Quantification of Within-Field Spread of Soybean Mosaic Virus in Soybean Using Strain-Specific Monoclonal Antibodies

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Accepted for publication 10 June 1998.

ABSTRACT

Nutter, F. W., Jr., Schultz, P. M., and Hill, J. H. 1998. Quantification of within-field spread of soybean mosaic virus in soybean using strain-specific monoclonal antibodies. *Phytopathology* 88:895-901.

Strain-specific monoclonal antibodies were used to follow the temporal increase and spatial spread of soybean mosaic virus (SMV) strain G-5 released from a point source. The use of strain-specific monoclonal antibodies allowed discrimination of within-field temporal and spatial spread of SMV strain G-5 from non-G-5 SMV isolates that originated from exogenous field sources. SMV isolates originating from exogenous sources have potential to alter the temporal and spatial pattern of within-field virus spread, which could potentially affect the choice of models used to quantify within-field pathogen spread. Analysis of SMV epidemics in field-plot experiments

indicated that the logistic model was the most appropriate model to describe and compare the temporal spread of SMV among years. On the basis of ordinary runs analyses, within-field spread of SMV strain G-5 was random in 1991 and 1994, but was mostly aggregated in 1992 and 1993. Non-G-5 SMV isolates arising from exogenous sources displayed a random spatial pattern over time. This is the first study in which pathogen incidence (detection of SMV using strain-specific monoclonal antibodies) as opposed to disease incidence (based on symptoms) was employed to monitor and model SMV spread in time and space.

Additional keywords: potyvirus, risk assessment, statistical modeling, virus epidemiology.

Soybean mosaic virus (SMV), which belongs to the virus family Potyviridae (39), causes a disease of soybean (*Glycine max* (L.) Merr.) that is present in all the major soybean-growing areas of the United States (10,13,22,23). SMV is seedborne and is nonpersistently transmitted by more than 20 species of aphids (13,23,26,39). Significant reductions in yield and quality as a consequence of SMV epidemics have been well documented (13,17,23,25,34,36–38). SMV also adversely affects both oil content and nodulation (8,23,42). Although SMV may cause severe mosaic symptoms on susceptible cultivars, distinct mosaic symptoms are not evident in most soybean cultivars currently grown in the Midwest (24). In spite of advances in breeding soybean cultivars with reduced symptom expression, yield reductions of up to 24% have been documented in soybean cultivars with reduced symptom expression (F. W. Nutter, Jr. and J. H. Hill, *unpublished data*).

Field studies to quantify the temporal and spatial dynamics of plant virus epidemics have largely relied upon the visual assessment of plants or plant parts to assess disease incidence within host populations. A useful operational definition for disease incidence is the number of plant units visibly diseased divided by the total number of plant units sampled and assessed (27,29). It is very important to clearly distinguish disease incidence from pathogen incidence, as these concepts involve two different populations. Disease incidence is based upon visual disease symptoms of individuals within a host population (sampling units), whereas pathogen incidence is based upon the detection of the pathogen within individual sampling units of a population. Since disease incidence is based on the presence of visual symptoms resulting from the interaction of host and pathogen (virus) populations in time and space, true disease incidence may be underestimated when disease symptoms are not fully expressed in all sampling units visually assessed (29).

Reduced or altered symptom expression may be due to the effects of environment, host resistance (or tolerance) that reduces symptom expression, and virus strains that are less aggressive in terms of reduced symptom expression. In virus pathosystems in which disease incidence cannot be accurately and precisely assessed, the validity of epidemiological studies based on visual assessment of disease incidence may be dubious (29). For example, if the rate of plant-to-plant spread (r) is used as a measure of epidemic risk (the higher the value of r , the higher the risk), the method employed to assess virus incidence must be both accurate and precise in order to obtain realistic estimates of epidemic risk. Geering (11) and Nutter (29) found that visual assessments for disease incidence of cucumber mosaic comovirus (CMV) underestimated the rate of plant-to-plant spread in lupine (*Lupinus angustifolius*) by nearly 50% ($r = 0.03$) as compared with rates based on enzyme-linked immunosorbent assay (ELISA) ($r = 0.06$). Irwin and Shultz (24) reported that assessment of SMV based on visual symptoms "can be unreliable" and that "symptom expression can vary according to weather conditions and be masked during periods of high temperature." The reliability of assessing SMV disease incidence is further confounded, because many soybean cultivars currently grown (including the cultivar employed in this study) do not exhibit distinctive SMV symptoms, and accurate assessments for "disease" incidence based upon visual symptoms are impossible to obtain. Moreover, the lack of distinctive disease symptoms greatly inhibits research efforts to quantify the temporal and spatial spread of SMV and, thus, the application of advanced statistical procedures and population growth models to such data is highly questionable (27,29).

Before any management strategy targeted towards control of SMV can be evaluated properly, sound sampling methods (7,27) coupled with new virus-detection tools (3,4,6,7,16,21,35,44) and computer models (29,30,32) will be needed. More specifically, knowledge of the spatial dynamics of SMV spread originating from a point source is essential in determining the appropriate plot size required for field experiments, estimating sample size, and determining the most appropriate sampling procedure (arbitrary, random,

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stratified, etc.) and sampling pattern ('x' pattern, 'w' pattern, transect, etc.) for temporal disease and yield loss studies (11,14,30). This is particularly important if a rate-reducing resistance strategy is to be deployed, because this strategy requires researchers to accurately and precisely track and quantify the temporal spread of specific virus strains in the field.

The use of epidemiological models coupled with new SMV-detection tools should enhance our understanding of the biological impact of different resistance strategies on plant virus epidemics in soybean. The objectives of this study were to employ strain-specific monoclonal antibodies and a computer program, EPIVIRUS (F. W. Nutter, Jr., *unpublished data*), to (i) detect, differentiate, and map the spatial pattern of SMV strain G-5 and non-G-5 SMV isolates; (ii) quantify the temporal (within-field) spread of SMV G-5 pathogen incidence in relation to the temporal spread of exogenous (non-G-5 isolates); and (iii) analyze the spatial pattern of SMV G-5 spread versus non-G-5 spread by ordinary runs analysis.

MATERIALS AND METHODS

Field plots. Field plots were established near Ames, IA, during the years 1991 to 1994. The soybean cultivar Corsoy 79 was seeded (six seeds per 30 cm of row) on 10 June 1991, 17 June 1992, 29 June 1993, and 18 May 1994. Planting dates varied from year to year based upon weather conditions (e.g., 1993 floods). Two weeks after seeding, plots were thinned to four plants per 30 cm of row. Plot sizes were six rows by 8.3 m in length, except in 1994 when rows were 6.1 m long. Each year, row spacing was 0.76 m, and each plot was bordered by an additional row of soybean and an additional 2.3 to 3.8 m of row on each end of the plot. Wooden stakes were used to delineate 150 quadrats in each plot. Each quadrat was 30 cm long and one row wide (25 quadrats per row $\times$ six rows). In 1994, there were 20 quadrats per row $\times$ six rows (120 quadrats). Four replicate plots were established in 1991, and three replicate plots were used in 1992 through 1994.

SMV strain G-5 (ATCC PV-720) was used throughout this study to monitor and model within-field spread of SMV in time and space. SMV G-5 was maintained on the soybean cultivar Williams 82 (18). Approximately 4 to 5 weeks after planting (three to four trifoliate stage), the two youngest fully expanded leaves of plants located in the middle quadrats (quadrat position 13) of the two center rows (rows 3 and 4) of each plot were mechanically inoculated with the aphid-transmissible SMV G-5. SMV G-5 was introduced into plots on 8 July 1991, 14 July 1992, 6 August 1993, and 24 June 1994. For each experiment, previously inoculated plants were reinoculated 2 to 3 days later to ensure a local source of SMV G-5 for acquisition and dissemination by aphids.

TABLE 1. Logistic model parameters for the increased incidence of soybean mosaic virus (SMV) caused by all SMV isolates, non-G-5 isolates, and SMV G-5 during 1991 to 1994 in Ames, IA

SMV isolates/year	Intercept	Slope	SE _y ^a	R ² (%)
G-5 plus other isolates				
1991	-11.091	0.115	0.268	98.4
1992	-6.654	0.095	0.487	94.1
1993	-9.546	0.122	0.745	88.5
1994	-7.002	0.078	0.473	88.3
Non-G-5 SMV isolates				
1991	... ^b	...	...	...
1992	...	...	...	...
1993	-8.070	0.063	0.576	77.3
1994	-9.461	0.081	0.262	96.5
SMV G-5				
1991	-11.091	0.115	0.268	98.4
1992	-6.493	0.091	0.507	93.2
1993	-10.355	0.128	1.131	78.6
1994	-6.699	0.071	0.559	81.5

^a Standard error of the estimate for y .

^b Insufficient incidence of infection by SMV isolates other than G-5.

Data collection. Weekly sampling to determine pathogen incidence (the presence or absence of SMV G-5 in all 30-cm quadrats) within each plot (i.e., census) began 2 to 3 weeks after introducing SMV G-5 into field plots and every week thereafter. All quadrats were sampled by removing one of the youngest, fully expanded leaves from each soybean plant within a quadrat (three or four leaflets) and storing these in plastic bags at 4°C. There were five to eight sampling dates each year, depending upon the planting date, seasonal weather conditions, and crop maturity. Pathogen incidence (quadrats in which SMV was detected divided by the total number of quadrats per plot $\times$ 100) was determined for each sampling date.

Virus detection. Sap was extracted from leaves within 1 day after sampling. Bagged leaves from each quadrat were placed into a leaf press (Ravenel Specialties Corp., Seneca, SC) with 3 to 4 ml of a solution of 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.2. Plant sap was stored in plastic 1.5-ml microcentrifuge tubes at -40°C until used for testing by ELISA.

ELISA. Biotin-avidin ELISA (9) was used to track within-field spread of SMV G-5 and to differentiate G-5 from other exogenous sources of the virus. For the initial assay, monoclonal antibodies S1 (21) or S4 (18) were used as the capture antibody to test all samples. The biotinylated antibody S2-G12, designed to identify all SMV strains, was a mixture of monoclonal antibodies S2 and S7 (18). Monoclonal antibody S7 detects all SMV strains tested; whereas the high-affinity S2 detects all SMV strains except G-5 (18). All samples that tested positive in the initial assay were re-assayed, but this time using S1 or S4 as the capture antibody and biotinylated monoclonal antibody S2 as the second antibody. Thus, samples that tested positive with biotinylated antibody S2-G12 and negative with S2 were regarded as SMV G-5.

S1 or S4 monoclonal antibodies were used at a concentration of 2.5 μ g/ml, and biotinylated S2-G12 and biotinylated S2 were used at 2.5 μ g/ml and 1.0 μ g/ml, respectively, as determined by calculation of maximum signal to noise ratio (20). All incubation steps were performed at 26°C, and alkaline phosphate-conjugated Extravidin (Sigma Chemical Co., St. Louis) was used in place of conjugated avidin. Known positive and negative controls were included in each assay. Samples with absorbance values (wavelength = 405 nm) that exceeded the mean plus two standard deviations of the negative control (sap from noninoculated soybean plants) were regarded as positive.

Data analysis. For each plot, the increase in pathogen (SMV) incidence over time and tests for randomness of individual quadrats found to test positive for SMV were performed separately for SMV G-5 (within-field spread), non-G-5 SMV isolates, and for all SMV isolates combined for each sampling date. Pathogen incidence data from each plot were left untransformed (linear model) and also were transformed using the monomolecular, exponential, Gompertz, and logistic population growth models using the computer program EPIMODEL (30). Coefficients of determination (R^2), standard errors of the estimate for pathogen incidence (y), and the subjective evaluation of plots of standardized residuals versus predicted values were used to evaluate the goodness of fit of each population growth model (16,30,32). Once a growth model was chosen that best described all SMV epidemics, pathogen progress data were transformed and regressed against time using ordinary least-squares regression. The slope parameter obtained from regression equations was taken as a quantitative measure of the temporal infection rate of spread. Infection rates were calculated for within-field spread (SMV G-5), for spread of exogenous strains (non-G-5 isolates), and for all SMV isolates combined (EPIVIRUS software; F. W. Nutter, Jr., *unpublished data*).

The spatial position (row, column) of each SMV-infected quadrat within each plot was recorded for each assessment date. The computer program EPIVIRUS was then used to analyze the spatial pattern of infected quadrats over time using ordinary runs analysis for combined rows (16,28). This analysis can be used to determine randomness (null hypothesis) or degree of clustering (alternative

hypothesis) among quadrats testing positive for SMV. A run is defined as a sequence of like events (28) that, in our study, were sequences of contiguous quadrats in which SMV was detected or not detected (not infected). If the SMV spread among quadrats is nonrandom, the observed number of runs will be less than the number expected to occur by random chance (28). The test for clustering (nonrandomness) was one sided, and a negative z score greater than 1.64 indicates nonrandomness ($P \leq 0.05$). These calculations are automatically performed by EPIVIRUS. Ordinary runs analyses also were performed for SMV isolates other than G-5.

RESULTS

Although SMV G-5 progress for specific years was best described by different models, the logistic model provided the best overall fit to all data to describe and compare pathogen progress curves among all 4 years (based on the evaluation criteria for linear regression models). Overall, the logistic model explained more of the variation in the change in pathogen incidence with respect to time (higher R^2 values) than any other model tested (monomolecular, exponential, and Gompertz) and best linearized the relationship for transformed Y (incidence) and time (27,29). Moreover, inspection of residual plots indicated that the logistic model best fit the SMV epidemics that were quantified in our 4-year study. Therefore, the logistic model was selected to quantify and compare SMV infection rates (slopes) and intercepts (Table 1) among years and isolates. Coefficients of determination (R^2) ranged from 77.3 to 98.4%, and the standard errors for estimated Y were low (0.268 to 1.131). Pathogen progress curves of SMV G-5 in 1991 to 1994 are shown in Figure 1A. The rate of SMV G-5 infection was fastest in the years 1991 and 1993 ($r = 0.115$ and 0.128 logits per day, respectively), although SMV G-5 epidemics began earlier and reached a higher level of incidence (88%) by the end of the season in 1992. Rates of SMV G-5 temporal spread with respect to time were 0.091 and 0.071 in 1992 and 1994, respectively.

Non-G-5 SMV isolates were not detected in 1991, and the incidence of non-G-5 isolates in 1992 was too low (less than 2%) to calculate the rate of temporal spread (Fig. 1B). In 1993 and 1994, however, final SMV incidence of non-G-5 isolates was 12.7 and 11.3%, respectively, and rates of spread for non-G-5 isolates were calculated. In 1993, non-G-5 isolates of SMV spread only half as fast as SMV G-5. This may have been due to (i) environmental conditions favoring faster spread of G-5 early in the season relative to weather conditions that occurred later in the season during the time period that non-G-5 strains appeared and began to spread, or (ii) non-G-5 strains were less aggressive (lower acquisition and transmission efficiencies) compared with G-5.

Spatial maps of pathogen incidence by time of assessment were generated for SMV G-5 and non-G-5 isolates, and these maps were generated and analyzed using the EPIVIRUS software program. For example, spatial pattern maps of SMV G-5 and isolates other than G-5 in 1992 and 1993 are shown in Figures 2 and 3, respectively. The red quadrats indicate infection by G-5, the yellow quadrats indicate infection by non-G-5 SMV isolates, and the green quadrats indicate quadrats in which SMV was not detected. The numbers within quadrats indicate the assessment period in which SMV was first detected within a specific quadrat. EPIVIRUS generates a bar graph (Fig. 4) for each assessment that depicts the number of runs that could be expected to occur by chance (bars with diagonals) (based on the incidence of infected sampling units for each assessment date) and the number of runs that were actually observed (solid bars). The EPIVIRUS program also calculated and produce a table of z scores based on the t statistic (one sided) for each assessment date to indicate if the number of observed runs is significantly lower ($P \leq 0.05$) than the number of runs that would occur by chance. Spatial patterns of infected quadrats, as determined by

Fig. 1. Increase in pathogen incidence of quadrats of soybean mosaic virus (SMV) in 'Corsoy 79' soybean during 1991 to 1994 in Ames, IA, infected with **A**, all SMV isolates; **B**, non-G-5 isolates; and **C**, SMV strain G-5. Pathogen progress is based on incidence of infected soybean plants in quadrats versus time.

ordinary runs analyses, were generally random throughout 1991 and most of 1994 (Table 2). In 1992 and 1993, however, the spatial pattern of SMV G-5 was aggregated throughout most of both seasons (Table 2). In most cases, quadrats infected with non-G-5 SMV isolates arising from exogenous sources displayed random spatial patterns over time (Table 3).

DISCUSSION

This study is the first quantitative analysis of a virus pathosystem in which within-field spread of a released virus strain is differentiated from virus inoculum arising from exogenous sources. Moreover, this is the first study involving SMV in which temporal and spatial analyses were based upon pathogen incidence (detection of SMV using strain-specific monoclonal antibodies) as opposed to monitoring disease incidence (presence of SMV symptoms), which is highly unreliable for most soybean cultivars that are currently being grown.

The ability to accurately detect and differentiate specific virus strains is important for assessing the biological impact (e.g., yield, rate of temporal spread, and initial inoculum) of any virus disease management strategy, particularly those aimed at reducing the rate of plant-to-plant spread within the crop (27,29). Randles (35) emphasized the importance of identifying virus isolates to specific strains to estimate parameters that may be used to quantify virus spread. The ability to differentiate strains is also paramount in quantifying the aggressiveness of specific strains with respect to their rate of spread in time and space and the relative yield reductions they cause. Using the sampling, detection, and modeling techniques developed in our study, it is now possible to evaluate the effectiveness of specific disease management tactics on the temporal and spatial spread of SMV epidemics in the field. It is also now possible to quantify the impact of specific SMV strains on soybean yield and quality. Although a number of researchers have developed biological and molecular tools to differentiate virus strains (3,4,6,16,18), epidemiological studies involving plant viruses have mostly utilized non-strain-specific tools (such as polyclonal antibodies) to detect all strains of a virus group.

Moreover, molecular and serological tools that have a broad spectrum of activity across virus strains (or pathotypes) do not have the specificity required to differentiate virus isolates arising from potentially different sources (i.e., sources of virus inoculum arising from within-field sources such as infected seed, infected weed hosts, or infected volunteer plants as opposed to those arising from exogenous field sources). Such ecological information is critical to the development of effective integrated disease management programs (27,29).

In general, SMV strains are serologically homogeneous and difficult to differentiate by conventional techniques applicable to field collections (19,31,39,40). Consequently, a single monoclonal antibody has not been identified that detects a single strain. For this reason, we utilized three antibodies that included a monoclonal antibody that does not detect SMV G-5 as evidence for the presence of SMV G-5 within 30-cm quadrats. Our approach (using molecular tools to differentiate virus strains) could be applied to other virus pathosystems to quantify the effects that specific virus disease management strategies (e.g., host resistance, use of insecticides, eradication of alternative weed hosts, etc.) may have on the temporal rate and spatial spread of plant viruses. Such an approach could also be used as the framework to evaluate integrated virus disease management programs.

In addition to producing spatial maps of disease (or pathogen) incidence for each assessment period (Figs. 2 and 3), the computer software program EPIVIRUS can produce graphs (and tables) depicting disease (or pathogen) progress with respect to time of assessment, and EPIVIRUS also automatically calculates the area under the disease (or pathogen) progress curve. In addition to ordinary runs analysis, EPIVIRUS can also perform doublets analysis (28). Information concerning EPIVIRUS can be obtained from F. W. Nutter, Jr.

In our study, the rate of SMV spread with respect to time was best described by the logistic model (29) and, thus, this was the most appropriate model to compare rates of within-field spread of SMV among years and isolates. In general, this model best describes plant virus epidemics that are initiated by a source of inoculum followed by plant-to-plant (within-field) spread (11,16,27,

Fig. 2. Spatial map generated by the software program EPIVIRUS depicting the spatial spread of soybean mosaic virus (SMV) at various sampling times within a field plot of 'Corsoy 79' soybeans in 1992. Spread of SMV within the plot is depicted at successive sampling times from left to right and top to bottom. Numbers within boxes indicate the sampling period in which SMV was first detected within a quadrat. Red quadrats depict the detection of SMV G-5 released from a point source in the plot, the yellow quadrats depict non-G-5 SMV isolates arising from exogenous sources, and the green quadrats depict the nondetection of SMV.

29,30). In our study, the immigration of exogenous SMV inoculum into field plots occurred randomly. Although the immigration of non-G-5 isolates did not affect the overall pattern of logistic temporal spread, inoculum arising from exogenous sources has the potential to alter the shape of a pathogen (or disease) progress curve, which could impact on the population growth model that best describes pathogen progress. In our study, the logistic model was adequate to describe the temporal spread of SMV, even though different modes and scales of dispersal were probably operating to facilitate SMV spread within and among soybean fields.

The immigration of exogenous inoculum also has the potential to mask the presence of virus gradients arising from local (within-field) sources (14). Because our study employed a complete census of sampling units over time, no sampling design was utilized. However, based on the aggregated patterns of SMV spread in our experiments, sampling designs that take into account the spatial aggregation of SMV over time would be recommended to provide unbiased estimates of the change in SMV incidence over time (27,29).

Polycyclic diseases including those caused by plant viruses can be successfully managed by (i) reducing initial inoculum (y_0) to levels approaching zero, (ii) reducing the rate of temporal virus progress (r), and (iii) reducing the time span (t) that host and patho-

gen populations interact. Among these three strategies, reducing the amount of effective inoculum (y_0) to zero is the strategy often sought by plant breeders and molecular biologists, because this "extreme" form of resistance is qualitative, easy to identify, and usually is conferred by a single gene or a small number of genes (2,12,15,27,43). However, for a number of plant-virus pathosystems (15,33,41), including the SMV-soybean pathosystem (5,22,23,26), disease-resistant cultivars have not remained durable when this strategy has been used. Thus, for SMV and related virus pathosystems in which the pathogen population is highly variable, alternative strategies that are strain (race)-nonspecific, such as host resistance that reduces the rate of plant-to-plant spread, may provide greater benefits in terms of efficacy and durability (1,32). To assess the biological impact of the rate-reducing resistance strategy on the rate of temporal spread and soybean yield, strain-specific virus-detection tools, sampling designs, and computer programs similar to the ones developed in our study will be necessary. Regardless of the resistance strategy used (i.e., reducing y_0 versus r), the biological and economic viability of future biotechnology-derived resistant host populations cannot be properly assessed until accurate and reliable virus detection and sampling protocols are developed.

Fig. 3. Spatial map generated by the software program EPIDUR depicting the spatial spread of soybean mosaic virus (SMV) at various sampling times within a field plot of 'Corsoy 79' soybeans in 1993. Spread of SMV within the plot is depicted at successive sampling times from left to right. Numbers within boxes indicate the sampling period in which SMV was first detected within a quadrat. Red quadrats depict the detection of SMV G-5 released from a point source in the plot, the yellow quadrats depict non-G-5 SMV isolates arising from exogenous sources, and the green quadrats depict the nondetection of SMV.

Fig. 4. Bar graph generated by the software program EPIVIRUS depicts the number of runs that could be expected to occur by chance (bars with diagonals) versus the number of runs that were actually observed (solid bars) for each date in 1992 that soybean quadrats (cultivar Corsoy 79, replication 1) were tested for the presence of SMV (all isolates).

TABLE 2. z scores^a for ordinary runs analyses for soybean quadrats testing positive for soybean mosaic virus G-5 in Ames, IA, during 1991 to 1994

Assessment date (day of year)	Plot number			
	1	2	3	4
1991				
224	1.09	1.08	1.20	1.09
239	-1.16	1.45	0.29	-1.42
246	-0.22	2.18	0.64	-2.34*
253	-2.69*	-1.18	-1.32	-2.79*
260	-5.09*	-0.77	-1.02	-0.58
1992				
209	-3.56*	-0.93	1.19	
216	-5.09*	-3.82*	-2.20*	
223	-4.94*	-5.39*	-1.87*	
230	-5.28*	-5.39*	-3.18*	
237	-4.13*	-3.49*	-5.76*	
244	-3.39*	-2.69*	-4.85*	
251	-4.61*	-2.15*	-4.33*	
258	-2.95*	-3.24*	-4.38*	
1993				
244	-4.32*	-2.36*	-4.79*	
251	-1.39	-0.91*	-4.35*	
258	-1.17	0.24	-4.02*	
265	-1.66*	0.62	-1.91*	
272	-3.61*	0.06	-1.17	
1994				
192	-0.26	1.16	1.10	
199	-0.26	-2.51*	0.10	
206	0.15	-2.20*	-0.58	
213	-0.86	-2.20*	-0.86	
220	-0.46	-2.12*	-1.60	
227	-1.42	-1.57	-3.54*	

^a A z score followed by * identifies a value less than -1.65 ($P = 0.05$), which indicated nonrandomness.

TABLE 3. z scores^a for ordinary runs analyses for soybean quadrats testing positive for non-G-5 soybean mosaic virus in Ames, IA, during 1993 to 1994^b

Assessment date (day of year)	Plot number		
	1	2	3
1993			
244	-1.41	LI	LI
251	-1.41	LI	LI
258	-1.11	-1.20	LI
265	-0.43	-1.20	-0.06
272	-0.69	-0.68	-0.23
1994			
192	LI	LI	LI
199	LI	-1.82*	LI
206	-1.82*	-1.16	LI
213	-0.73	-1.10	LI
220	-0.73	-1.10	LI
227	-1.30	-0.85	-0.59

^a A z score followed by * identified a value less than -1.65 ($P = 0.05$), which indicated nonrandomness. LI = incidence of non-G-5 SMV isolates too low to analyze by ordinary runs.

^b No non-G-5 isolates were detected in 1991 and the incidence of non-G-5 isolates in 1992 was too low ($<2\%$) to analyze.

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ACKNOWLEDGMENTS

Research was supported by State and Hatch Funds allocated to the Iowa Agriculture and Home Economics Experiment Station and, in part, by the Iowa Soybean Promotion Board, Pioneer Hi-Bred International, Inc., and the Iowa Center for Advanced Technology Development. Journal paper no. 16769 of the Iowa Agriculture and Home Economics Experiment Station, project no. 3394.

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