Soybean Mosaic Potyvirus Enhances the Titer of Two Comoviruses in Dually Infected Soybean Plants

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ABSTRACT

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Soybean plants dually infected with soybean mosaic potyvirus (SMV) and either one of two comoviruses, cowpea mosaic virus (CPMV) or bean pod mottle virus (BPMV), showed greatly increased symptom severity over that induced by the individual viruses. The titer of the comovirus (but not of SMV) in the dually infected plants was significantly higher than that in singly infected plants. Electron microscopic examination of thin sections of leaves from dually infected plants revealed that both SMV and CPMV (or BPMV) were present in the same cells. Unlike SMV, the potyviruses bean yellow mosaic virus and peanut mottle virus did not interact synergistically with BPMV in dually infected soybean plants. The increase in accumulation of comovirus coat protein and RNA components in dually infected plants compared to singly infected plants was monitored by enzyme-linked immunosorbent assay, western blotting, and RNA dot blot hybridization. The results indicated that the levels of these two viral components increased to the same extent in the dually infected soybean plants.

In nature, mixed viral infections are common in higher plants, including soybeans (8,24). It is well accepted that pairs of related or unrelated viruses can replicate in the same cells and, depending on the combination, the viruses may interact synergistically or antagonistically (22,24). As a consequence of these interactions, a different disease with more severe symptoms than those produced by single infections may develop, and/or significant changes in the concentration of either or both viruses may occur (2,16, 17,23).

Soybean mosaic potyvirus (SMV) and bean pod mottle comovirus (BPMV) interact synergistically in soybean and result in more severe symptoms than the sum of the effects of single infections (2,25). The titer of BPMV in singly and dually infected soybean plants correlates well with symptom severity and is significantly higher in dually infected than in singly infected plants (2). The mechanism of synergism between SMV and BPMV is not understood. In order to gain better understanding of synergistic virus interactions, we have addressed the questions of whether SMV may enhance the concentrations of comoviruses other than BPMV in dually infected soybean plants, and whether potyviruses other than SMV may interact synergistically with comoviruses. Our results suggest that the outcome of dual infections involving potyviruses and comoviruses is determined by the coinfecting potyvirus.

MATERIALS AND METHODS

Viruses. The G-7 Kentucky isolate of BPMV (11) was propagated in soybean (*Glycine max* (L.) Merrill cv. Essex), and the SB strain of cowpea mosaic comovirus (CPMV) (obtained from O. W. Barnett, Clemson University) was increased in *Vigna unguiculata* (L.) Walp. cv. Early Ramshorn. A subculture of the SMV K-1 isolate, which belongs to the G-6 group of SMV strains (3,13), was used. Peanut mottle potyvirus (PMV) strain M-2 (4) was supplied by J. M. Demski, University of Georgia. The RC isolate of bean yellow mosaic potyvirus (BYMV) was a subculture of an isolate previously used (28).

Virions of BPMV and CPMV were purified by the procedure of Semancik and Bancroft (26) as modified by Ghabrial and Schultz (12). Virions of SMV were purified as described by Calvert and Ghabrial (2).

Synergism experiments. The severity of symptoms induced by dual infections of soybean with four pairs of unrelated viruses were compared with those resulting from infections with the individual viruses. The following virus pairs were used: SMV/BPMV, SMV/CPMV, BYMV/BPMV, and PMV/BPMV. Inocula were applied simultaneously to the unifoliate primary leaves of soybean seedlings 7 days after planting or sequentially by applying the potyvirus component of the virus pair to the primary leaves and the comovirus (except for the BYMV/BPMV pair) to the first trifoliolate leaves 1 wk later. In the case of the BYMV/BPMV system, BYMV was inoculated onto the primary leaves and BPMV was applied to the second trifoliolate leaves 10 days later (at which time symptoms due to BYMV first appeared). In all treatments involving single virus inoculations, the same inocula used for dual inoculations were concurrently applied to comparable leaves of the test plants. The accumulation of virions, viral RNAs, and coat proteins in the youngest fully or nearly fully expanded trifoliolate leaves of singly and dually inoculated plants were monitored by enzyme-linked immunosorbent assay (ELISA), RNA dot blot hybridization, and western blotting at various intervals during the 6- to 26-day period post-inoculation.

Seeds of the soybean cv. Essex were germinated in 10.2-cm plastic pots containing Pro-Mix BX (Premier Brand Inc., New Rachelle, NY). The plants were grown in a greenhouse under daylight supplemented with fluorescent light for a 14-h photoperiod. The temperature in the greenhouse varied between 20 and 25 C. Virus inocula were prepared by grinding systemically infected leaves from soybean plants infected with the individual viruses (1:5 w/v) in a mortar with a pestle in 0.05 M potassium phosphate buffer, pH 7.0. All inoculations were made by rubbing Carborundum-dusted leaves with inoculum-soaked cheesecloth pads. For simultaneous inoculations with two viruses, the inocula of the respective viruses were mixed 1:1 (v:v) before inoculation. A control of mock-inoculated plants was also included. In all experiments, at least four plants were used per treatment.

ELISA. The direct, double antibody-sandwich form of ELISA, as described by Ghabrial and Schultz (12), was used for monitoring the antigen titers of BPMV, CPMV, SMV, BYMV, and PMV in singly or dually infected soybean plants. The antisera to BPMV,

SMV, BYMV were produced in our laboratory, and their applications in ELISA were as described previously (2,11,12). The antiserum to PMV was a gift from J. W. Demski.

An antiserum to CPMV, strain SB, was produced in rabbits by a series of subcutaneous injections. Purified immunoglobulin G (IgG) was prepared (12) and used at a concentration of 0.5 μg/ml for coating the microtiter plates. The IgG-alkaline phosphatase conjugate was prepared as described before for the antiserum against BPMV (12) and used at a dilution of 1:2,000.

Western blots. Extracts were prepared from infected and mock-inoculated Essex soybean plants as described by Fransen et al (9) for the preparation of the 30,000 *g* supernatant fraction, hereafter referred to as the 30S fraction. The proteins in 500 μl samples of the 30S fraction, obtained from the various treatments, were precipitated by adding 4 vol of cold acetone, and the pellets were dried under vacuum. The pellets were resuspended in Laemmli buffer (20) containing 4% w/v SDS, 20% glycerol, and 10% v/v 2-mercaptoethanol. Samples were boiled for 3 min and electrophoresed in 12.5% SDS-polyacrylamide gel at 50 V. Purified virions were used as a control. Proteins were electrophoretically transferred onto an immobilon PVDF membrane (Millipore, Bedford, MA) using a transblot cell (Bio-Rad, Richmond, California). The transfer was performed at 70 V for 4 h using 25 mM Tris/192 mM glycine buffer, pH 8.2, containing 15% v/v methanol (29). After electro-transfer the membrane was incubated in blocking buffer (5% low fat milk in TBS [50 mM Tris base, 200 mM sodium chloride, pH 7.4] and 0.1% sodium azide) at 50 C for 12 h. The membrane was incubated in blocking buffer containing 1 μg/ml of rabbit anti-BPMV IgG for 4 h at room temperature. The membrane was washed three times in TBS and incubated in blocking buffer containing 2 μg/ml of antirabbit IgG-alkaline phosphatase conjugate for 2 h at room temperature. The membrane was washed three times in TBS and incubated in 30 ml of substrate solution (75 mg of nitroblue tetrazolium in *N,N*-dimethylformamide, 5 mg of bromo-4 chloroindolyl phosphate in *N,N*-dimethylformamide, and 3 ml of 10 × alkaline phosphatase buffer [12.1 g Tris base, 5.8 g NaCl and 1.02 g $MgCl_2$], pH 9.5) for 15–20 min. The membrane was rinsed with water and air-dried.

RNA dot blot hybridization. RNA samples were extracted from singly and dually infected soybean plants essentially as described by Garger et al (10). Frozen leaf tissue (500 mg wet weight per sample) was ground in liquid nitrogen, and 1 ml of STE buffer (50 mM Tris-HCl, pH 7.2, containing 100 mM NaCl, and 1 mM ethylene diamine tetra-acetate, EDTA) was added. The mixture was clarified by centrifugation at 10,000 *g* for 5 min, and the supernatant was extracted with an equal volume of phenol:chloroform:isoamyl alcohol mixture (25:24:1). Following centrifugation at 10,000 *g* for 5 min, the aqueous phase was removed and reextracted with equal volume of chloroform. The RNA samples were precipitated by adding 2.5 volumes of ice-cold 95% ethanol, incubated for 24 h at −20 C, and resuspended in 50 ml of a solution containing 50% deionized formamide and 6% formaldehyde. The mixtures were then incubated at 50 C for 1 h, and the denatured RNA samples were spotted onto GeneScreen Plus membranes (Du Pont Company, Wilmington, DE) by using a dot blot manifold (Schleicher and Schuell, Keene, NH). The membrane was air-dried, baked at 80 C for 2 h, and prehybridized at 42 C for 12 h in 6× SSC (1× is 0.15 M NaCl, 0.015 M sodium citrate) containing 0.25% (w/v) nonfat dry milk, 50% formamide, and 1% sodium dodecyl sulfate (SDS). Hybridization was performed using the same conditions as prehybridization plus including oligonucleotide-^{32}P-labeled probes (7) derived from cloned cDNA to CPMV and BPMV RNAs (21, and *unpublished*, respectively). Following washing, the membranes were exposed to XAR 5 X-ray film using intensifying screens at −70 C.

Electron microscopy. Leaf samples (1- × 2-mm) were fixed in 0.1 M sodium phosphate buffer, pH 7, containing 1% glutaraldehyde and 4% paraformaldehyde for 2 h at 4 C. The samples were rinsed in 0.1 M phosphate buffer, pH 7.0, and postfixed in 1% osmium tetraoxide in the same phosphate buffer for 1 h at 4 C. The samples were then subjected to dehydration in an ethanol series followed by embedding in Spurr's medium (27). The plasmolysis procedure described by Hatta and Matthews (19) was used. Thin sections (70 nm) were cut with an LKB Ultrotome III, stained with uranyl acetate, and examined with a Philips 400 electron microscope.

RESULTS

Comparative symptom severity in singly and dually infected plants. Soybean plants singly infected with BPMV developed two flushes of mottling symptoms separated by a recovery period, as described in earlier studies (2,14). CPMV induced more severe mottling symptoms in soybean than BPMV. Unlike BPMV-induced symptoms, those induced by CPMV did not vary with leaf position and no recovery phase was observed. Of the three potyviruses used in dual inoculations, only SMV interacted synergistically with the two comoviruses, BPMV and CPMV.

Comparative BPMV (or CPMV) antigen titer in singly and dually infected plants. The BPMV antigen titer in plants simultaneously or sequentially inoculated with SMV and BPMV was significantly higher than that in plants singly infected with BPMV (Table 1). BPMV antigen titer varied with leaf position, and this difference was more pronounced in singly than in dually infected plants (Table 1).

Likewise, the CPMV antigen titer was significantly higher in plants dually infected with SMV and CPMV than in those singly

TABLE 1. Comparison of bean pod mottle virus (BPMV) antigen titer in Essex soybean plants singly and dually infected with BPMV and soybean mosaic virus (SMV) as determined by enzyme-linked immunosorbent assay (ELISA)

Treatment	Time[a]	Position of trifoliolate leaf tested[b]	ELISA values (A_{405nm})[c]		Ratio dual/single
			BPMV	BPMV + SMV	
Simultaneous inoculation[d]	6	1st	1.617 ± 0.072	1.740 ± 0.083	1.16
	11	2nd	1.505 ± 0.107	1.697 ± 0.085	1.13
	15	3rd	0.245 ± 0.031	1.600 ± 0.074	6.40
	20	4th	0.507 ± 0.040	1.512 ± 0.131	2.96
Sequential inoculation[e]	6	2nd	1.227 ± 0.110	1.425 ± 0.096	1.16
	12	3rd	0.547 ± 0.060	1.497 ± 0.153	2.73
	17	4th	0.207 ± 0.033	1.175 ± 0.081	5.67
	23	5th	0.677 ± 0.051	1.775 ± 0.125	2.60

[a] Days postinoculation. The leaf assayed was the youngest fully expanded trifoliolate leaf at the times indicated.
[b] The 1st leaf position refers to the first trifoliolate leaf that developed above the unifoliate leaf, the 2nd leaf position indicates the second trifoliolate leaf that developed above the unifoliate leaf, and so on.
[c] Values are means for four plants ± standard error. Leaf extracts were prepared with PBS-PVP-Tween-20 at a dilution of 1:250 w/v. Purified virions of BPMV at concentrations of 4, 8, 16, 32, 63, and 125 ng/ml, which were included in the same ELISA plates, gave readings of (mean for 3 wells) 0.083, 0.157, 0.347, 0.777, 1.583, and >2.0, respectively. Leaf extracts of comparable trifoliolates from plants singly infected with SMV or healthy control gave negligible ELISA values (0.004–0.012).
[d] Mixed inoculum of SMV and BPMV was applied to the primary unifoliate leaves.
[e] SMV inoculum was applied to the primary unifoliate leaves and BPMV to the first trifoliolate leaves 1 wk later.

infected with CPMV (Table 2). CPMV antigen titer, unlike that of BPMV, did not vary with leaf position in plants singly inoculated with CPMV. On the other hand, significant differences in viral antigen titer between singly and dually infected plants were detected at all leaf positions, even at the first leaf position (Tables 1 and 2). To further quantify the synergism in symptom severity induced by dual infection of soybean with CPMV and SMV, the plant heights of singly and dually infected plants were measured. Plants dually infected had a mean height ± standard deviation of 10.5 ± 0.8 cm. This height was significantly less than that of plants singly infected with CPMV or SMV or that of mock-inoculated plants, which had mean heights of 17.0 ± 0.3 cm, 21.7 ± 0.6 cm, and 22.5 ± 0.4 cm, respectively. No significant differences in height were detected between SMV-infected and mock-inoculated plants.

It was of interest to determine whether the enhancement in CPMV (BPMV) antigen titer, as measured by ELISA, may reflect accumulation of the coat proteins-precursor, as well as of the mature viral capsid proteins. The antiserum used in ELISA is known to react strongly with the 60K precursor to the coat proteins (21). For this purpose, the 30S fraction of leaf extracts (see Materials and Methods) was examined by western blotting. The results indicated a marked enhancement in the levels of CPMV coat proteins in plants dually infected with CPMV and SMV when compared to plants singly infected with CPMV (Fig. 1). No accumulation of the 60K precursor was detected. The antiserum to CPMV was not reactive with proteins extracted from healthy tissue (Fig. 1, lane a). The results of western blot analysis were consistent with the ELISA data.

Western blot analysis was also performed with protein samples from soybean plants singly and dually infected with BPMV and SMV. The results of western blotting (data not shown) were consistent with those obtained by ELISA (Table 1).

Specificity of the enhancement by potyviruses of BPMV concentration. In contrast to the marked enhancement in BPMV antigen titer detected in soybean plants dually infected with BPMV and SMV (Table 1), the BPMV antigen titers in plants dually inoculated with BYMV and BPMV or with PMV and BPMV were not significantly different from those in plants singly infected with BPMV (Tables 3 and 4). The enhancement induced by SMV infection is apparently strain-independent, because all SMV strains tested, namely G-2, G-6, and N (3,6,13), interacted synergistically with BPMV in dually infected plants (data not shown).

Comparative antigen titers of SMV, BYMV, or PMV in singly and dually infected plants. No significant differences in SMV, BYMV, or PMV antigen titers were observed between corresponding trifoliolates of dually and singly infected plants. The ratios for relative SMV antigen titer in plants dually infected with SMV and BPMV and singly infected with SMV ranged from 0.93 to 1.03 in plants simultaneously inoculated and from

TABLE 2. Comparison of cowpea mosaic virus (CPMV) antigen titer in Essex soybean plants singly and dually infected with CPMV and soybean mosaic virus (SMV) as determined by enzyme-linked immunosorbent assay (ELISA)

Days after inoculation[a]	Position trifoliolate leaf tested[b]	ELISA values (A_{405nm})[c] CPMV inoculum	CPMV + SMV inoculum	Ratio dual/single
8	1st	0.134 ± 0.024	0.442 ± 0.017	3.29
14	2nd	0.136 ± 0.026	0.685 ± 0.029	5.03
20	3rd	0.142 ± 0.023	1.195 ± 0.125	8.40
26	4th	0.131 ± 0.020	0.595 ± 0.037	4.50

[a] Mixed inoculum of SMV and CPMV was applied to the primary unifoliate leaves. The leaf assayed was the youngest fully expanded trifoliolate leaf at the times indicated.

[b] The 1st leaf position refers to the first trifoliolate leaf that developed above the unifoliate leaf, the 2nd leaf position indicates the second trifoliolate leaf that developed above the unifoliate leaf, and so on.

[c] Values are the means for four plants ± standard error. Leaf extracts were prepared with PBS-PVP-Tween-20 at a dilution of 1:500 w/v. Purified virions of CPMV at concentrations of 16, 32, 63, 125, 250, and 500 ng/ml, which were included in the same ELISA plates, gave readings of (mean for 3 wells) 0.062, 0.137, 0.353, 0.798, 1.474, and >2.0, respectively. Leaf extracts of comparable trifoliolates from plants singly infected with SMV or healthy control gave negligible ELISA values (0.002–0.006).

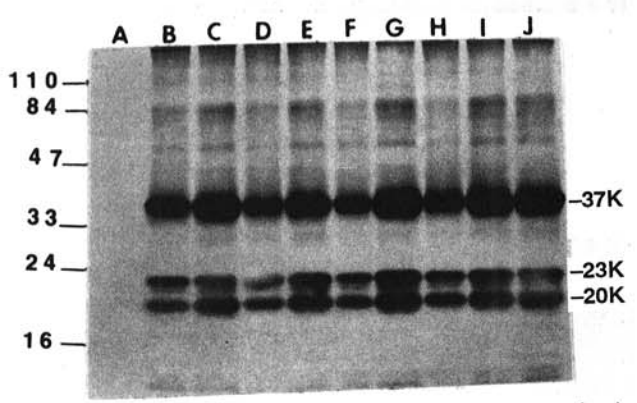

Fig. 1. A western blot showing accumulation of cowpea mosaic virus (CPMV) coat proteins at various leaf positions in soybean plants singly and dually infected with CPMV and soybean mosaic virus (SMV). The protein samples were from: mock-inoculated plant, lane A; first through fourth trifoliolate leaves from plants singly inoculated with CPMV, lanes B, D, F, and H, respectively; first through fourth trifoliolate leaves from plants dually inoculated with CPMV + SMV, lanes C, E, G, and I, respectively; purified CPMV, lane J. The western blot was reacted with rabbit antibodies raised against CPMV virions. Positions of prestained protein standards are indicated on the left. Positions of the viral capsid proteins 37K, 23K, and 20K are indicated on the right.

TABLE 3. Comparison of bean pod mottle virus (BPMV) antigen titer in Essex soybean plants singly and dually infected with BPMV and bean yellow mosaic virus (BYMV), as determined by enzyme-linked immunosorbent assay (ELISA)

Days after inoculation[a]	Position trifoliolate leaf tested[b]	ELISA values (A_{405nm})[c] BPMV inoculum	BPMV + BYMV inoculum	Ratio dual/single
6	3rd	1.062 ± 0.071	1.137 ± 0.152	1.07
14	4th	1.655 ± 0.142	1.582 ± 0.093	0.96
20	5th	1.512 ± 0.114	1.482 ± 0.050	0.98
26	6th	1.271 ± 0.093	1.357 ± 0.071	1.06

[a] BYMV inoculum was applied to the primary unifoliate leaves and BPMV to the second trifoliolate leaves 10 days later. The leaf assayed was the youngest fully expanded trifoliolate leaf at the times indicated.

[b] The 3rd leaf position refers to the third trifoliolate leaf that developed above the unifoliate leaf, the 4th leaf position indicates the fourth trifoliolate leaf that developed above the unifoliate leaf, and so on.

[c] Values are means for four plants ± standard error. Leaf extracts were prepared with PBS-PVP-Tween-20 at a dilution of 1:250 w/v.

TABLE 4. Comparison of bean pod mottle virus (BPMV) antigen titer in Essex soybean plants singly and dually infected with BPMV and peanut mottle virus (PMV), as determined by enzyme-linked immunosorbent assay (ELISA)

Days after inoculation[a]	Position trifoliolate leaf tested[b]	ELISA values (A_{405nm})[c] BPMV inoculum	BPMV + PMV inoculum	Ratio dual/single
5	2nd	1.312 ± 0.167	1.317 ± 0.093	1.00
10	3rd	1.152 ± 0.106	1.065 ± 0.090	0.92
16	4th	0.260 ± 0.021	0.295 ± 0.125	1.15
22	5th	0.605 ± 0.060	0.592 ± 0.037	0.98

[a] PMV inoculum was applied to the primary unifoliate leaves and BPMV to the first trifoliolate leaves 1 wk later. The leaf assayed was the youngest fully expanded trifoliolate leaf at the times indicated.

[b] The 2nd leaf position refers to the second trifoliolate leaf that developed above the unifoliate leaf, the 3rd leaf position indicates the third trifoliolate leaf that developed above the unifoliate leaf, and so on.

[c] Values are means for four plants ± standard error. Leaf extracts were prepared with PBS-PVP-Tween-20 at a dilution of 1:250 w/v.

0.98 to 1.05 in plants sequentially inoculated (data not shown). Similar results were obtained with the SMV/CPMV virus pair (Table 5). For BYMV the ratios for dually/singly infected plants ranged from 1.02 to 1.10, and for PMV they ranged from 0.91 to 1.18 (data not shown).

Accumulation of viral RNA in plants dually infected with BPMV or CPMV and SMV. Results of dot blot hybridization analysis of RNA samples from four trifoliolates collected from soybean plants singly infected with BPMV or dually infected with SMV and BPMV (Fig. 2) were consistent with the ELISA data (Table 1) and the results of western blot analysis (data not shown). The amount of BPMV-RNA detected varied with leaf position in singly infected plants but not in dually infected plants. There were few or no differences in viral RNA content of first and second trifoliolates from singly and dually infected plants (compare dots B1 and B2 with dots C1 and C2, Fig. 2). There were, however, marked differences at the third and fourth leaf positions (compare dots B3 and B4 with dots C3 and C4). No hybridization was detected with RNA from mock-inoculated plants spotted in positions B5 and C5 (Fig. 2).

The concentration of CPMV-RNA extracted from singly infected plants, unlike that of BPMV-RNA, did not vary with leaf position (Fig. 3, row C). There were significant differences, however, between the respective trifoliolates of singly and dually infected plants at all leaf positions (compare rows B and C, Fig. 3). These results are consistent with the ELISA and western blotting data (Table 2 and Fig. 1). No hybridization was detected with RNA from mock-inoculated plants spotted in positions B5 and C5 (Fig. 3).

Ultrastructural evidence that both SMV and CPMV (BPMV) replicate in the same cells. Although isometric particles similar in size to BPMV or CPMV can be seen scattered in the cytoplasm of SMV-infected cells containing cylindrical inclusions (Fig. 4A, arrows), it is somewhat difficult to distinguish the viruslike particles from ribosomes. Aggregates of CPMV or BPMV particles, however, can be readily discerned in the vacuoles of such cells (Fig. 4A). The observation of rows of isometric virus particles in plasmadesmata traversing the cell wall between two adjoining cells, each of which contained cylindrical inclusions (Fig. 4B–D), was also considered as evidence of dual infection of cells with SMV and CPMV (or BPMV). We utilized the plasmolysis procedure of Hatta and Matthews (19) in order to induce the virus particles to form crystalline arrays, which are then easily distinguished in thin sections (Fig. 4E). The plasmolysis protocol, however, appears to destroy the cylindrical inclusions, as we were unable to observe such inclusions in any of the infected cells that were processed by this procedure.

TABLE 5. Comparison of soybean mosaic virus (SMV) antigen titer in Essex soybean plants singly and dually infected with SMV and cowpea mosaic virus (CPMV), as determined by enzyme-linked immunosorbent assay (ELISA)

Days after inoculation[a]	Position trifoliolate leaf tested[b]	ELISA values (A_{405nm})[c] SMV inoculum	SMV + CPMV inoculum	Ratio dual/single
8	1st	0.545 ± 0.057	0.575 ± 0.056	1.05
14	2nd	0.650 ± 0.066	0.722 ± 0.060	1.11
20	3rd	0.735 ± 0.036	0.775 ± 0.045	1.05
26	4th	0.882 ± 0.047	0.927 ± 0.036	1.06

[a] Mixed inoculum of SMV and CPMV was applied to the primary unifoliate leaves. The leaf assayed was the youngest fully expanded trifoliolate leaf at the times indicated.

[b] The 1st leaf position refers to the first trifoliolate leaf that developed above the unifoliate leaf, the 2nd leaf position indicates the second trifoliolate leaf that developed above the unifoliate leaf, and so on.

[c] Values are means for four plants ± standard error. Leaf extracts were prepared with PBS-PVP-Tween-20 at a dilution of 1:250 w/v.

DISCUSSION

Previous studies (2) have shown that the synergistic interaction between SMV and BPMV in dually infected soybean plants is characterized by a marked increase in the accumulation of BPMV antigen, as determined by ELISA. Furthermore, the increase in BPMV antigen titer is paralleled by an increase in the titer of infectious viral nucleoprotein particles (2). The present study confirmed and extended these findings, as SMV was shown to interact synergistically with an additional comovirus, CPMV. The results of ELISA, western blotting, and RNA dot hybridization experiments demonstrated that the levels of capsid proteins in the dually infected plants increased to about the same extent as that of viral RNA (Tables 1 and 2; Figs. 1–3). These results support the conclusion that the enhancement by SMV of CPMV or BPMV antigen titers reflects an increase in the accumulation of virus nucleoprotein particles rather than empty capsids or coat proteins precursor.

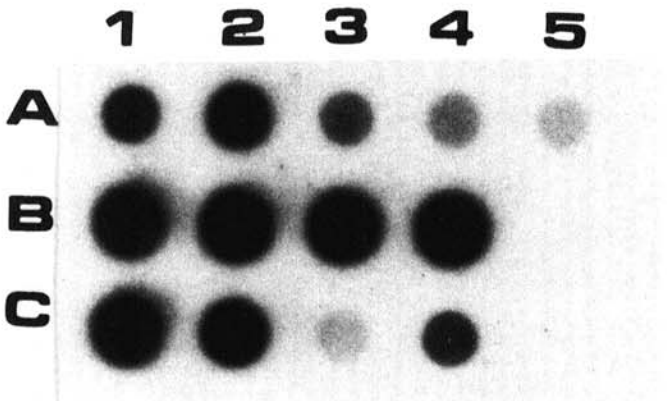

Fig. 2. Dot blot hybridization analysis of RNA samples extracted from plants inoculated with bean pod mottle virus (BPMV) alone or with a mixture of BPMV and soybean mosaic virus. Row A: dots 1–5 represent purified BPMV M-RNA spotted in amounts of 50, 100, 25, 12.5, and 6.3 ng, respectively; row B: dots 1–4 represent RNA extracted from 1st, 2nd, 3rd, and 4th trifoliolates, respectively, of dually infected plants; row C: dots 1–4 represent RNA extracted from 1st, 2nd, 3rd, and 4th trifoliolates, respectively, of plants singly infected with BPMV. RNA samples from mock-inoculated plants were spotted in positions B5 and C5. The blot was hybridized to an oligonucleotide ^{32}P-labeled probe derived from cloned cDNA to BPMV M-RNA.

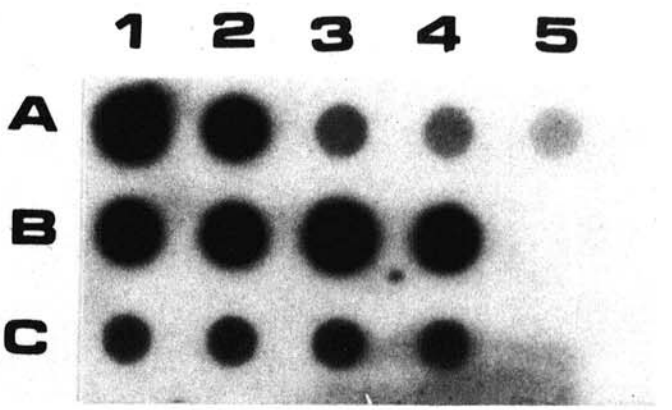

Fig. 3. Dot blot hybridization analysis of RNA samples extracted from plants inoculated with cowpea mosaic virus (CPMV) alone or with a mixture of CPMV and soybean mosaic virus. Row A: dots 1–5 represent purified CPMV B-RNA spotted in amounts of 100, 50, 25, 12.5, and 6.3 ng, respectively; row B: dots 1–4 represent RNA extracted from 1st, 2nd, 3rd, and 4th trifoliolates, respectively, of dually infected plants; row C: dots 1–4 represent RNA extracted from 1st, 2nd, 3rd, and 4th trifoliolates, respectively, of plants singly infected with CPMV. RNA samples from mock-inoculated plants were spotted in positions B5 and C5. The blot was hybridized to an oligonucleotide ^{32}P-labeled probe derived from cloned cDNA to CPMV B-RNA.

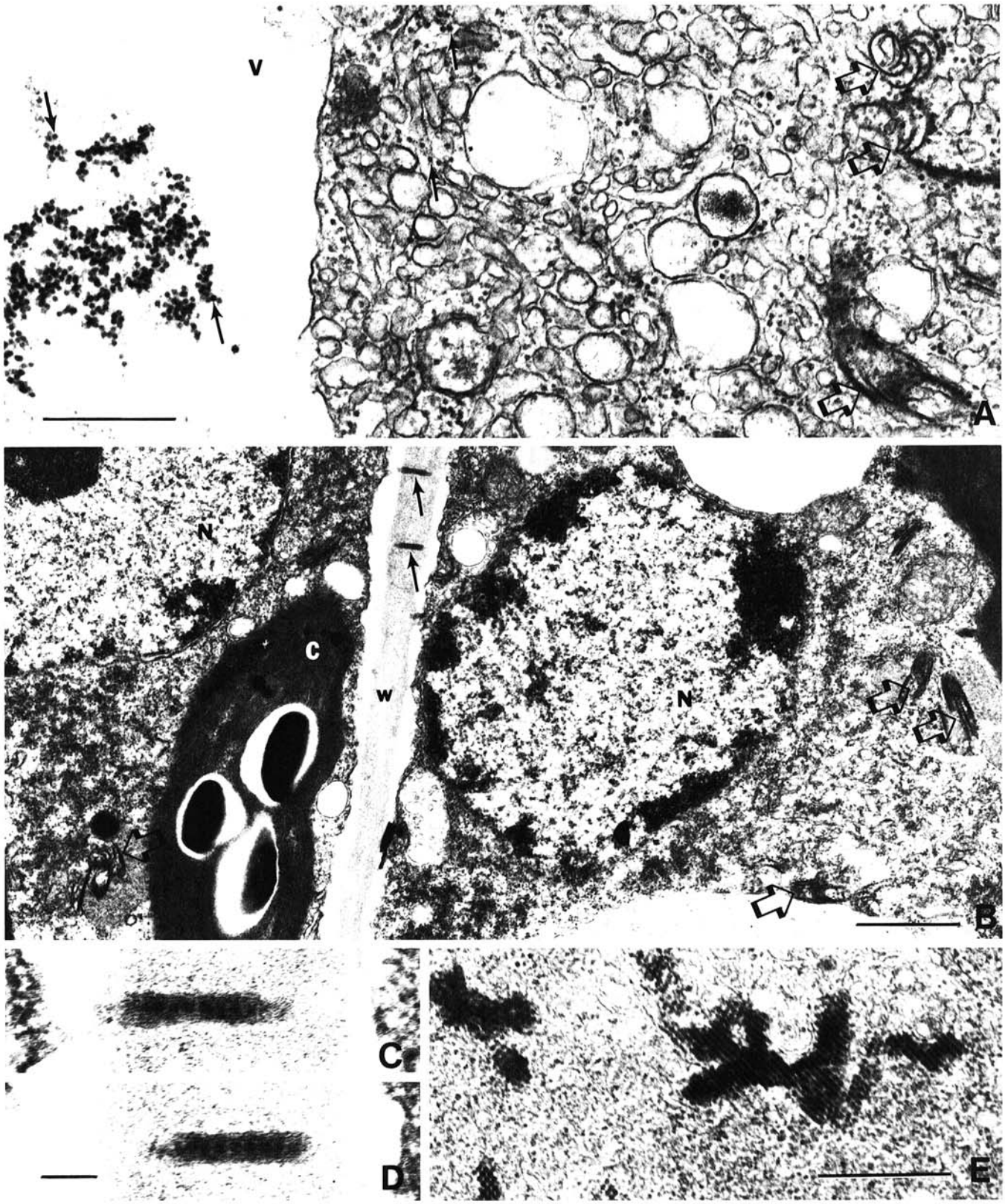

Fig. 4. Electron micrographs of thin sections of soybean leaves dually infected with soybean mosaic virus (SMV) and either bean pod mottle virus (BPMV) or cowpea mosaic virus (CPMV). **A,** a micrograph showing a portion of a mesophyll cell from a plant dually infected with SMV and BPMV. Small aggregates of isometric particles (smaller arrows) are present in the vacuole (V) as well as in the cytoplasm. Open arrows indicate cytoplasmic pinwheel inclusions characteristic of infection with SMV. Bar = 500 nm. ×46,000. **B,** a micrograph showing portions of two adjacent mesophyll cells from a plant dually infected with SMV and CPMV. Cytoplasmic inclusions (open arrows) in both cells are indicative of SMV infection, and the two plasmadesmata (smaller arrows) traversing the cell wall (W) are filled with isometric viruslike particles (for detail, see **C** and **D**). N = nucleus; C = chloroplast. Bar = 1,000 nm. ×18,000. **C** and **D,** enlarged views of the plasmadesmata shown in **B**. Note the rows of isometric particle with similar size to CPMV particles. Bar = 100 nm. ×95,000. **E,** crystalline inclusions in the cytoplasm of a mesophyll cell from a plant dually infected with SMV and CPMV. Bar = 500 nm. ×46,000.

The findings that SMV enhanced the titers of both BPMV and CPMV and that SMV (but not BYMV or PMV) interacted synergistically with comoviruses suggest that synergism in this system is determined by the coinfecting potyvirus. BYMV and PMV should provide excellent controls for investigations on the cellular and molecular basis of synergism between SMV and comoviruses. To investigate whether the lack of synergism between either BYMV or PMV and comoviruses is related to the frequency of dual infection of cells in dually infected plants, we experimented with an ultrastructural approach based on the formation of crystal arrays of comoviruses (Fig. 4E) in cells containing potyvirus-induced cylindrical inclusions. Because the plasmolysis procedure used for this purpose apparently destroyed the cylindrical inclusions, this approach was not pursued.

Several nonstructural gene products of comoviruses and potyviruses (including RNA dependent RNA polymerase and protease) share significant similarities in amino acid sequences (5,15,18). A possible explanation for the enhancement of comoviruses in dually infected plants may thus be related to the ability of the comoviruses to utilize SMV replication machinery for their own multiplication. Our results do not support such a mechanism (*unpublished data*). Alternatively, the putative SMV-encoded movement protein may enhance the transport of comoviruses, and as a result the number of infected cells in dually infected plants is higher than that in singly infected plants. This possibility is currently being investigated in our laboratory.

There are several known examples of pairs of viruses, in addition to SMV and BPMV (or CPMV), that interact synergistically in dually infected plants. These include potato virus Y (PVY) and potato virus X (PVX), maize dwarf mosaic virus and maize chlorotic mottle virus, PVY and potato leaf roll virus, and zucchini yellow mosaic virus and cucumber mosaic virus (1,16,17,23). These pairs of viruses have two features in common: first, each pair of viruses is comprised of a potyvirus and a nonpotyvirus member, and second, the concentration of the nonpotyvirus member of the pair is significantly increased in the dually infected plants. The concentration of the potyvirus component, on the other hand, remains essentially unchanged. With the exception of the comoviruses, the nonpotyvirus members of these pairs differ from the potyviruses in genome organization and expression strategies. With this in mind, it is likely that more than one mechanism is involved in synergism between unrelated viruses. Recent studies with the PVX/PVY system have shown that the level of PVX (−) strand RNA increased dramatically in dually infected plants (30). These results suggest that synergism between PVX and PVY in dually infected tobacco plants may involve an alteration in the normal regulation of the relative levels of PVX (+) and (−) strand RNAs during viral replication (30). No such differential accumulation in the level of comovirus (−) strand RNA over that of (+) strand RNA was observed in soybean plants dually infected with SMV and CPMV (or BPMV), suggesting that still a different mechanism for synergism is involved in this system (Ghabrial, *unpublished*).

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