortion, and rugosity. Midwest plants showed mild mosaic; MIN plants were rated 3, whereas MIP plants, which showed mild mosaic and some stunting, were rated 4. Occasional Amsoy 71 plants were slightly stunted (rating 2), whereas the remaining

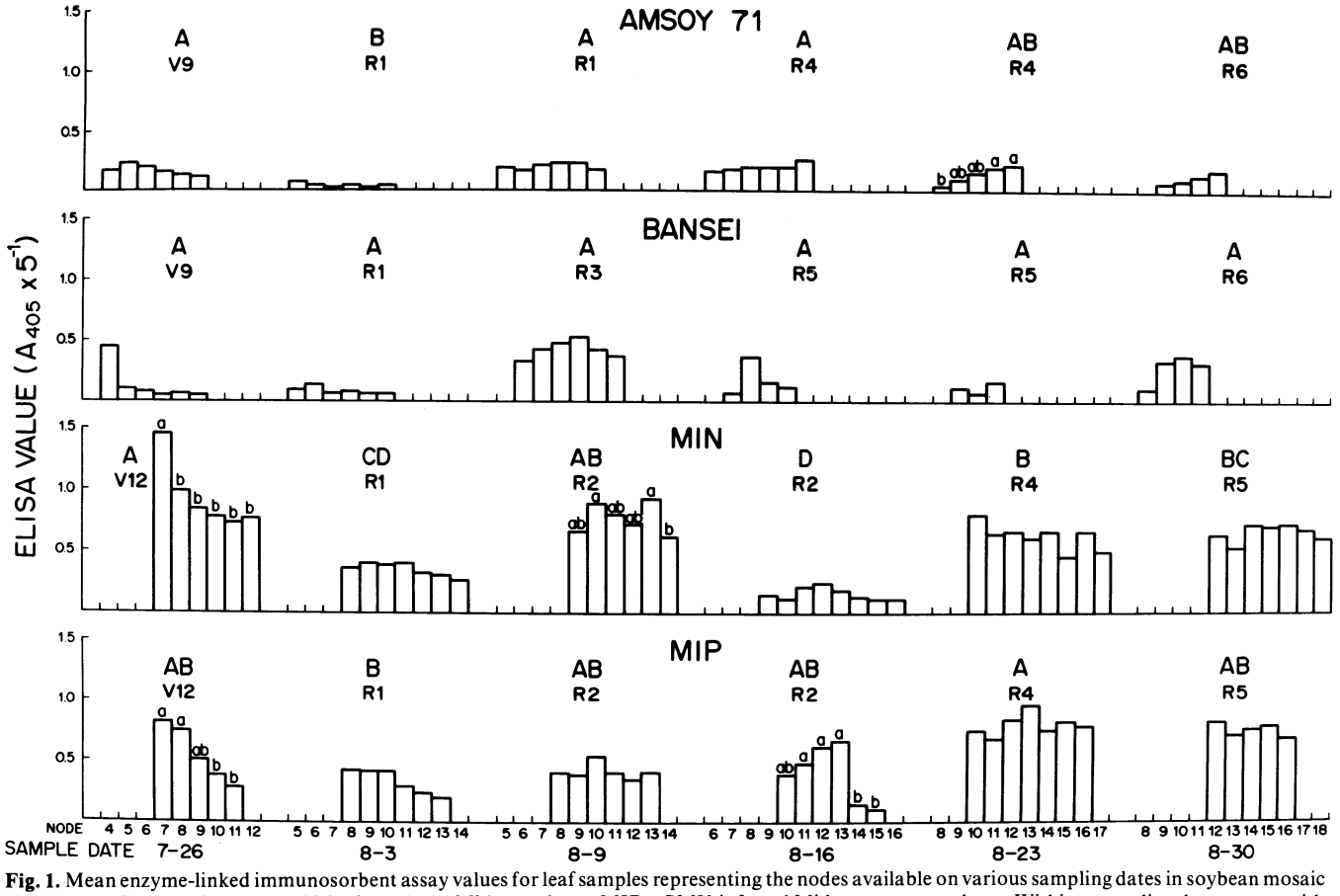


**Fig. 1.** Mean enzyme-linked immunosorbent assay values for leaf samples representing the nodes available on various sampling dates in soybean mosaic virus (SMV) field studies. MIN = SMV-inoculated Midwest plants; MIP = SMV-infected Midwest progeny plants. Within a sampling date, means with the same lower case letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test; between sampling dates, overall means with the same upper case letter are not significantly different. V9, R1, etc., indicate growth stages at the time of sampling.

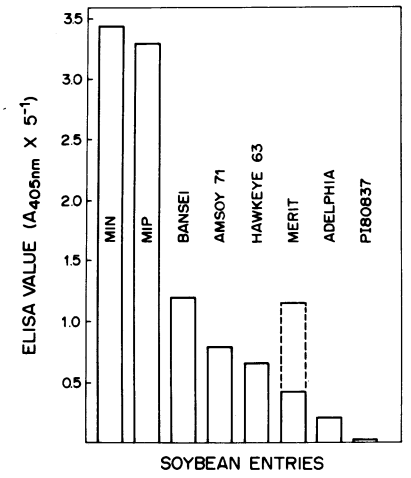


**Fig. 2.** Ranking of entries by sums of overall means of enzyme-linked immunosorbent assay values over the period of sampling in soybean mosaic virus field studies. For Merit, the bar outlined with dashes represents the mean including all values; the bar outlined with a solid line excludes some values regarded as anomalously high.

cultivars were symptomless (rating 1). Thus, virus contents as indicated by ELISA did not parallel symptom severity.

**TRSV field and greenhouse studies.** ELISA values obtained in field and greenhouse studies with TRSV indicated very little node-to-node variation in virus content, suggesting that if a "recovery" phenomenon (8) occurs in soybean, its effects were not obvious in our samplings.

Overall rankings of entries by mean ELISA values were generally similar for both field and greenhouse tests, but some differences occurred (Table 2). For example, Harosoy gave the highest mean ELISA value of all entries in the field test but a lower mean value than four other entries in the greenhouse test. Cutler 71 had the lowest apparent amount of virus in the field tests but the highest mean ELISA value of all entries in the greenhouse test. Comparisons by Duncan's new multiple range test also grouped other entries differently in the two experiments (Table 2).

By analogy with the results for the SMV field studies, such differences may result from time of sampling, especially of the field plants. It is also possible that the results for field samples discriminated differently between entries because of the relatively greater environmental variation and stress. Certainly, the range of ELISA values for field samples was wider than that for greenhouse samples (Table 2). Comparisons of the values with those for virus dilutions indicated a fivefold range in average virus concentration for the field samples but only a twofold range for the greenhouse samples. A further difference related to the timing of sampling. All samples were taken at the R3 or R4 stage of development; entries reached this stage almost concurrently in the field but several weeks apart in the greenhouse study. The entries were therefore developing at different relative rates in the two studies.

For some entries, virus titers as indicated by ELISA were reasonably consistent with symptom ratings (Table 2). Again, however, obvious exceptions occurred; eg, for CX 655-3-3-3, a high indicated virus titer for the field plants was coupled with a low symptom rating.

Although ELISA measures specific antigen concentration rather than infectivity, it provides a much simpler means of estimating virus content than infectivity assay. It is also highly reproducible, consistent, and in general can discriminate between smaller differences in virus concentration than can infectivity assay. ELISA is thus ideal for quantitative as well as qualitative measurements of virus in plants. In combination with symptom observations, such information may aid in understanding many aspects of the interactions between viruses and their hosts, including defining sources of various types of resistance and understanding their inheritance. For example, in relation to virus production, Bansei appeared to be more resistant than Midwest to SMV, but the reverse was true in relation to symptom production, on which basis Bansei was highly susceptible. Again, although PI 80837 was apparently immune to SMV-MI (confirming unpublished results of T. S. Abney, based on infectivity tests), it would be ranked with Adelphia, Hawkeye-63, and Merit, which are susceptible but apparently tolerant to SMV-MI, on the basis of symptoms. A low symptom rating with relatively high ELISA values for field plants of CX 655-3-3-3 inoculated with TRSV also indicates tolerance.

Our results indicate that assessing virus contents by ELISA requires an understanding of the distribution of virus throughout the plant during development and under various environmental conditions so that appropriate sampling procedures can be developed. Comparing virus contents of representative plant samples collected once or twice during development may give valuable information (6), but it may not be adequate for detailed comparisons unless based on knowledge of the cycle of virus multiplication and its relationship to environment. In this way, virus content assessment even by ELISA is more demanding than symptom assessment, which represents the sum of many virus/host interactions (of which virus content is only one) and can be quickly done on an empirical basis. However, symptom assessment is far less precise than ELISA, and therefore less discriminating. For this reason, as well as to improve our understanding of resistance, exploring the potential for such ELISA applications further seems warranted.


## ACKNOWLEDGMENTS

We thank T. Moore for skilled technical assistance and G. E. Shaner and F. A. Laviolette for advice.


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