Temporal and Spatial Spread of *Soybean mosaic virus* (SMV) in Soybeans Transformed with the Coat Protein Gene of SMV

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ABSTRACT

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Soybean lines transformed with the coat protein (CP) gene of *Soybean mosaic virus* (SMV) were evaluated for SMV resistance by quantifying the temporal and spatial spread of SMV strain AL-5 released from a point source in the field. The temporal spread of SMV within field plots during 1999 and 2000 was quantified by enzyme-linked immunosorbent assay. The Gompertz model most appropriately described temporal spread. Two SMV CP transformed lines (genotypes) had significantly lower infection

rates and significantly lower final SMV incidence values ($P \leq 0.05$) compared with controls that did not contain the CP gene. Ordinary runs analysis revealed that the spatial pattern of SMV-infected quadrats was more clustered in plots with higher SMV infection rates. Soybean lines with the lowest infection rates had significantly higher yields in 2000 and significantly less seed coat mottling compared with the controls. To our knowledge, this is the first field study demonstrating the effectiveness of pathogen-derived resistance on the temporal and spatial dynamics of pathogen spread in soybean.

Additional keywords: potyvirus, virus epidemiology.

Soybean mosaic virus (SMV) causes one of the most common viral diseases of soybean and is found throughout most soybean production areas in the world (9,14,21). Infection by SMV can result in a broad array of symptoms ranging from visually undetectable to quite severe (7,10). Symptoms may be masked at temperatures above 27°C, although many soybean cultivars do not express strong symptoms regardless of temperature (12,25,49).

Infection by SMV may result in severe yield losses due to abortion of flowers, reduced pod set, reduction in the number and weight of seeds (21,43), and early plant infection usually results in greater losses (3,19,43). Reduced grain quality, as a result of SMV-induced seed discoloration (often described as hilum bleeding or seed coat mottling), can also occur. The presence of seed coat mottling is important because financial penalties may be assessed when mottled grain is brought to the grain elevator (3). In the major soybean-production areas of the northern United States, the primary inoculum source of SMV is thought to originate from SMV-infected seed (23). Secondary spread may occur within and among soybean fields through the activity of more than 30 different aphid species that can acquire and transmit SMV (16,20,30).

Several single resistance genes to SMV infection have been identified (4,7,10,21,26,29); however, resistance-breaking SMV strains may emerge due to the strong directional selection pressure that is created when single resistance genes are deployed (8,9,11). An alternative approach to the single gene resistance strategy is a strategy that exerts a rate-reducing resistance that is effective against all SMV strains. This strategy allows some infection within a plant population, yet reduction in the rate of plant-to-plant pathogen spread minimizes any adverse effects on grain yield and quality (37,38). Reductions in pathogen spread may be due to reduced host receptivity, slowed virus replication within cells, reduced translocation within infected host cells and plants, and/or

a reduction in acquisition or transmission efficiencies by insect vectors (34). For example, this resistance strategy effectively reduced the rate of *Tobacco etch virus* epidemics in bell pepper without a breakdown of resistance. Presumably this was due to decreased selection pressure for the emergence of new virus strains (37).

In addition to the more traditional approaches of plant breeding programs that introduce single resistance genes into adapted host cultivars, disease resistance may also be achieved by the insertion of genes, gene sequences, or sequences of nucleic acids from the genome of a plant pathogen (44). This form of resistance, termed pathogen-derived resistance, typically involves insertion of viral coat protein (CP) genes. The effectiveness of this method to obtain resistance to plant viruses was first demonstrated with transgenic tobacco expressing the CP of *Tobacco mosaic virus* (39). The development and use of pathogen-derived resistance has subsequently been achieved by other researchers (1,27,28,32,45–48); however, the resistance selection criteria have typically been for plant genotypes that express near immunity to the pathogen, resulting in potentially strong directional selection for resistance-breaking strains (40,45–48).

A chimeric CP gene of SMV strain N was recently transformed into soybean cv. 9341 to yield six lines (50). Four of the lines retained the CP gene, while two of the lines were segregants that did not retain the CP gene. Three of the four transformants that retained the insertion expressed CP, whereas one line (line 7B-11) did not. Greenhouse and growth chamber tests with mechanically transmitted SMV showed at least two of the transformed soybean lines had reduced virus incidence and delayed symptom development when challenged with four different SMV strains. It was our hypothesis that some of these transgenic soybean lines may provide adequate levels of rate-reducing resistance at the host population level in which varying numbers of viruliferous aphids and SMV strains may challenge the pathogen-derived resistance throughout the season (40). Therefore, it was deemed necessary that these soybean lines be evaluated using epidemiological models to quantify and compare the effects of transgenic soybean lines on SMV epidemics in the field (35,36). The objectives of

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this study were to conduct field experiments to quantify and compare the effect of transgenic and nontransgenic soybean lines on the temporal and spatial dynamics of SMV epidemics and to quantify the effects of transgenic resistance and nontransgenic soybean lines on components of soybean yield and seed quality.

MATERIALS AND METHODS

Field plots. Field plots were planted on 26 May 1999 and 24 May 2000 at the Iowa State University Curtiss Farm in Ames, Iowa. SMV-free seed of five transformed lines (3-24, 3-3, 7B-11, 6-13-1, and 7B-10-9), as well as the nontransgenic isoline (cv. 9341), were planted each year (total of six treatments). One soybean line (7B-10-9) was transformed, but was selected as a segregant (treatment control) because it did not retain the SMV CP transcript (50).

The six treatments (soybean genotypes) were planted in a randomized complete block design with three replications. Each plot consisted of eight rows, 10.5 m long and 6 m wide, with a row spacing of 0.75 m. A 9 m area of tilled earth was maintained between plots in order to reduce interplot interference in 1999, while two rows of soybean cv. Kenwood 94 were planted within each buffer area in 2000 to serve as trap plants (insecticide-treated) for bean leaf beetles (*Cerotoma trifurcata* [Forster]), which were prevalent in Iowa during both years of the study. Seed was planted within plots at a density of six seeds per 30 cm, and later thinned to four plants per 30 cm. Plant borders for each plot consisted of the two outside rows, as well as 1.5 m of row surrounding both ends of each plot; thus, there were six sampling rows, each 7.5 m long. Sampling rows were divided with wooden stakes into 30 cm quadrats (25 per row). The soybean seed in quadrat position 13 of rows three and four were removed after planting and replaced with SMV susceptible cv. Williams 82. After soybean plants had reached growth stage V-3 (two trifoliate nodes above the unifoliate node with fully developed leaves) (17), the 'Williams 82' plants (in the 13th quadrats) were mechanically inoculated with aphid-transmissible SMV strain AL-5 (GenBank Accession No. AF242844) on 25 and 26 June 1999 and on 3, 4, and 22 July 2000. These SMV-inoculated plants served as point sources of inoculum for pathogen (SMV) spread within each plot.

Data collection. This study utilized a systematic, quadrat-based sampling design with all quadrats within a plot being sampled (36). Each quadrat (30-cm section of row) was considered a sampling unit, and all individual plants within a quadrat were sampled and combined according to the methods of Nutter et al. (36). This involved recording the row and quadrat position of each quadrat sampled within a soybean plot (150 quadrats per plot). Sampling was done by removing the newest fully expanded trifoliate leaf (near the plant apex) from each plant within a quadrat and placing these leaves into prelabeled plastic bags. Each quadrat was sampled weekly beginning 7 to 10 days after inoculation of the 'Williams 82' quadrats, with 10 sampling dates in both 1999 and 2000.

Virus assay. Quadrat samples were refrigerated until sap was expressed from leaves with a sap extractor (Ravenel Specialties Corp., Seneca, SC). Sap from leaves collected from each quadrat was diluted in approximately 5 ml of 0.05 M sodium phosphate buffer, pH 7.2, and samples were frozen at -20°C until testing in duplicate wells by biotin-avidin double antibody sandwich enzyme-linked immunosorbent assay (ELISA) (15). As previously shown (50), expression of SMV CP in soybean leaves of lines 3-3, 3-24, and 6-13-1 was not detectable by ELISA.

The ELISA used anti-SMV monoclonal S4 capture antibody (1.0 $\mu\text{g/ml}$) (22), coated onto Immulon 1B polystyrene microtiter plates (Dynex Technologies, Inc., Chantilly, VA), and biotinylated polyclonal anti-SMV antibody (0.5 $\mu\text{g/ml}$). Alkaline phosphatase conjugated Extravidin (1:40,000) (Sigma Chemical Co., St. Louis) was followed by *p*-nitrophenyl phosphate (1 mg/ml). A quadrat

sample was considered positive for SMV if the absorbance value of duplicate wells was equal to or greater than twice the value of the mean of the negative controls (sap from healthy soybean leaves of plants grown in the greenhouse). It is important to note that SMV CP expression in leaves can be detected by a sensitive-enhanced western blot assay; however, SMV CP could not be detected in leaves by ELISA (50). Pathogen incidence for each soybean plot was determined for each sampling date by dividing the number of quadrats testing positive for SMV by the total number of quadrats in each plot (150) and multiplying by 100.

Temporal analysis. To aid in the determination of which pathogen growth model was most appropriate to quantify and compare all SMV epidemics, the percentages of infected quadrats versus time (as affected by soybean line) and the change in virus incidence versus time (dy/dt) were first graphed for each epidemic (plot), and then examined (33,35). Based on the shapes of these curves, the mean values for pathogen incidence were transformed (18) using three potential models that might best fit all data (33, 35). These models were the linear (nontransformed), the logistic, and the Gompertz population growth models (Statistical Analysis System, SAS Institute, Cary, NC) and the EPIMODEL program (35). Simple linear regression of linear and transformed incidence values versus time were used to estimate model parameters and statistics. The *F* statistic, the coefficient of determination (R^2), the standard error of the estimate for transformed pathogen incidence (SEE_t), and a visual inspection of residual plots were used to determine the goodness of fit of each population growth model (33,35). After selecting the most appropriate model, the slope of each regression line was used as a quantitative measure of temporal pathogen progress (infection rate of SMV). Slopes were tested for significance by testing for parallelism (18). In order to further compare the effect of soybean lines on temporal SMV dynamics, the day of year of epidemic onset for each epidemic was operationally defined as the day of year on which pathogen incidence reached the 0.05 level (5% incidence).

Spatial analysis. Maps of SMV-infected quadrats over time were used to analyze the spatial pattern of infected quadrats in each plot (epidemic). The EPIVIRUS program (F. W. Nutter, Jr., unpublished data) was used to perform ordinary runs analyses (6,31). A run was defined as a series of like events, in this case, a series of infected or noninfected quadrats (31). The number of observed runs was compared with the number of expected runs that could occur by random chance. This was done by calculating a *z* score for a one-sided *t* test, with a *z* score of less than -1.64 indicating rejection of the null hypothesis of randomness in favor of clustering ($P \leq 0.05$).

Soybean yield, yield components, and quality. Prior to harvest, the number of plants in each row was counted. After plant senescence and drying in the field, soybean plots were hand-harvested on 14 to 17 October 1999 and 13 October 2000 by cutting the plants at the soil line with pruning shears and placing all plants within a row in a burlap bag. The four central rows of each plot were individually harvested, except 'Williams 82' quadrats were harvested separately. Following harvest, plants in bags were further dried on a greenhouse bench. Soybean grain was cleaned with a stationary thresher and weighed. The 100-seed weight and the incidence of seed coat mottling (percent) were obtained for each soybean row. To determine the effect of pathogen-derived transgenic resistance on grain quality, an additional 100-seed subsample from each row was passed through an infrared grain analyzer (Infratec 1229; Tecator, Hoganas, Sweden) for protein and oil analysis.

The 100-seed weight sample from each row was combined for each plot and a 100-seed subsample of the 400 seeds from each plot was tested using immunocapture reverse transcription (RT) polymerase chain reaction (PCR) to determine the presence or absence of seedborne SMV (50). For detection of potential seedborne *Bean pod mottle virus* (BPMV), the same 100-seed

subsample was tested using a biotin-avidin ELISA as described for SMV, except anti-BPMV polyclonal antibody was substituted for anti-SMV antibodies.

Data analysis. Mean separations for the date of epidemic onset, final SMV incidence, yield, yield components, and quality assessments were tested for using the least significant difference (LSD) test ($P \leq 0.05$) (SAS Institute). In addition to analysis of variance (ANOVA), linear regression was used to quantify the relationship between final SMV incidence (x) and dependent variables (y) such as yield, yield components, and seed quality.

RESULTS

Pathogen progress curves for 1999 and 2000 (Fig. 1) demonstrated that there were significant differences among soybean genotypes with regards to the day of year that epidemic onset began (Table 1). For both years, epidemic onset was delayed the most by transgenic lines 3-24 and 7B-11 and began the earliest in

transgenic lines 6-13-1 (day of year 141 in 1999 and day of year 200 in 2000) and 3-3 (day of year 167 in 1999 and day of year 198 in 2000). The dates of epidemic onset for nontransgenic control cv. 9341 and segregant 7B-10-9 (transformed control) were similar (day of year 201 and 204 in 1999, and day of year 202 and 204 in 2000, respectively). The dates of epidemic onset in transgenic lines 3-24 and 7B-11 were significantly ($P \leq 0.05$) delayed beyond the date of epidemic onset in transgenic line 6-13-1 in 1999, whereas lines 3-24 and 7B-11 significantly delayed epidemic onset ($P \leq 0.05$) when compared with all other soybean lines in 2000 (Table 1).

Although final SMV incidence was somewhat lower for all soybean lines at the end of the 1999 growing season compared with the 2000 season, the order of final SMV incidence levels among lines was highly consistent. Final pathogen incidence in 1999 was highest in soybean line 3-3, with 35% of the quadrats infected by SMV at seasons end, whereas lines 3-24 and 7B-11 had significantly ($P \leq 0.05$) lower final SMV incidence values of approximately 8% (Table 1). Nontransgenic cv. 9341 had a final pathogen incidence of 21% in 1999, whereas final incidence in lines 7B-10-9 and 6-13-1 were 23 and 26%, respectively. In 2000, all soybean lines had higher levels of final incidence relative to the 1999 season, with the highest final incidence (68%) occurring in nontransformed control cv. 9341. Final incidence was significantly lower in lines 3-24 (23%), 7B-11 (26%), and 6-13-1 (37%) in 2000 compared with all other soybean lines (Table 1). Final pathogen incidence was 53% in transgenic line 7B-10-9 (the transformed control) and 56% in transgenic line 3-3, both of which were not significantly different from nontransgenic control cv. 9341.

Overall, the rate of temporal SMV infection was faster in 2000 compared with the 1999 growing season. Based on the evaluation criteria for model selection (F test, R^2 , lowest SEE, and examination of residuals), the 1999 and 2000 SMV epidemics were best described by the Gompertz model (33,35). F statistics ranged from 64.37 to 640.59, and all were highly significant ($P \leq 0.0001$), indicating a very strong linear relationship between the change in gompit pathogen incidence and time. Coefficients of determination (R^2) were high, ranging from 0.87 to 0.99, indicating that time explained 87 to 99% of the variation in the change in gompit pathogen incidence (33) (Table 2). Standard errors of the estimate for y were low (0.03 to 0.18 gompits), which indicated that time could be used to precisely predict pathogen increase with respect to time (as affected by soybean genotype and year).

In 1999, infection rates ranged from 0.005 gompits per day (line 3-24) to significantly higher ($P \leq 0.05$) rates of spread in control line 7B-10-9 (0.015 gompits per day) and nontransformed control cv. 9341 (0.014 gompits per day) (Table 2; Fig. 2). Transgenic lines 7B-11 and 6-13-1 had significantly lower infection rates of 0.007 and 0.010 gompits per day, respectively, compared with control cv. 9341. Transgenic line 3-3 had an infection rate of

Fig. 1. Soybean mosaic virus (SMV) incidence in transgenic soybean lines and nontransgenic cv. 9341 during **A**, 1999 and **B**, 2000 in Ames, IA. Dashed line indicates the 5% incidence level, operationally defined as epidemic onset.

TABLE 1. Time to 5% Soybean mosaic virus (SMV) incidence (day of year) and final incidence for transgenic soybean lines and nontransformed cv. 9341 in the 1999 and 2000 epidemics in Ames, IA^a

Soybean line	Time to 5% incidence		Final incidence	
	1999	2000	1999	2000
3-24	211 a	225 a	8.4 c	23.1 d
3-3	167 ab	198 b	35.1 a	56.0 ab
7B-11	230 a	221 a	8.2 c	26.0 cd
6-13-1	141 b	200 b	26.0 ab	37.1 b-d
9341 ^y	201 ab	202 b	20.7 b	68.0 a
7B-10-9 ^z	204 ab	204 b	22.7 b	53.3 a-c

^a Numbers followed by the same letter within the same column are not significantly different from each other at $P \leq 0.05$ according to least significant difference.

^y Nontransgenic soybean cultivar used as a control.

^z Transformed soybean line, selected as a segregant that did not retain the SMV coat protein transcript, used as a control.

0.012 gompits per day, which was not significantly different from the controls (cv. 9341 and 7B-10-9).

Infection rates in the 2000 epidemics were much faster than in 1999, with infection rates ranging from 0.015 gompits per day (line 3-24) to a significantly higher rate of 0.040 gompits per day in the nontransgenic control cv. 9341 ($P \leq 0.05$) (Table 2; Fig. 2). Transgenic lines 6-13-1 and 7B-11 had significantly slower infection rates (0.022 gompits per day and 0.017 gompits per day, respectively) than line 3-3 and the controls 7B-10-9 and cv. 9341. The infection rates of 0.031 and 0.033 gompits per day in lines 3-3 and 7B-10-9, respectively, were approximately twice as fast as rate-reducing lines 3-24 and 7B-11.

Spatial maps were generated by mapping the x - y coordinates of infected quadrats using the software program EPIVIRUS. For both years, SMV spatial patterns were somewhat variable; however, within-field spread was generally more clustered in plots with the highest temporal rates of pathogen spread (Tables 2 and 3). In 1999, rate-reducing transgenic line 3-24 showed random spatial patterns in all three replications as did rate-reducing transgenic line 6-13-1 (Table 3). Virus epidemics in the transgenic rate-reducing line 7B-11 plots were mostly random except for replication two, which displayed limited clustering between day of year 214 and 239 (2 August to 27 August). Soybean line 3-3, a non-rate-reducing transgenic line, also exhibited mostly random spatial patterns; however, some clustering did occur after day of year 239 (27 August) in replicate two. The susceptible nontransgenic control cv. 9341 exhibited strong clustering in all replicates, beginning on day of year 207 in replication one. Control line 7B-10-9 also exhibited clustering of virus-infected quadrats as the season progressed, but significant clustering occurred somewhat later in the season compared with cv. 9341.

As in 1999, ordinary runs analysis of the 2000 epidemics showed random spatial patterns of infected quadrats for all three replications of rate-reducing transgenic line 3-24 (Table 4). Virus-infected quadrats in rate-reducing transgenic line 6-13-1 were generally randomly distributed throughout time and space. Rate-reducing transgenic line 7B-11 showed some clustering, but only very late in the season (after day of year 250, 7 September) for all three replications. Soybean transgenic line 3-3 had clustered spatial patterns beginning on day of year 220 and 227 for replications one and two, respectively, whereas spatial patterns of infected quadrats in replication three were random during the entire sea-

son. Nontransformed soybean cv. 9341 showed little clustering in replications one and two, but significant clustering occurred in replication three beginning day of year 220 until the end of the season. Control line 7B-10-9 exhibited significant clustering of infected quadrats beginning on day of year 220 in replications one and three, whereas SMV-infected quadrats in replication two were randomly distributed throughout the season.

In 1999, soybean yields among transgenic and control lines showed few significant differences among treatments (ANOVA), with rate-reducing transgenic line 6-13-1 having the highest yield (746.3 kg/ha) (Table 5). This line was significantly different ($P \leq 0.05$) from line 3-3, which had a yield of 656.2 kg/ha (Table 5). None of the remaining soybean lines were significantly different from either of these two lines. In 2000, however, two rate-reducing transgenic lines (6-13-1 and 3-24) had significantly higher yields (370.2 and 383.4 kg/ha, respectively) than nontransformed control soybean cv. 9341 (Table 5). Regression of final SMV incidence on yield revealed that there was not a significant linear relationship between SMV incidence and yield in 1999. In 2000,

TABLE 2. Gompertz model parameters and statistics describing the progress of *Soybean mosaic virus* (SMV) strain AL-5 in transgenic soybean lines and nontransformed cv. 9341 during 1999 and 2000 in Ames, IA

Year and soybean line	Intercept	Slope ^w	R ²	SEE _y ^x
1999				
3-24	-2.29	0.005 a	0.94	0.04
3-3	-3.24	0.012 b	0.99	0.04
7B-11	-2.66	0.007 a	0.96	0.04
6-13-1	-2.71	0.010 c	0.97	0.05
9341 ^y	-3.80	0.014 b-d	0.89	0.12
7B-10-9 ^z	-4.07	0.015 bd	0.95	0.08
2000				
3-24	-4.45	0.015 a	0.87	0.13
3-3	-7.29	0.031 b	0.95	0.18
7B-11	-4.91	0.017 ac	0.95	0.09
6-13-1	-5.49	0.022 ac	0.92	0.16
9341 ^y	-9.19	0.040 d	0.97	0.15
7B-10-9 ^z	-7.81	0.033 bd	0.95	0.18

^w Numbers followed by the same letter within the same column and year are not significantly different from each other at $P \leq 0.05$ according to least significant difference.

^x Standard error of the estimate for y .

^y Nontransgenic soybean cultivar used as a control.

^z Transformed soybean line, selected as a segregant that did not retain the SMV coat protein transcript, used as a control.

Fig. 2. Gompertz transformed *Soybean mosaic virus* (SMV) incidence in transgenic soybean lines and nontransgenic cv. 9341 during A, 1999 and B, 2000 in Ames, IA.

however, there was a significant linear relationship ($P \leq 0.10$, $R^2 = 0.54$), with soybean yield decreasing by 1.87 kg/ha for each 1% increase in final SMV incidence (Fig. 3A).

Using ANOVA and LSD mean comparisons, few significant differences occurred among soybean lines in 1999 for the number of seeds per plant or weight of seeds per plant. One hundred-seed weight, however, was significantly greater in transgenic line 7B-10-9 (13.7 g) compared with line 7B-11 (13.3 g) (Table 5). In 2000, the number of seeds per plant was significantly greater ($P \leq 0.05$) in rate-reducing lines 3-24 and 6-13-1 (169.6 and 170.5 seeds per plant, respectively) compared with the nontransgenic control cv. 9341 (105.5 seeds per plant) (Table 5). Seed weight per plant had similar results with rate-reducing lines 3-24 (at 21.2 g of seed per plant) and 6-13-1 (at 20.2 g of seed per plant) having sig-

nificantly higher seed weights than the nontransgenic control cv. 9341 (12.7 g of seed per plant).

Regression of final SMV incidence on yield components (number of seeds per plant, seed weight per plant, and 100-seed weight) revealed no significant relationships in 1999. In 2000, however, regression of final SMV incidence on seed weight per plant was significantly linear ($P \leq 0.09$, $R^2 = 0.56$) with seed weight per plant decreasing by 0.13 g for each 1% increase in final SMV incidence (Fig. 3B).

Using ANOVA and LSD mean comparisons, seed quality (as determined by measuring the percentage of soy protein and soy oil in the harvested grain) showed no significant differences among soybean lines in 1999. In 2000, however, soy oil content was significantly higher ($P \leq 0.05$) in all soybean lines relative to the

TABLE 3. Ordinary runs analyses (z scores) for *Soybean mosaic virus* (SMV) epidemics as affected by transgenic soybean lines and nontransgenic cv. 9341 in Ames, IA, during the 1999 growing season^a

Soybean line	Replication	Day of year									
		187	194	200	207	214	221	228	239	249	259
3-24	1	18.149	18.149	18.149	18.149	11.706	11.706	7.136	3.224	1.574	1.574
	2	2.759	-0.959	-0.683	-0.683	-0.462	-0.462	-0.462	-0.256	-0.061	-0.061
	3	7.133	2.759	0.623	0.623	0.806	0.806	0.806	0.806	0.899	0.899
3-3	1	0.973	0.973	1.289	1.041	0.708	0.538	-0.434	-0.196	-0.487	0.579
	2	1.714	0.794	0.000	-0.692	-0.385	-1.388	-0.918	-1.797*	-1.766*	-2.457*
	3	3.872	3.872	0.522	-0.887	-1.417	-0.473	-0.331	-1.376	-1.234	-1.429
7B-11	1	18.149	18.149	8.783	8.783	3.224	1.552	0.225	0.225	0.225	0.225
	2	2.044	-0.892	-0.892	-0.892	-2.804*	-2.804*	-2.239*	-1.796*	-1.124	-0.462
	3	18.149	18.149	18.149	7.136	6.097	6.097	6.097	5.394	1.610	1.090
6-13-1	1	0.680	-0.496	-0.085	-0.085	-0.085	-0.085	-0.836	-0.845	-0.845	-0.387
	2	5.798	4.440	4.061	3.361	3.245	0.516	-0.115	0.515	0.515	0.308
	3	1.610	1.658	1.714	0.794	0.180	-0.445	-0.620	-0.692	-1.002	-1.002
9341 ^y	1	2.044	-0.892	-0.892	-1.796*	-2.086*	-2.501*	-2.540*	-2.693*	-3.480*	-3.156*
	2	18.149	7.099	4.984	3.791	-1.569	-1.315	-2.302*	-2.596*	-2.596*	-2.297*
	3	11.706	11.706	1.551	1.658	-2.281*	-2.542*	-2.542*	-2.196*	-2.558*	-2.723*
7B-10-9 ^z	1	18.149	18.149	8.783	6.097	2.768	1.304	1.440	-1.289	-1.805*	-1.784*
	2	1.784	1.784	1.784	-0.579	-0.920	-0.920	-1.387	-1.944*	-1.834*	-2.254*
	3	7.133	5.414	1.577	-0.123	0.794	-2.826*	-3.119*	-3.317*	-3.525*	-2.960*

^a A z score followed by * indicates a value less than -1.64, indicating nonrandomness (i.e., clustering).

^y Nontransgenic soybean cultivar used as a control.

^z Transformed soybean line, selected as a segregant that did not retain the SMV coat protein transcript, used as a control.

TABLE 4. Ordinary runs analyses (z scores) for *Soybean mosaic virus* (SMV) epidemics as affected by transgenic soybean lines and nontransgenic cv. 9341 in Ames, IA, during the 2000 growing season^a

Soybean line	Replication	Day of year									
		192	199	206	213	220	227	234	241	250	259
3-24	1	18.149	18.149	18.149	18.149	18.149	18.149	18.149	4.061	0.000	-1.257
	2	18.149	6.097	6.097	5.394	4.897	4.897	2.768	2.876	2.150	3.135
	3	43.856	11.706	11.706	8.783	8.783	8.783	2.178	1.678	1.678	-0.389
3-3	1	43.856	1.577	0.994	-0.393	-3.480*	-4.499*	-4.300*	-4.003*	-3.853*	-3.829*
	2	43.856	8.783	8.783	0.378	-0.743	-2.420*	-2.587*	-2.353*	-1.892*	-1.634
	3	5.798	-0.445	-0.239	0.273	-0.320	-0.236	0.171	0.319	0.514	0.469
7B-11	1	0.000	0.000	0.000	7.133	7.133	7.133	7.133	0.060	-3.068*	-3.480*
	2	0.000	43.856	18.149	4.984	3.458	3.458	3.458	3.255	-1.970*	-1.717*
	3	0.000	7.133	1.647	1.647	1.647	0.714	-2.876*	-2.873*	-2.873*	-2.106*
6-13-1	1	0.000	2.387	-0.045	-2.083*	-1.824*	-1.313	-1.313	-1.313	-0.933	-0.792
	2	43.856	8.783	8.783	8.783	7.136	1.552	-1.329	-1.267	-1.267	-0.356
	3	18.149	4.984	-2.126*	-0.678	-0.185	-0.098	0.495	-0.474	-0.417	-0.690
9341 ^y	1	8.783	7.136	3.224	-1.531	-3.738*	-1.940*	1.468	1.162	0.708	0.517
	2	8.783	8.783	2.923	1.304	0.365	-2.475*	-1.133	-0.446	-0.118	-0.172
	3	18.149	18.149	7.133	0.000	-3.719*	-3.177*	-3.823*	-3.117*	-3.117*	-3.209*
7B-10-9 ^z	1	43.856	7.133	4.460	1.489	-1.766*	-3.829*	-3.660*	-4.325*	-3.922*	-3.768*
	2	18.149	5.414	3.872	1.179	-1.634	0.440	-0.357	-0.805	-0.892	-0.458
	3	18.149	18.149	18.149	0.225	-2.448*	-2.998*	-3.442*	-2.169*	-1.790*	-1.116

^a A z score followed by * indicates a value less than -1.64, indicating nonrandomness (i.e., clustering).

^y Nontransgenic soybean cultivar used as a control.

^z Transformed soybean line, selected as a segregant that did not retain the SMV coat protein transcript, used as a control.

nontransgenic control cv. 9341, whereas soy protein was significantly higher ($P \leq 0.05$) in the nontransformed control cv. 9341 compared with all other soybean lines (Table 6).

As with ANOVA, regression of final SMV incidence on soy oil (percent) and soy protein (percent) in 1999 revealed no significant relationships; however, regression of final SMV incidence on soy oil (percent) in 2000 showed a significant negative linear relationship ($P \leq 0.01$, $R^2 = 0.82$) as oil content decreased 0.02% for each 1% increase in final SMV incidence (Fig. 4A). Regression of final SMV incidence on protein (percent) in 2000 also revealed a significant (but positive) linear relationship ($P \leq 0.03$, $R^2 = 0.73$) with soy protein increasing by 0.03% for every 1% increase in final SMV incidence (Fig. 4B).

Seed coat mottling, an important soybean quality factor in that low seed coat mottling is desirable, was significantly reduced by the rate-reducing transgenic lines in both 1999 and 2000. Generally, mottling of seeds from all transgenic lines was less than that observed on the transgenic control line 7B-10-9 and the nontransformed control cv. 9341. Based on regression analysis, the relationship between final SMV incidence and percentage of seed coat mottling in 1999 was not significant; however, in 2000, there was a significant linear relationship ($P \leq 0.06$, $R^2 = 0.62$) showing that the percentage of seed coat mottling increased by 0.30% for each 1% increase in final SMV incidence (Fig. 4C).

In 1999, seedborne SMV was detected by immunocapture RT-PCR only in the nontransgenic control cv. 9341; however, in 2000, seedborne SMV was detected in all lines except rate-reducing transgenic lines 3-24 and 7B-11. Harvested seed was also tested by quantitative ELISA for the presence of BPMV, which was found in all soybean seed harvested from the plots in 1999 and 2000. There was a significant linear relationship between the relative amount of BPMV detected and the incidence of seed mottling in both 1999 ($P \leq 0.09$, $R^2 = 0.54$) and 2000 ($P \leq 0.06$, $R^2 = 0.61$) (Fig. 5). Relative amounts of seedborne BPMV were higher in 2000 than in 1999, when final SMV incidence was lower.

DISCUSSION

The slope of the Gompertz-transformed SMV incidence values regressed against time can be used as an estimate of the rate of pathogen progress with respect to time to allow for the quantitative comparison of SMV epidemics as influenced by host genotype (33). Clearly, the highest rates of pathogen progress in 2000 occurred in soybean cv. 9341 and the control segregant 7B-10-9. Although considerably slower in 1999, the highest infection rates

occurred in the same lines in both years. Temporal rates of SMV infection in lines 3-24 and 7B-11 were significantly reduced, resulting in significantly higher yields than the controls in 2000. Although the overall rate of SMV spread in line 6-13-1 was also moderately reduced, epidemic onset occurred the earliest in this line in 1999. Epidemic onset in rate-reducing lines 3-24 and 7B-11 was consistently delayed. In addition to having the lowest infection rates in this study compared with soybean cv. 9341 in 1999, epidemic onset in line 3-24 occurred 10 days later. In 2000, epidemic onset in line 3-24 occurred 23 days later compared with the nontransgenic control cv. 9341. Epidemic onset in line 7B-11 was delayed by 29 days compared with cv. 9341 in 1999, whereas line 7B-11 delayed onset in 2000 by 19 days (compared with cv. 9341).

Fig. 3. Relationship between final *Soybean mosaic virus* (SMV) incidence in transgenic and nontransgenic soybeans during 1999 and 2000 in Ames, IA and **A**, yield and **B**, seed weight per plant. Points without a regression line indicate nonlinear relationships.

TABLE 5. Yield and yield components for transgenic soybean lines and nontransgenic cv. 9341 as affected by *Soybean mosaic virus* (SMV) during 1999 and 2000 in Ames, IA^x

Year	Soybean line	Yield/plot (kg/ha)	Number of seeds/plant	Seed weight/plant (g)	100-seed weight (g)
1999	3-24	696.6 ab	168.0 a	22.6 a	13.5 ab
	3-3	656.2 b	158.6 a	21.3 a	13.5 ab
	7B-11	679.6 ab	170.3 a	22.5 a	13.3 ab
	6-13-1	746.3 a	174.0 a	23.1 a	13.3 ab
	9341 ^y	698.5 ab	164.1 a	21.9 a	13.3 ab
	7B-10-9 ^z	715.3 ab	159.6 a	21.9 a	13.7 a
2000	3-24	383.4 a	169.6 a	21.2 a	12.5 a
	3-3	329.1 ab	145.3 ab	17.0 ab	11.7 a
	7B-11	315.3 ab	140.4 ab	16.7 ab	11.9 a
	6-13-1	370.2 a	170.5 a	20.2 a	11.9 a
	9341 ^y	254.4 b	105.5 b	12.7 b	12.1 a
	7B-10-9 ^z	323.7 ab	148.4 ab	17.6 ab	12.0 a

^x Numbers followed by the same letter within the same column and year are not significantly different from each other at $P \leq 0.05$ according to least significant difference.

^y Nontransgenic soybean cultivar used as a control.

^z Transformed soybean line, selected as a segregant that did not retain the SMV coat protein transcript, used as a control.

Seed transmission of SMV is greatly reduced if infection occurs after the onset of flowering (2). We suggest that rate-reducing lines that delay the onset of SMV epidemics until well after flowering (as occurred in our study) would significantly reduce seed transmission. In 1999 and 2000, flowering occurred in 50% of the soybean plants on day of year 207 and 205, respectively. Lines 3-24 and 7B-11 reached epidemic onset 4 and 23 days post-flowering in 1999 and 20 and 16 days postflowering in 2000, respectively. Conversely, seedborne SMV was detected in all other lines when epidemic onset of these lines occurred prior to flowering during both 1999 and 2000. Results of this study are consistent with this hypothesis because no seedborne SMV was detected in lines 3-24 and 7B-11. The delay in epidemic onset for both 3-24 and 7B-11, relative to time of flowering, should significantly reduce (or possibly eliminate) virus transmission to seed from season to season. This may substantially affect the amount of initial inoculum available for future epidemics.

The data are very consistent with regards to the rank order of final SMV incidence. The highest final SMV incidence (68%) occurred in 2000 on the nontransgenic control cv. 9341. In 1999, final incidence was highest in line 3-3 (35%). The final incidence levels are comparable to those reported by Nutter et al. (36) in experiments with the soybean cv. Corsoy 79, performed from 1991 to 1994 with SMV strain G-5. Using the same sampling design and testing procedures, the final SMV incidence for transgenic line 3-3 in 1999 was similar to the approximate 45% final incidence reported on 'Corsoy 79' in 1994 (36). The final incidence on nontransgenic cv. 9341 in 2000 was similar to the approximate 65% final incidence reported by Nutter et al. (36) in 1991. Variation in the SMV epidemics that occurred in 1999 versus 2000 may be related to differing aphid species and populations, differences in relative transmission efficiencies of aphid vectors, the timing of aphid flights, different soybean cultivars, and/or environmental factors that affect plant growth and development. It is also possible that differences such as relative aggressiveness of the predominant infecting SMV strains might have led to the different temporal patterns of virus spread from season to season. It is also possible that the effect of the origin of the rate-reducing SMV CP genes may be different depending on the predominant infecting SMV strains present in the field. However, we do not expect the efficacy of rate-reducing resistance to vary significantly with SMV strain because of the high sequence identity in the CP gene of SMV strains (50).

Clustering of infected quadrats, as revealed by ordinary runs analyses, may have been influenced by the relative susceptibility

of a given line in terms of the rate of SMV disease progress. The amount of clustering compares well with rates of pathogen progress, because higher amounts of clustering in this study appear to be associated with higher rates of temporal virus spread. A susceptible genotype should have a greater probability of infection when challenged with a virus, resulting in a greater probability of clustering if vectors predominately travel short distances after acquiring the virus (24). There does not appear to be any pattern as to how the soybean field plots with significant patterns of clustering were arranged in the field in either 1999 or 2000.

TABLE 6. Quality factors for transgenic soybean lines and nontransgenic cv. 9341 as affected by *Soybean mosaic virus* (SMV) during 1999 and 2000 in Ames, IA^x

Year	Soybean line	Oil (%)	Protein (%)	Mottled seed (%)
1999	3-24	17.0 a	35.2 a	9.8 c
	3-3	17.2 a	35.3 a	8.3 c
	7B-11	17.1 a	35.4 a	11.2 c
	6-13-1	17.2 a	35.0 a	24.0 b
	9341 ^y	17.2 a	35.2 a	33.0 a
	7B-10-9 ^z	17.0 a	35.3 a	21.3 b
2000	3-24	18.0 a	34.0 c	16.1 bc
	3-3	17.5 b	34.9 b	21.8 b
	7B-11	17.9 ab	34.6 bc	21.3 b
	6-13-1	17.8 ab	34.4 bc	15.3 c
	9341 ^y	16.9 c	35.8 a	31.5 a
	7B-10-9 ^z	17.7 ab	34.6 bc	30.2 a

^x Numbers followed by the same letter within the same column and year are not significantly different from each other at $P \leq 0.05$ according to least significant difference.

^y Nontransgenic soybean cultivar used as a control.

^z Transformed soybean line, selected as a segregant that did not retain the SMV coat protein transcript, used as a control.

Fig. 4. Relationship between final *Soybean mosaic virus* (SMV) incidence in transgenic and nontransgenic soybeans during 1999 and 2000 in Ames, IA and A, percent oil; B, percent protein; and C, percent mottling incidence. Points without a regression line indicate nonlinear relationships.

In 2000, ordinary runs analyses identified predominant clustering only in the non-rate-reducing lines (3-3, 9341, and 7B-10-9). Significantly fewer clustered quadrats were found in rate-reducing lines 7B-11 and 6-13-1, and none were found in rate-reducing transgenic line 3-24. Random, as opposed to clustered spatial patterns, may have important implications for yield compensation. When SMV-infected soybean quadrats occur randomly in the field, as they did in the rate-reducing soybean lines, there is greater potential for yield compensation by neighboring healthy (non-SMV-infected) quadrats. Conversely, the clustering of SMV-infected quadrats within a field would limit the crops' ability to compensate.

Using ANOVA and mean separation analyses, few significant yield differences occurred among soybean lines in 1999. However, in 2000, when final SMV incidence and infection rates were higher, regression analyses revealed a significant linear relationship between final incidence and soybean yield, with final SMV incidence explaining 54% of the variation in soybean yield. The remaining 46% of the variation in yield was presumably due to other factors such as insects (e.g., bean leaf beetle feeding) and other pathogens (such as BPMV). During the years this study was conducted, populations of the bean leaf beetle, a vector of BPMV, were unusually high and BPMV was epidemic throughout Iowa. The presence of BPMV in our experimental plots was confirmed by detection of the seedborne virus in soybean seed harvested from the plots. Previous reports have shown that BPMV and SMV act synergistically (5) and yield of soybeans infected by both viruses may be more adversely affected (41–43). In 2000, the yield of nontransgenic cv. 9341 was significantly lower compared with the yields of the two best rate-reducing lines. The absence of any relationship between final virus incidence and 100-seed weight, and the variation in reports of effects of virus infection on 100-seed weight, are consistent with previous results (3,41–43).

Oil and protein content of seed showed few differences when tested by ANOVA, but regression of final SMV incidence for all soybean lines versus oil and protein in 2000 showed significant linear relationships, with final SMV incidence explaining 82 and 73% of the variation in soy oil and protein content, respectively. Previous work has also shown that virus infection can affect soy oil and protein (13).

Fig. 5. Relationship between the relative quantities of seedborne *Bean pod mottle virus* (BPMV) on incidence of seed mottling in soybean lines during 1999 and 2000 in Ames, IA. A ratio indicating the relative amounts of seedborne BPMV was calculated by dividing the average absorbance value from enzyme-linked immunosorbent assay for a specific soybean line (four wells per plot) by the average absorbance value of the negative controls.

Both SMV and BPMV induce seed coat mottling that is indistinguishable. Increased seed coat mottling in 2000 may be related to a synergistic interaction (5) between BPMV and the higher incidence of SMV in 2000 compared with 1999. The increased final incidence of SMV in 2000 could result in increased BPMV replication and the higher relative amounts of seedborne BPMV measured in 2000 compared with 1999. Increased relative amounts of seedborne BPMV detected in harvested seed were associated with detection of seedborne SMV (one of six lines in 1999, four of six lines in 2000). Consistent with Wang et al. (50), seedborne SMV was never detected in rate-reducing lines 3-24 and 7B-11. Coinfection of plants by SMV and BPMV may allow higher amounts of seedborne BPMV.

It is likely that BPMV was associated with seed mottling in both 1999 and 2000, whereas SMV incidence had an effect only in 2000 (Figs. 4C and 5). In 1999, there was not a linear relationship between percentage of seed coat mottling and final SMV incidence. In 2000, however, 62% of the variation in seed coat mottling could be explained by final SMV incidence (Fig. 4C). Therefore, mottling was attributed to factors other than SMV in 1999 (such as BPMV), whereas in 2000, 38% of the mottling was not explained by SMV incidence. In both 1999 and 2000, analyses demonstrated both a linear relationship between percent mottling and relative amounts of seedborne BPMV and relatively higher amounts of seedborne BPMV in 2000 than in 1999 (Fig. 5). We suggest that the higher mottling observed in 2000 was, at least in part, consistent with the higher final incidence of SMV, because the probability of coinfection by both viruses was very high. Since Wang et al. (50) reported that no mottling occurred in seeds harvested from SMV-inoculated plants of lines 3-24 and 7B-11 and no seedborne SMV was ever detected in these lines, we propose that most of the virus-induced mottling observed in these lines was likely caused by BPMV.

Results of this study suggest an alternative approach for evaluation of transgenic resistance to plant viruses. As opposed to the effect of transgenic genotypes that confer near-immunity to a pathogen, this study demonstrates that transgenic crop plants can reduce pathogen spread but allow some random infections in the plant population. In contrast to near-immunity, some SMV infection in the rate-reducing lines occurred, but most infection occurred late (after flowering) and late infection results in little or no effect on yield and quality (34,38). In addition, the random distribution of infected plants or quadrats allows the potential for yield compensation from neighboring healthy plants and quadrats. The rate-reducing transgenic tactic could increase durability, defined as the length of time during which a resistance tactic remains usable (37,38), and may be most useful in annual crop plants.

Further, the high sequence identity of the SMV CP gene suggests, as discussed by Wang et al. (50), that the rate-reducing resistance shown in lines 3-24, 7B-11, and 6-13-1 should remain durable. This previous report did not reveal potential for rate-reducing resistance in line 6-13-1. However, data from this study have shown that this line has some rate-reducing effects, although the rate of infection was not reduced to the extent of lines 3-24 and 7B-11. This report is, to our knowledge, the first demonstration of pathogen-derived resistance to any pathogen of soybeans in the field.

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