Enhancement by Soybean Mosaic Virus of Bean Pod Mottle Virus Titer in Doubly Infected Soybean

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ABSTRACT

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The concentration of bean pod mottle virus (BPMV) in soybean plants doubly infected with BPMV and soybean mosaic virus (SMV) was significantly higher than that in singly infected plants. The enhancement of BPMV titer in doubly infected plants was evident in both greenhouse and field-grown plants and was independent of the virus assay method used. The enhancement phenomenon was detected in doubly infected plants regardless of the timing, sequence, or means of inoculation with the two viruses. No significant differences in SMV concentration were detected between singly and doubly infected plants. The BPMV titer in singly infected plants, as determined by enzyme-linked immunosorbent assay (ELISA), varied with leaf position and could be correlated with symptom severity. In doubly infected plants, however, the variation in BPMV titer with leaf position was less pronounced. Although the BPMV titer, as determined by ELISA, remained relatively constant in the individual fully expanded trifoliolate leaves of singly and doubly infected plants, a decline in infectivity with age of infection was detected by local lesion assays. The yields of field-grown doubly infected plants of two soybean cultivars were significantly lower than that of plants singly inoculated with either SMV or BPMV.

In nature, mixed viral infections often occur in plants of soybean as well as in many other crops and their biological and epidemiological implications are beginning to be recognized (3,11). It is now well established that pairs of related or unrelated viruses can often replicate in the same cells and, depending on the combination, the viruses may interact synergistically or antagonistically (8,10). As a consequence of these interactions a new disease with more severe symptoms than those produced by single infections may develop, and/or significant alteration in the concentration of either or both viruses may occur (8,11).

Soybean mosaic virus (SMV) and bean pod mottle virus (BPMV) interact synergistically in soybean and result in more severe symptoms than the sum of the effects of single infections (13). Earlier reports on the effect of this interaction on the concentration of SMV and BPMV in doubly infected plants yielded conflicting results (9,12,16). The virus assay methods used in these earlier studies (9,16) were far less sensitive and less accurate than presently available methods. The enzyme-linked immunosorbent assay (ELISA) was found to be highly sensitive and reliable in detecting and quantitating BPMV and SMV in infected plants (5). In this paper, ELISA as well as other assay methods were used to monitor the virus titer in singly and doubly infected soybean plants grown under greenhouse and field conditions. The relationship between virus concentration and symptom severity of doubly infected plants is discussed.

MATERIALS AND METHODS

Viruses. A subculture of the G-7 Kentucky isolate of BPMV (4) was used. The virus was maintained and increased in soybean (*Glycine max* (L.) Merrill 'York'). Purification of BPMV was by the procedure of Semancik and Bancroft (14) except that final purification was made by centrifugation on sucrose density gradients (100–400 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0) for 150 min in a Spinco SW27 rotor. The pooled middle and bottom components constituted the purified preparation.

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An isolate of SMV obtained from infected soybean seeds of plant introduction PI 85-663 was used. The virus culture was maintained and increased in the soybean cultivar Dare. Purification of SMV was by the following procedure: Infected leaves were homogenized (1:2, w/v) with 0.165 M disodium phosphate-0.018 M trisodium citrate buffer containing 0.01 M disodium EDTA, 0.1% sodium diethyldithiocarbamate, and 0.5% 2-mercaptoethanol. The final pH of the extraction buffer was 7.6. The homogenate was strained through two layers of cheesecloth and the filtrate was adjusted to 2% (v/v) with Triton X-100. The mixture was stirred overnight at 4 C and then centrifuged for 15 min at 8,000 *g*. The supernatant was layered on a cushion of 8 ml of 30% sucrose in 0.05 M sodium phosphate buffer, pH 7.6, containing 0.01 M EDTA and 0.5% Triton X-100 (PET buffer) and centrifuged for 3.5 hr at 27,000 rpm in a Spinco 30 rotor. The pellets were suspended in PET buffer and clarified by centrifugation at 6,500 *g* for 10 min. Further purification was made by two cycles of equilibrium zonal density gradient centrifugation in CsCl (initial density of 1.36 g/ml).

Serology. Antisera to BPMV and SMV were produced in rabbits by a series of intravenous and subcutaneous injections. The microprecipitin titers of these antisera were 1/8,192 and 1/1,024, respectively. The double antibody form of ELISA was the main virus assay method used in this study. Procedures for ELISA of BPMV and SMV were as previously described (5). Leaf extracts were prepared for the ELISA test by homogenization of tissues with an extraction buffer (PBS-PVP-T) comprised of 0.02 M phosphate buffered saline (PBS) containing 2.0% polyvinylpyrrolidone (PVP, MW 40,000), 0.05% Tween-20, and 0.02% sodium azide. Tissue was homogenized in a Polytron homogenizer and using a PT20-generator (Brinkmann Instruments Inc., Westbury, NY 11590).

Greenhouse experiments. Two experimental schemes were used to compare SMV and BPMV titers in singly and doubly infected soybean plants. In the first, the virus titer in the youngest fully expanded trifoliolate leaf was monitored by ELISA at various intervals during a 45-day period following inoculation of the primary leaves with SMV, BPMV, or both. Samples of single leaflets were collected 13, 18, 22, 28, 35, and 45 days after inoculation. These dates corresponded to the times when all plants had reached the V2, V3, V4, V5, V6, and V7 stages, determined according to Fehr's system (2). Thus, leaflets sampled on day 13

after inoculation were from first trifoliolate leaves and those tested on day 45 after inoculation were from sixth trifoliolate leaves. In the second scheme, the virus titer in each of the first through the fifth trifoliolate leaves of singly and doubly infected plants was simultaneously measured by ELISA 40 days after inoculation.

The soybean cultivars Williams and Essex were used. Seeds were germinated in 10.2-cm (4-in.)-diameter plastic pots (two to four seeds per pot) containing Pro-Mix BX (Premier Brands Inc., New Rochelle, NY 10801). The plants were grown in a greenhouse under daylight supplemented with fluorescent light for a 14-hr photoperiod. The temperature in the greenhouse varied between 20–30 C. Treatments included inoculation with SMV alone, BPMV alone, or with a mixture of the two viruses. A control of uninoculated plants was also included. Virus inocula were prepared by grinding systemically infected leaves from soybean plants infected with either virus in a mortar and pestle with 0.05 M potassium phosphate buffer, pH 7.0. All inoculations were made by rubbing Carborundum-dusted leaves with inoculum-soaked cheesecloth pads. Inocula of SMV, BPMV, or a mixed inoculum of both viruses, were applied to the primary leaves of test plants 9–10 days after planting. In some experiments, the double inoculation treatments were performed sequentially by applying the first virus to the primary leaves and the second virus to the first trifoliolate leaves one week later. In all experiments, six plants were used per treatment.

Virus assay by density gradient centrifugation. The virus concentration in clarified extracts from singly and doubly infected plants was estimated by centrifugation in density gradients. Composite leaf samples comprised of all systemically infected leaves were collected from soybean plants 47 days after inoculation with SMV, BPMV, or both. The double inoculation treatments were applied sequentially as described before. Tissue samples of 25 g each were processed by the purification procedure for SMV and/or BPMV. To compare SMV concentration in singly and doubly infected plants, the partially purified preparations from the respective tissues were adjusted to equal volumes and 1-ml samples were layered over 4 ml of 36% (w/w) CsCl in 0.05 M sodium phosphate buffer, pH 7.6. The gradient tubes were centrifuged for 17 hr at 45,000 rpm in a Spinco SW65 rotor. In the case of BPMV, the respective partially purified preparations were layered over linear-log sucrose density gradients (1) and centrifuged for 90 min at 37,000 rpm in a Spinco SW40 rotor. In all cases, the centrifuged gradients were fractionated with an ISCO density gradient fractionator equipped with an ultraviolet (UV) analyzer absorbing at 254 nm. The weight of the chart paper beneath the virus peaks was determined and used as an index for virus concentration. Subsamples of clarified extracts from all preparations were also tested by ELISA and the results of the two assay methods were compared.

Specific infectivity of BPMV. The effect of age of infection on BPMV titer in singly and doubly infected plants was evaluated by both ELISA and infectivity assays on *Phaseolus vulgaris* L. Pinto, a local lesion host for BPMV. For this purpose, single leaflets of the same trifoliolate leaf from each test plant (six plants per treatment) were tested at different intervals after inoculations. Leaf extracts were prepared in PBS-PVP-T (1:50, w/v) and rubbed on eight half-leaves of the assay plant. The presence of SMV in extracts from doubly infected plants did not pose problems in scoring the results since the necrotic local lesions produced by BPMV infection were easily differentiated from the diffuse chlorotic lesions produced by SMV. For ELISA, samples of the leaf extracts were tested at a dilution of 1:1,500 (w/v).

Field experiment. The field experiment was conducted at a University of Kentucky farm in Lexington. The soybean cultivars Williams, Essex, and York were planted on 29 May 1980 in hill plots (15) consisting of 20 seeds planted within a linear distance of 45 cm. The hills were spaced 90 cm in one direction and 76 cm in the other. Four virus treatments were applied: SMV alone, BPMV alone, SMV followed by BPMV, and BPMV followed by SMV. In addition, a control of uninoculated plants was included. The cultivar/treatment combinations were completely randomized in a block design with three blocks containing three replications each. Thus, each cultivar/treatment combination was replicated nine times. Inocula of SMV or BPMV were applied to the primary leaves of the appropriate test plants 20 days after planting. In treatments involving inoculation with the two viruses, the second virus was applied to the first trifoliolate leaf 1 wk later. Two weeks after inoculation, the success of mechanical inoculation was evaluated and the hills were thinned to nine plants each. The percentage of virus-infected plants, as judged from symptom development, was 100% in all hills except those of the control and the York/SMV combination. The SMV isolate used in this study did not infect plants of cultivar York (S. A. Ghabrial, *unpublished*).

The SMV or BPMV titers in singly and doubly infected plants were monitored by ELISA at various intervals during the growing season. At each sampling date, single leaflets from young fully expanded trifoliolate leaves were collected from representative plants in each cultivar/treatment combination. Leaf extracts were prepared by homogenizing the tissues with PBS-PVP-T (1:20, w/v).

Seeds were harvested from all plants at maturity. Several harvests were required because of differing cultivar maturities. The seeds were dried to a moisture content of 8–9%, cleaned by hand, and weighed.

RESULTS

Symptoms on singly and doubly infected plants. Greenhouse-grown soybean plants, inoculated at the primary leaf stage with SMV, developed mosaic symptoms on all systemically infected trifoliolate leaves. Transient vein clearing followed by moderate to severe mosaic appeared on expanding young leaves; the symptoms tended to fade on mature leaves. Leaf rugosity, distortion, and/or puckering symptoms were sometimes observed on older infected plants.

Plants singly inoculated with BPMV showed two flushes of symptom development separated by a recovery period. The first flush occurred during the development of the first and second trifoliolates. These leaves were stunted and showed mottling symptoms; the leaf margins were generally curved upward. The mottling symptoms faded with age and were completely masked by the time the experiments were terminated (40–50 days after

TABLE 1. Comparison of bean pod mottle virus (BPMV) titer in the youngest, fully expanded trifoliolate leaves of cultivar Williams soybean plants singly and doubly infected with BPMV and soybean mosaic virus (SMV) as determined by enzyme-linked immunosorbent assay (ELISA) at various intervals following inoculation of the primary leaves

Days after inoculation	Trifoliolate leaf tested	ELISA values (A_{405nm}) for plants inoculated with[a]		Ratio[b] (BPMV + SMV)/ BPMV
		BPMV	BPMV + SMV	
13	1st	0.237 ± 0.021[c] (0.179 to 0.295)[d]	0.318 ± 0.044 (0.196 to 0.440)	1.34
18	2nd	0.129 ± 0.016 (0.085 to 0.173)	0.303 ± 0.020 (0.247 to 0.359)	2.35
22	3rd	0.036 ± 0.005 (0.022 to 0.050)	0.221 ± 0.037 (0.118 to 0.324)	6.14
28	4th	0.096 ± 0.023 (0.037 to 0.155)	0.433 ± 0.019 (0.384 to 0.482)	4.51
35	5th	0.264 ± 0.014 (0.228 to 0.300)	0.599 ± 0.059 (0.447 to 0.751)	2.27
45	6th	0.164 ± 0.021 (0.110 to 0.218)	0.530 ± 0.046 (0.412 to 0.648)	3.23

[a] Leaf extracts were prepared by homogenization with PBS-PVP-T and dilutions of 1:1,500 (w/v) were tested by ELISA. Leaf extracts of comparable trifoliolates from plants singly infected with SMV served as controls and gave negligible ELISA values (0.001–0.006).

[b] Ratios for BPMV titer in doubly:singly infected plants.

[c] Values are means ± standard error for leaf extracts from five or six plants with each extract tested in duplicate wells; the plate was read with a Titertek Multiskan photometer (Flow Laboratories, McClean, VA) 10 min following addition of substrate.

[d] Values represent the 95% confidence intervals.

inoculation). The third trifoliolate leaves showed either no symptoms or only very faint mottling. The fourth trifoliolate leaves developed mild mottling, which quickly faded. The period during which the third and fourth trifoliolate leaves developed may be considered the recovery period. The fifth and sixth trifoliolates developed during the second flush of symptoms and showed mottling symptoms.

Plants doubly inoculated with SMV and BPMV (simultaneously or sequentially) were severely stunted and all systemically infected leaves showed severe mottling, distortion, and varying degrees of necrosis. The stunting of the doubly infected plant was a result of shorter internode lengths rather than slower development of the vegetative stages. Top necrosis occurred in some of the doubly infected plants, particularly those simultaneously inoculated with the two viruses. The frequency of top necrosis varied greatly from one experiment to another. Symptoms on doubly infected plants, unlike singly infected plants, did not fade during the experimental period.

Comparative BPMV titer in singly and doubly infected plants. The BPMV titer in the youngest fully expanded trifoliolate leaves from singly and doubly infected Williams soybean plants varied with leaf position, with the third trifoliolate leaves showing the lowest virus titer (Table 1). The variation in virus titer with leaf position was more pronounced with singly than with doubly infected plants. With the exception of the first trifoliolates, all successive trifoliolate leaves of doubly infected plants had significantly higher BPMV titers than did the corresponding trifoliolates of singly infected plants (Table 1). The ratios for BPMV titer in doubly:singly infected plants ranged from 1.34 to 6.14 for the various trifoliolate leaves with a mean value of 3.0 (Table 1).

The enhancement in BPMV titer in doubly infected plants was also detected when the virus titers in each of the first through the fifth trifoliolate leaves from singly and doubly infected plants were simultaneously measured by ELISA 40 days after inoculation. In these experiments, the ratios for BPMV titer in doubly:singly infected plants for the first through the fifth trifoliolate leaves were: 1.65, 4.57, 4.84, 3.57, and 2.77, respectively. These values are comparable to those shown in Table 1, in which each of the trifoliolate leaves was tested separately on different dates.

BPMV antigen concentration in extracts of composite leaf samples from singly and doubly infected plants was determined by ELISA. The BPMV antigen concentration in the test samples was determined by interpolation in a standard curve. An average of four- to fivefold increase of BPMV antigen concentration was detected in doubly infected plants (Table 2).

SMV titer in singly and doubly infected plants. No significant differences in SMV titer were detected between corresponding

TABLE 2. Comparative bean pod mottle virus (BPMV) antigen concentration in extracts from composite leaf samples of singly and doubly infected cultivar Essex soybean plants as determined by enzyme-linked immunosorbent assay (ELISA)

Virus treatment[a]	Leaf extract dilution (w/v)	ELISA values ($A_{405\ nm}$) for sample no.[b]		Antigen concentration[c] (ng/ml) for sample no.	
		1	2	1	2
BPMV	1:1,000	1.423 ± 0.063[d]	1.091 ± 0.100	142.0(14.2)[e]	78.0(7.8)
	1:5,000	0.303 ± 0.010	0.197 ± 0.021	24.0(12.0)	15.6(7.8)
	1:25,000	0.056 ± 0.006	0.040 ± 0.006	4.0(10.0)	3.0(7.5)
BPMV + SMV	1:1,000	>2.0	>2.0	ND[f]	ND
	1:5,000	1.258 ± 0.044	0.869 ± 0.057	110.0(55.0)	66.0(33.0)
	1:25,000	0.243 ± 0.037	0.163 ± 0.014	20.0(50.0)	13.2(33.0)
Control[g]	1:20	0.007 ± 0.006			

[a] Essex soybean seedlings were inoculated at the primary leaf stage with BPMV or with a mixed inoculum of BPMV and SMV.
[b] The second, third, and fourth trifoliolate leaves were collected from each plant 25 days after inoculation and the combined leaf samples for each plant were homogenized and diluted with PBS-PVP-T.
[c] Determined by interpolation in a standard curve made with a series of dilutions of purified BPMV tested in the same ELISA plate with the test samples.
[d] Values are means for quadruplicate wells ± standard deviation; absorbance readings were made 30 min following the addition of substrate.
[e] Values in parentheses are milligrams of virus per 100 g of tissue.
[f] ND = not determined.
[g] Comparable composite leaf samples from uninoculated plants.

TABLE 3. Comparison of soybean mosaic virus (SMV) titer in the youngest fully expanded trifoliolate leaves of Williams soybean plants singly and doubly infected with SMV and bean pod mottle virus (BPMV) as determined by enzyme-linked immunosorbent assay (ELISA) at various intervals following inoculation of the primary leaves

Days after inoculation	Trifoliolate leaf tested	ELISA absorbance ($A_{405\ nm}$) for plants inoculated with[a]		SMV + BPMV[b] / SMV
		SMV	SMV + BPMV	
13	1st	0.392 ± 0.022[c] (0.331 to 0.453)[d]	0.397 ± 0.026 (0.325 to 0.469)	0.097
18	2nd	0.347 ± 0.025 (0.278 to 0.416)	0.382 ± 0.049 (0.246 to 0.518)	1.10
22	3rd	0.135 ± 0.009 (0.110 to 0.160)	0.169 ± 0.030 (0.086 to 0.252)	1.25
28	4th	0.247 ± 0.034 (0.160 to 0.334)	0.216 ± 0.020 (0.165 to 0.267)	0.87
35	5th	0.332 ± 0.029 (0.257 to 0.407)	0.427 ± 0.027 (0.358 to 0.496)	1.29
45	6th	0.291 ± 0.030 (0.214 to 0.368)	0.331 ± 0.051 (0.200 to 0.462)	1.14

[a] Leaf extracts were prepared by homogenization with PBS-PVP-T and dilutions of 1:200 w/v were tested by ELISA. Leaf extracts of comparable trifoliolates from plants singly infected with BPMV served as controls and gave negligible ELISA absorbance values (0.001–0.015).
[b] Ratios for SMV titer in doubly:singly infected plants.
[c] Values are means ± standard error for leaf extracts from five or six plants with each extract tested in duplicate wells; the plate was read 20 min after addition of substrate.
[d] Values in parentheses represent the 95% confidence intervals.

trifoliolates of singly and doubly infected plants (Table 3). The ratios for SMV titer in doubly:singly infected plants for the various trifoliolate leaves ranged from 0.87 to 1.29 with a mean value of 1.1 (Table 3).

Quantitative ELISA was used to estimate SMV antigen concentration in stem terminals of Essex soybean plants 4 wk after inoculation. In one experiment, values of 240 and 165 μg/g tissue were estimated for singly and doubly infected plants, respectively.

TABLE 4. Comparison of virus concentration in leaf extracts from Essex soybean plants singly and doubly infected with soybean mosaic virus (SMV) and bean pod mottle virus (BPMV) as determined by density gradient centrifugation and enzyme-linked immunosorbent assay (ELISA)

Virus tested	Virus treatment[a]	Density gradient index[b]	ELISA[c] absorbance values at 405 nm	ELISA ratios for doubly:singly infected plants
BPMV	BPMV	1.0	0.322[d] ± 0.025	...
	SMV→BPMV	2.90	0.923 ± 0.029	2.87
	BPMV→SMV	2.54	0.892 ± 0.028	2.77
SMV	SMV	1.0	0.346 ± 0.024	...
	SMV→BPMV	0.68	0.260 ± 0.012	0.75
	BPMV→SMV	1.18	0.417 ± 0.021	1.21

[a] The primary leaves of all test plants were inoculated with BPMV or SMV 10 days after planting; in the case of double inoculation treatments, the second virus was applied to the first trifoliolate leaves 1 wk later.
[b] The weight of chart paper beneath the virus peak (both middle and bottom component peaks, in the case of BPMV) for preparations from singly infected plants was taken as unity.
[c] Subsamples of leaf extract corresponding to the various treatments were tested by ELISA at dilutions of 1:500 and 1:1,000 (w/v) for SMV and BPMV, respectively.
[d] Values are means for quadruplicate wells ± standard deviation; absorbance measurements were made 10 and 20 min after substrate addition for BPMV and SMV, respectively.

Virus assay by density gradient centrifugation. To determine whether the higher concentration of BPMV antigen, as revealed by ELISA in doubly over singly infected plants, reflected an increase in viral nucleoprotein concentration, the virus concentration in clarified extracts was estimated by both density gradient centrifugation and ELISA. The two assay methods showed excellent agreement in evaluating the relative concentration of SMV or BPMV (Table 4). When analyzed by density gradient centrifugation, purified preparations of BPMV from singly and doubly infected plants had similar titer ratios of middle:bottom components and contained only traces of top component (empty protein capsids).

Effect of age of infection on virus titer. Age of infection had little or no effect on SMV titer, as determined by ELISA, in either singly or doubly infected plants (Table 5). The BPMV titer in singly infected plants also remained unchanged during the testing period (Table 5). The BPMV titer in doubly infected plants, however, showed some variation. This was particularly evident in tests made 40 days after inoculation with leaflets from second trifoliolate leaves compared with tests made 18 or 28 days after inoculation (Table 5).

Decline of BPMV infectivity with age of infection. Although the BPMV titer, as determined by ELISA, remained relatively constant in the individual fully expanded trifoliolate leaves of singly and doubly infected plants, a decline in BPMV infectivity with age of infection was detected by local lesion assays (Table 6). At any given testing date, the results of local lesion assays with a set

TABLE 5. Effect of age of infection on titers of soybean mosaic virus (SMV) and bean pod mottle virus (BPMV) in singly and doubly infected cultivar Williams soybean plants as evaluated by enzyme-linked immunosorbent assay (ELISA)

Virus tested	Days after[a] inoculation	ELISA absorbance values ($A_{405\ nm}$) for extracts from[b]: Second trifoliolate leaves, Single infection	Second trifoliolate leaves, Double infection	Fourth (or fifth) trifoliolate leaves[c], Single infection	Fourth (or fifth) trifoliolate leaves[c], Double infection
SMV	18	0.348 ± 0.024[d]	0.342 ± 0.050	ND[e]	ND
	28	0.288 ± 0.021	0.310 ± 0.028	0.248 ± 0.034	0.275 ± 0.012
	35	ND	ND	0.218 ± 0.021	0.215 ± 0.017
BPMV	18	0.106 ± 0.022	0.294 ± 0.023	ND	ND
	28	0.118 ± 0.015	0.308 ± 0.019	ND	ND
	40	0.118 ± 0.029	0.510 ± 0.065	0.180 ± 0.028	0.502 ± 0.078
	50	ND	ND	0.140 ± 0.025	0.390 ± 0.051

[a] Williams soybean seedlings were inoculated at the primary leaf stage with SMV, BPMV, or with a mixed inoculum of both viruses.
[b] Leaf extracts prepared by homogenization and dilution with PBS-PVP-T were tested at dilutions of 1:200 and 1:1,500 (w/v) for SMV and BPMV, respectively.
[c] The fourth trifoliolate leaves were tested in the case of SMV, and the fifth trifoliolates in the case of BPMV.
[d] Values are means ± standard error for leaf extracts from six plants with each extract tested in duplicate wells; readings were made at 10 and 20 min, respectively, for the BPMV and SMV plates.
[e] ND = not determined.

TABLE 6. Effect of age of infection on bean pod mottle virus (BPMV) titer in second trifoliolate leaves of cultivar Williams soybean plants singly or doubly infected with BPMV and soybean mosaic virus (SMV) as determined by local lesion infectivity assay and enzyme-linked immunosorbent assay (ELISA)

Virus treatment[a]	Plant no.	Local lesions[b] for extracts tested at (days after inoculation): 18	28	ELISA values[c] (A_{405nm}) for extracts tested at (days after inoculation): 18	28	Specific infectivity[d] for extracts tested at (days after inoculation): 18	28
BPMV	1	18.2[e]	5.3	0.104[f]	0.117	175	45
	2	9.8	4.5	0.075	0.065	131	69
	3	23.5	8.2	0.140	0.142	168	58
BPMV + SMV	4	88.5	33.9	0.390	0.370	227	92
	5	58.7	28.2	0.314	0.298	187	95

[a] Soybean seedlings were inoculated at the primary leaf stage with BPMV or with a mixed inoculum.
[b] Soybean leaf extracts, prepared in PBS-PVP-T at a dilution of 1:50 (w/v), were rubbed on eight half-leaves of *Phaseolus vulgaris* 'Pinto' plants.
[c] Subsamples of the same extracts used for the local lesion assay were tested by ELISA at dilutions of 1:1,500, w/v.
[d] Computed by dividing mean local lesions per half-leaf by the corresponding ELISA absorbance value measured at 405 nm.
[e] Values are mean local lesion numbers per half-leaf.
[f] Values are averages for duplicate wells; plate reading was made 10 min after the addition of substrate.

TABLE 7. Results of enzyme-linked immunosorbent assay (ELISA) for soybean mosaic virus (SMV) and bean pod mottle virus (BPMV) in field-grown cultivar Williams soybean plants inoculated with SMV, BPMV, or both and tested at various intervals during the growing season

Virus treatment[a]	Plant no.	ELISA values (A_{405nm}) for extracts: Tested for SMV[b] at (days after planting)[c] 46	61	76	89	Tested for BPMV[d] at (days after planting)[c] 46	61	76	89
SMV	1	0.41[e]	0.41	0.19	0.42	0.02	0.01	0.01	0.01
	2	0.22	0.20	0.26	0.61	0.01	0.01	0.01	0.01
	3	0.44	0.39	0.26	0.71	0.01	0.01	0.01	0.01
BPMV	4	0.02	0.01	0.01	0.01	0.39	0.18	0.11	0.25
	5	0.02	0.15	0.14	0.36	0.46	0.74	0.64	0.83
	6	0.01	0.01	0.01	0.10	0.33	0.12	0.14	0.78
SMV→BPMV	7	0.31	0.36	0.26	0.83	1.20	0.46	0.55	0.71
	8	0.39	0.51	0.19	0.75	0.83	0.83	0.74	0.79
	9	0.50	0.45	0.24	0.65	0.86	0.75	0.66	1.07
BPMV→SMV	10	0.43	0.52	0.20	0.64	1.11	0.70	0.44	0.88
	11	0.37	0.26	0.36	0.73	1.27	0.47	0.63	0.76
	12	0.40	0.33	0.33	0.85	1.09	0.67	0.75	0.65

[a] Inocula of SMV or BPMV were applied to the primary leaves of all test plants 20 days after planting; in the case of double inoculation treatments, the second virus was applied to the first trifoliolate leaves 1 wk later.
[b] Leaf extracts were prepared in PBS-PVP-T and tested at dilutions of 1:200 (w/v); plate readings were made 20 min after substrate addition.
[c] Single leaflets of young fully expanded trifoliolate leaves from sixth, 10th, and 13th trifoliolate leaf position were tested at 46, 61, and 76 days after planting, respectively. Composite samples of three leaflets from the same leaf positions sampled earlier were tested at 89 days after planting.
[d] Leaf extracts were tested at dilutions of 1:1,000 (w/v); plate readings were made 10 min after the addition of substrate.
[e] Values are averages for duplicate wells.

TABLE 8. Effect of single or sequential inoculation with soybean mosaic virus (SMV) and bean pod mottle virus (BPMV) on yields of three soybean cultivars grown in hill plots

Virus treatment[y]	Yield (g/hill) for the soybean cultivars: Williams	Essex	York
Control	228.2 a[z]	236.8 a	251.4 a
SMV	183.8 b	145.1 b	249.0 a
BPMV	147.6 c	98.6 c	134.7 b
SMV→BPMV	56.1 d	57.6 d	168.4 b
BPMV→SMV	70.8 d	57.2 d	151.2 b

[y] The primary leaves of all test plants, except controls, were inoculated with SMV or BPMV 20 days after planting; in the case of double inoculation treatments the second virus was applied to the first trifoliolate leaves 1 wk later.
[z] Values are means for nine replications. For each cultivar means followed by the same letter are not significantly different ($\alpha = 0.05$) according to Duncan's multiple range test.

of leaf extracts from second trifoliolate leaves (Table 6) were highly correlated with those of ELISA made with samples of the same extracts ($r = 0.99$ and 0.98 for extracts tested at 18 and 28 days after inoculation, respectively). The specific infectivity (local lesions/ELISA absorbance at 405 nm) of BPMV declined by 64 and 55% in singly and doubly infected plants, respectively (Table 6). These experiments were repeated using the fifth trifoliolate leaves and results comparable to those shown in Table 6 were obtained. In this case, the decline in specific infectivity was 69 and 59% for BPMV in singly and doubly infected plants, respectively.

Field experiment. Monitoring field-grown plants for SMV and BPMV by ELISA enabled not only comparison of the virus titer in singly and doubly inoculated plants, but also evaluation of the secondary spread of these two viruses. ELISA results for representative Williams soybean plants tested at 46, 61, 76, and 89 days after planting are shown in Table 7. Plants doubly inoculated with BPMV and SMV had significantly higher titers of BPMV than did singly infected plants. The increase in BPMV titer was also detected in plants that were singly inoculated with BPMV and later became naturally infected with SMV (eg, plants 5 and 6, tested at 61 and 89 days after planting, respectively) (Table 7). The ratios for mean BPMV titer in doubly:singly infected plants ranged from 2.5 to 5.2 during the testing period. There was little or no difference in SMV titer between singly and doubly infected plants (Table 7). The ratios for mean SMV titer in doubly:singly infected plants ranged from 0.97 to 1.32 during the growing season.

Secondary spread of SMV and BPMV in the field was evaluated by ELISA monitoring of singly inoculated and uninoculated cultivar Williams and Essex plants (10 plants per each cultivar/treatment combination). The highest rate of secondary spread of both viruses occurred during the period from mid- to late August (76–89 days after planting). During this period, levels of natural infection with SMV increased from 33 to 90%, those for BPMV rose from 11 to 25%.

All virus treatments significantly reduced the yield of all cultivars when compared to respective uninoculated controls with the exception of the SMV treatment on York, a cultivar resistant to SMV (Table 8). In the case of Williams and Essex, the doubly inoculated plants had significantly lower yields than those inoculated with either virus alone (Table 8). The sequence of inoculation with the two viruses had little or no effect on the extent of yield reduction (Table 8).

DISCUSSION

Evidence is presented that supports the conclusion that the concentration of BPMV in soybean plants doubly infected with BPMV and SMV is significantly higher than that in singly infected plants. The enhancement of BPMV titer was consistently detected in doubly infected plants regardless of the cultivar or the virus assay method used. The enhancement phenomenon was evident in both greenhouse and field-grown plants and was independent of the timing, sequence, or means of inoculation with the two viruses. Ross (12), using a local lesion assay, reported a marked increase in BPMV concentration in doubly over singly infected plants. However, in a later publication, Lee and Ross (9) disclosed inconsistencies in detecting the enhancement of BPMV titer in doubly infected plants. Tu and Ford (16), using an infectivity dilution end point assay, were unable to demonstrate any significant differences in either BPMV or SMV titers between doubly and singly infected plants. The variation in BPMV titer with leaf position, as reported in the present study, may, at least in part, account for these discrepancies and/or inconsistencies.

Unlike BPMV, SMV concentration in doubly infected plants is not significantly different from that in singly infected ones. Despite the use of different virus assay methods and various experimental conditions we were unable to demonstrate any significant enhancement of SMV concentration in doubly infected plants.

The BPMV titer, as determined by ELISA, varied with leaf position in singly infected plants and appeared to be related to the severity of symptoms developed by the individual trifoliolate leaves. For example, the third trifoliolate leaves, which showed

mild or no mottling symptoms, had the lowest virus titer, whereas the first or fifth trifoliolate leaves that had developed severe mottling had the highest virus titers. The BPMV titer in doubly infected plants also varied with leaf position with the third trifoliolate leaves having the lowest titers (Table 1). The virus titer in that trifoliolate leaf, however, was still as high as or higher than that in any given trifoliolate leaf of singly infected plants. Thus, it appears that, in this system, the presence of relatively high concentrations of BPMV in all trifoliolate leaves is correlated with symptom severity of doubly infected plants.

The decline in BPMV infectivity in singly infected plants with age of infection was recognized earlier by Gillaspie and Bancroft (6). BPMV infectivity in doubly infected plants also declined with age of infection but at a slightly slower rate than that in singly infected plants (this report, and Table 6).

As was previously reported by Ross (13), field-grown soybean plants doubly inoculated with SMV and BPMV at the seedling stage had significantly lower yields than those inoculated with either virus alone. The reduction in yield of doubly inoculated cultivar Williams soybean plants was greater than the sum of yield reductions due to single inoculations (Table 8). The yield reduction in the case of doubly inoculated cultivar Essex plants, however, did not appear to be synergistic. Although a high percentage of the singly inoculated plants of either cultivar became doubly infected later in the season as a result of secondary spread, those of the cultivar Essex suffered disproportionately higher yield losses. This may have occurred because cultivar Essex (maturity group V) was at an earlier reproductive stage than those of cultivar Williams (maturity group III) during the period of rapid virus spread (mid- to late August). Greater yield losses were reported by Hepperley et al (7) for cultivar Williams soybean plants that were inoculated with SMV at earlier than at later reproductive stages.

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