Top Necrosis and Cellular Changes in Soybean Doubly Infected by Soybean Mosaic and Bean Pod Mottle Viruses

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ABSTRACT

Soybean plants inoculated with soybean mosaic virus (SMV) 1 week prior to bean pod mottle virus (BPMV) inoculation often develop top necrosis. Conditions favorable for top necrosis of doubly infected plants included diurnally fluctuating (27-21 C) and constant (27 C) temperatures, inoculation of seedlings rather than older plants, and use of inoculum containing high SMV titers. Concentrations of SMV were significantly higher in doubly infected plants than those in singly infected plants 3 and 4 weeks after SMV inoculation as determined by virus particle counts and by serological and infectivity dilution end points. Growth of SMV-infected and doubly infected plants was not increased by applications of gibberellic acid, whereas growth of BPMV-infected and healthy plants was increased. No consistent differences in number or size of SMV inclusion bodies between singly and doubly infected plants were detected by either light or electron microscope observations.

Inclusions of SMV and BPMV-like particles were found in the same cells. Starch granules were more numerous and larger in healthy than in singly or doubly infected plants. Osmiophilic globules appeared and increased in size in necrotic areas of doubly infected plants.

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Plant diseases caused by double infection with two unrelated viruses may be more severe and may manifest different symptoms than the diseases caused by either virus alone (6); virus concentrations may also be significantly altered compared to those in singly infected plants. Detailed information, however, on mixed infections of plants by unrelated viruses is limited. Studies on cellular host responses to combined infections by tobacco mosaic and etch viruses indicated that different viruses could coexist within the same cells (8); however, studies of infection with these two viruses by Fujisawa et al. (4) indicated that the challenge virus failed to form progeny when infection with the first virus had already been fully established.

Soybean mosaic virus (SMV) and bean pod mottle

virus (BPMV) are unrelated viruses which cause synergistic responses when coinfecting soybean (*Glycine max* [L.] Merr.) (9, 11). SMV-inoculated soybean seedlings initially develop chlorotic veinbanding and mild mottle on expanding leaves; subsequently, foliage becomes leathery and crinkled. Symptoms on BPMV-infected soybean are mainly limited to a mottling of expanding leaves; these symptoms become masked as the leaves expand to their final size. Previous research has shown that field-grown soybean doubly infected with these two viruses develops severe symptoms, including foliage distortion, chlorotic mottling, stunting, misshapen fruit, and necrosis (11). Soybean plants inoculated sequentially 6 days apart with SMV and BPMV, respectively, often develop top necrosis; and virus concentrations in doubly infected plants, as measured by local lesion assays, increase significantly above those in singly infected plants (10). Subsequent work, however, indicated that SMV local lesion assays on bean in the presence of BPMV are unreliable, since BPMV interferes with SMV local lesion formation (7, and J. P. Ross, *unpublished data*). The objective of this investigation was to obtain more information on the role of the two viruses in double infections by examining: (i) the effect of various factors on top necrosis; (ii) SMV concentrations in doubly and singly infected plants; and (iii) host responses and virus-associated structures detectable with light and electron microscopy.

MATERIALS AND METHODS.—The SMV isolate used was previously described as SMV-1 (12), and the BPMV isolate was recovered from commercial soybeans in North Carolina. Plants of soybean cultivar Hill were grown, two/4-inch pot, in a sandy loam provided with a complete fertilizer. Unless otherwise stated, plants were greenhouse-grown at average daily minimum and maximum temperatures of 19 and 32 C under a vegetative photoperiod provided by the interruption of the dark period with 2 hr of light.

Inoculum was produced by the grinding of leaves from soybean plants infected with either virus in 0.05 M phosphate buffer, pH 7.0. Infectivity of SMV and BPMV inoculum was determined in assays on primary leaves of greenhouse-grown *Phaseolus vulgaris* L. 'Kentucky Wonder' wax pole bean (KWP), a local lesion host for both viruses. All inoculations were made by rubbing inoculum-soaked cotton swabs on Carborundum-dusted leaves. Unless otherwise stated, in experiments dealing with sequential inoculations of soybean, fully expanded primary and first trifoliolate leaves were inoculated 1 week apart with SMV and BPMV, respectively, and were rinsed immediately after inoculation.

TABLE 1. Effect of temperature on development of top necrosis of Hill soybean inoculated sequentially with soybean mosaic and bean pod mottle viruses 1 week apart[a]

Temperature		
Day	Night	Plants with top necrosis
C	*C*	%
27	21	95
30	18	70
21	21	8
27	27	79

[a] Twenty plants/test.

RESULTS.—*Symptoms on doubly infected plants inoculated sequentially.*—Plants inoculated with BPMV 1 week after SMV developed top necrosis about 3 weeks after the second inoculation. The frequency of the necrotic reaction, however, varied considerably from one test to another. Nonnecrotic, doubly infected plants manifested severe mottling, foliar distortion, and dwarfing.

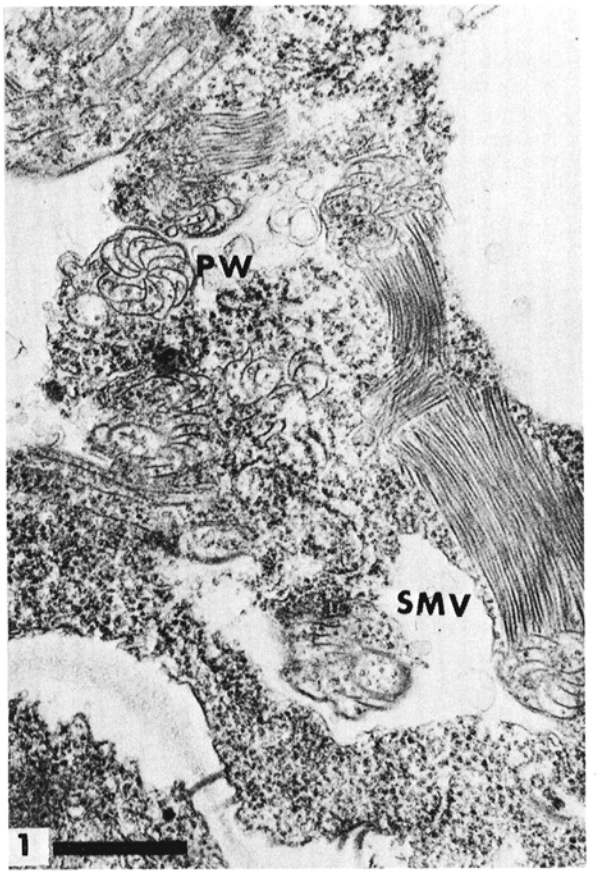
Effect of temperature on necrosis.—Soybean seedlings inoculated sequentially with SMV and BPMV were grown at various temperatures in either a growth chamber (Environator model E 3448 with 800 ft-c), temperature-controlled greenhouse chambers, or on the greenhouse bench. The growth chamber was set for 10-hr days, and greenhouse chambers were maintained at either 27 ± 2 or 21 ± 2 C. Data on top necrosis were obtained 3 weeks after BPMV inoculation. Each treatment contained 20 plants.

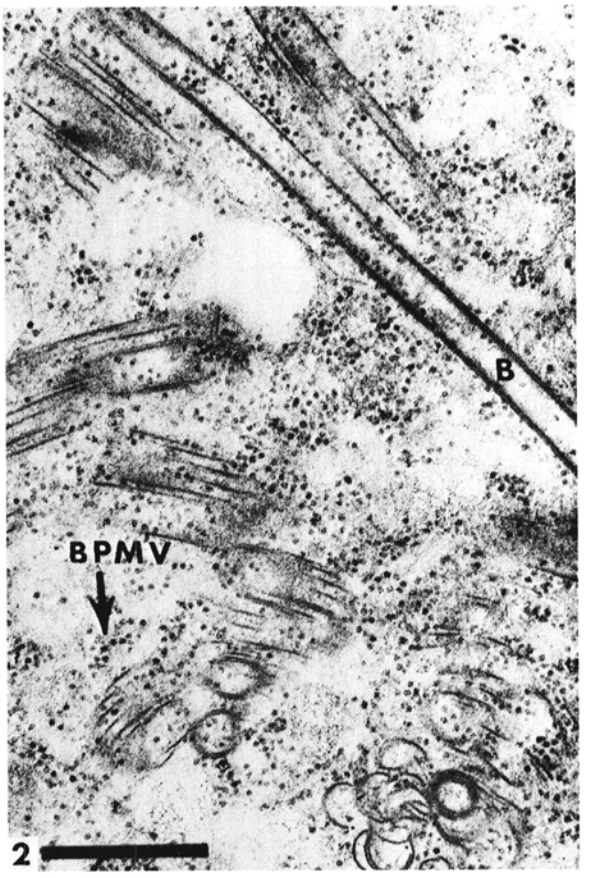
Top necrosis appeared in 70-95% of plants in the growth chamber with either diurnally fluctuating temperatures or constant 27 C, whereas, at constant 21 C, top necrosis developed on only 8% of the plants (Table 1).

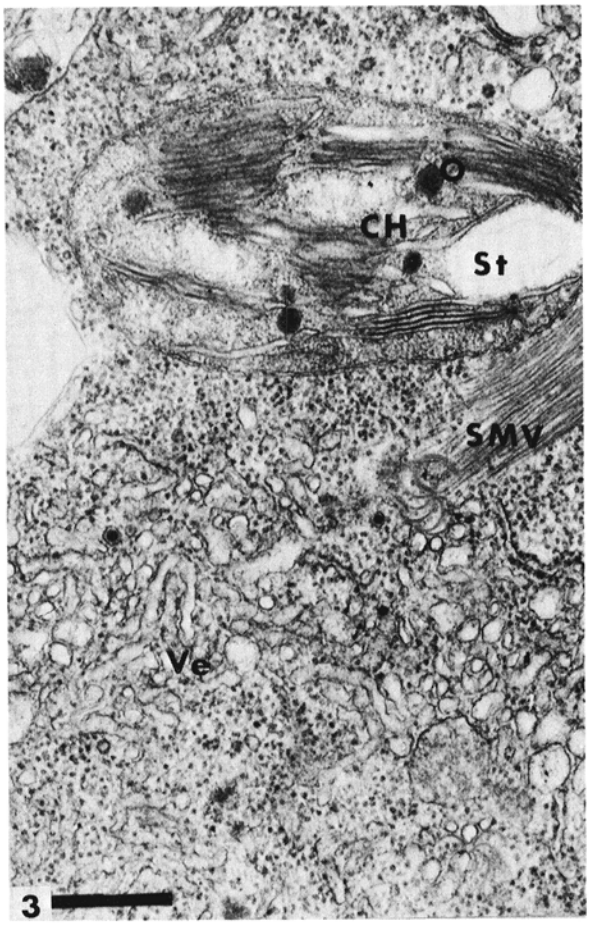
Effect of plant age on top necrosis.—Hill soybean plants, 9, 16, and 20 days old, were grown in the greenhouse and inoculated sequentially with SMV and BPMV 1 week apart, respectively, on the most recently expanded leaves. Necrosis developed on 90, 31, and 11% of the plants inoculated at 9, 16, and 20 days of age, respectively.

Effect of SMV inoculum concentrations.—Symptom development on greenhouse-grown soybeans was studied after inoculation of plants with various SMV

Fig. 1-4. Electron micrographs showing ultrastructure of soybean singly and doubly infected with soybean mosaic and bean pod mottle viruses. All magnification marks are 0.5 μ. B = bundle inclusion; BPMV = bean pod mottle virus; CH = chloroplast; O = osmiophilic globule; PW = pinwheel inclusion; SMV = soybean mosaic virus; St = starch; VA = vacuole; Ve = vesicle; W = cell wall. 1) Soybean stem parenchyma cell infected with SMV. 2) Bundle inclusions of SMV and scattered BPMV occurring within the same epidermal cell from a doubly infected plant. 3) Numerous vesicles in the cytoplasm of stem parenchyma cell from doubly infected soybean. 4) Numerous swollen osmiophilic globules in the chloroplast of a doubly infected leaf cell adjacent to the necrotic epidermis; an aggregate of BPMV lies in the vacuole; portions of the chloroplast membrane, lamellae, and starch granules have disappeared.


PW
SMV
1


B
BPMV
2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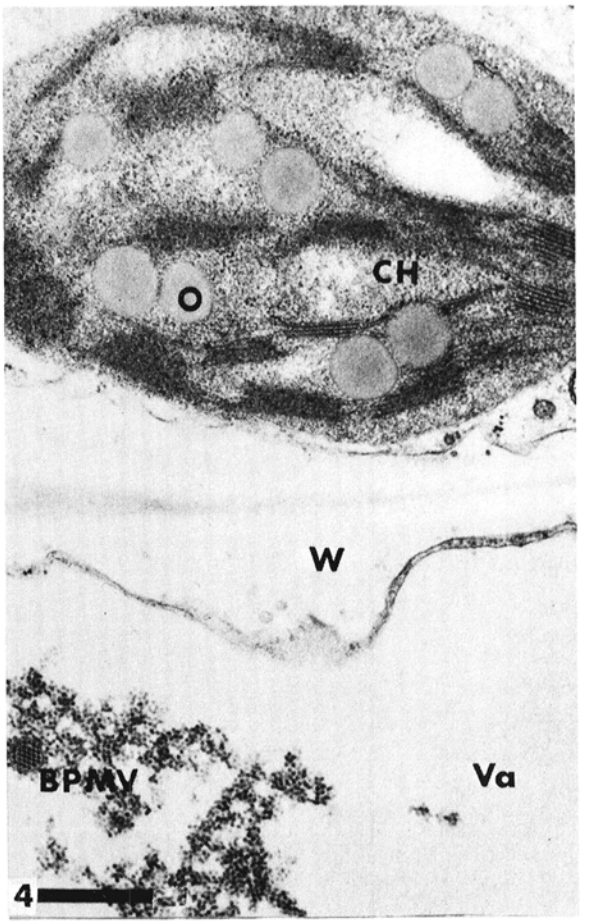
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TABLE 2. Effect of soybean mosaic virus (SMV) inoculum concentration on incidence of top necrosis developed by Hill soybean inoculated sequentially with SMV and bean pod mottle virus 1 week apart

SMV inoculum		
Dilution	Local lesion assay[a]	% Top necrosis
1:1	156	100
1:10	14	97
1:100	4	50
1:1,000	0.1	0

[a] Average lesions on eight half-leaves of Kentucky Wonder wax pole bean.

TABLE 3. Comparison of growth increases caused by gibberellic acid (GA) applied to Hill soybean inoculated with soybean mosaic virus (SMV), bean pod mottle virus (BPMV), or sequentially inoculated with SMV and BPMV 1 week apart

Treatment	Growth increase (cm) of plants infected with[a,b]			Growth increase (cm) of healthy control
	SMV	SMV + BPMV	BPMV	
GA	29.7 b	6.0 a	36.4 c	49.9 d
None	28.0 b	6.1 a	28.4 b	27.8 b
% Increase	6	0	28	79

[a] Each figure represents an average from 12 plants.
[b] Values with different letters are statistically different at the 5% level (Duncan's multiple range test).

concentrations, 1 week prior to BPMV inoculation. Incidence of top necrosis decreased as SMV inoculum titers were reduced (Table 2).

Time required for systemic infection of viruses.—Since systemic virus infection is necessary for necrosis, and this reaction is normally dependent on inoculation with SMV prior to BPMV, experiments were performed in the greenhouse chamber at 27 ± 2 C to determine the time necessary for systemic spread of the viruses from inoculated primary leaves to the main plant axis. Leaves were removed at 24-hr intervals after inoculation with either SMV or BPMV. Both viruses caused systemic infection of plants when inoculated leaves were removed 72 hr but not 48 hr after inoculation.

Effect of gibberellic acid on dwarfing symptom.—Since doubly infected soybean plants become severely dwarfed, the effect of the growth-promoting material, gibberellic acid (GA), was examined. Four days after primary leaves were inoculated with SMV, they were dipped in 50 mg/ml GA solution containing 0.05% $NaHCO_3$ and a wetting agent. Three days later, the first trifoliolate leaves were inoculated with BPMV, and another GA treatment was applied. Noninoculated controls consisted of GA-treated and nontreated plants; inoculated control plants were not treated with GA. Plant heights were measured at the time of BPMV inoculation and 6 weeks later. The growth of healthy and BPMV-infected plants was stimulated by GA, whereas doubly infected and SMV-infected plants failed to respond (Table 3). Doubly infected plants were severely stunted.

SMV concentration in singly and doubly infected plants.—Stem terminals (15 mm) or the youngest fully expanded trifoliolate leaves from 10 plants were

TABLE 4. Comparison of soybean mosaic virus (SMV) concentrations in Hill soybean inoculated with SMV alone and sequentially with SMV and bean pod mottle virus (BPMV) 1 week apart

Assay methods	Part assayed	Infection with	Weeks after SMV inoculation		
			2	3	4
			Virus particle counts[c]		
Electron-microscope counts	Stem[a]	SMV	118 a	309 b	17 a
		SMV + BPMV	230 a	621 c	389 b
	Leaf[b]	SMV	190 a	276 b	67 a
		SMV + BPMV	150 a	800 c	474 b
			Serological dilution end point[c]		
Microprecipitin test	Stem	SMV	1/2	1/6	1/2
		SMV + BPMV	1/4	1/16	1/4
	Leaf	SMV	1/4	1/8	1/4
		SMV + BPMV	1/4	1/32	1/8
			Infectivity dilution end point		
Infectivity test	Stem	SMV	10^{-3}	10^{-4}	10^{-3}
		SMV + BPMV	10^{-3}	10^{-4}	10^{-4}

[a] Terminal 1.5 cm of 10 stems were used for each assay.
[b] Sap from young expanded trifoliolate leaves from 10 plants.
[c] Each figure is the total of counts of particles between 400 and 800 nm on ten squares; figures with different letters are statistically different at the 5% level (Duncan's multiple range test).
[d] Highest serologically active dilution.

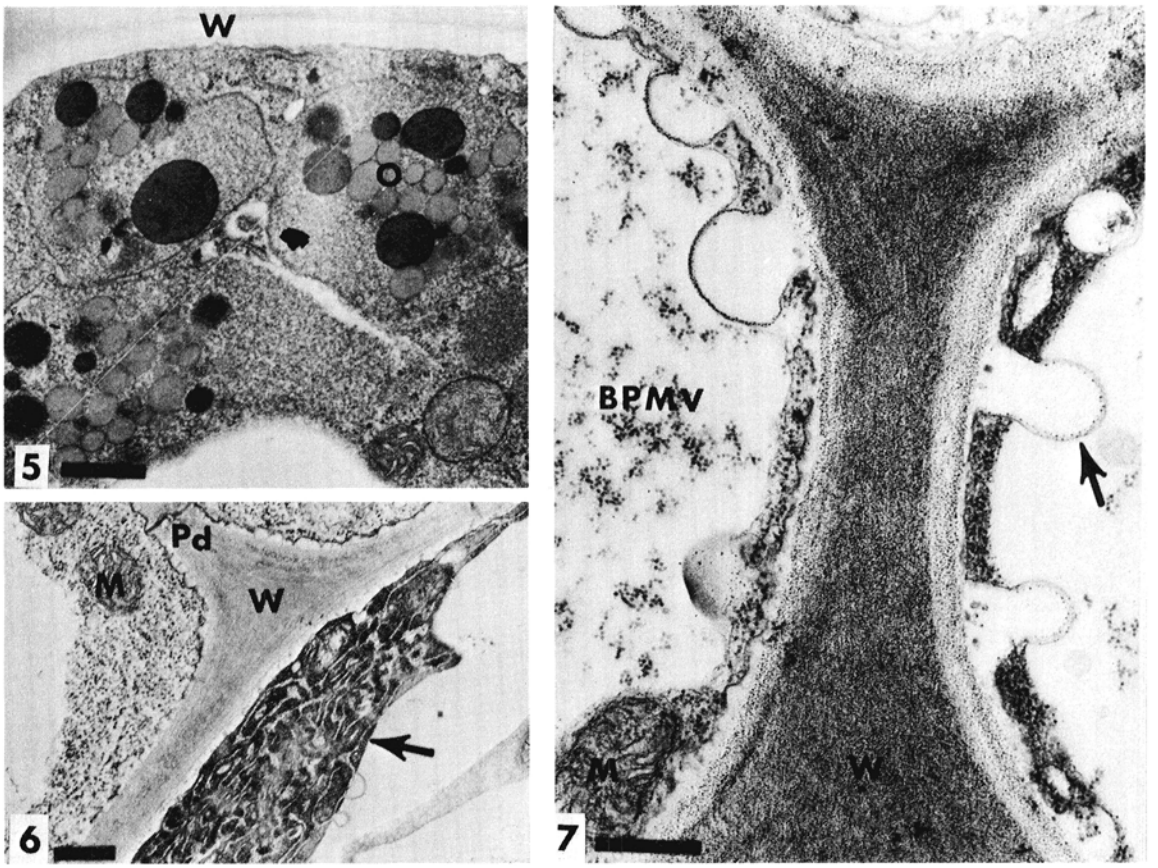

Fig. 5-7. Electron micrographs showing ultrastructure of soybean singly and doubly infected with soybean mosaic and bean pod mottle viruses. All magnification marks are 0.5 μ. BPMV = bean pod mottle virus; M = mitochondria; O = osmiophilic globule; Pd = plasmodesmata; W = cell wall. **5)** Doubly infected leaf cell showing large osmiophilic globules in the chloroplast. Starch and lamella have disappeared, chloroplast membranes ruptured, and osmiophilic globules escaped. **6)** Doubly infected soybean stem cell near a necrotic area. Note dense cytoplasm with endoplasmic reticulum (arrow). **7)** Plasmalemma protruding into the cytoplasm of a cortical cell from doubly infected plant.

assayed for virus concentration. One g of fresh tissue was ground in 1 ml of 0.05 M borate buffer, pH 8.3; the expressed sap was strained through cheesecloth and centrifuged at 12,000 *g* for 10 min. A portion of the supernatant fluid was stained in 2% phosphotungstic acid at pH 6.7, and drops of equal volume were placed on a 100-mesh grid coated with Formvar and carbon. After 2 min, each drop was drawn off with filter paper so that each grid contained a thin film of liquid. After drying, we observed the grids under the Siemens IA electron microscope (EM). Virus particles, 400 to 800 nm in length, were counted on each of 10 squares. The remaining supernatant fluid was used for microprecipitin serological dilution end point tests and for infectivity dilution end point tests utilizing primary leaves of 20 soybean plants for each dilution.

Counts of SMV particles from doubly infected plants were higher than those from singly infected plants 3 and 4 weeks after SMV inoculation (Table 4). Both singly and doubly infected tissues contained more SMV particles in the 3rd week than in the 2nd or 4th week after inoculation. Serological dilution-end point determinations confirmed results from virus particle counts. Infectivity dilution end points of sap from doubly infected plants were greater than those from singly infected plants only at 4 weeks after inoculation.

Electron microscopy studies.—Approximately 1-mm^2 samples of the youngest expanded leaves and pieces of stem terminals (less than 1 cm from apex) were taken from healthy, singly, and doubly infected soybean plants 2, 3, and 4 weeks after SMV inoculation. Tissues were excised in 3% glutaraldehyde in 0.05 M phosphate buffer at pH 7.2, postfixed in 1% osmium tetraoxide, and embedded in Epon 812. Sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed under the EM.

Particles of both viruses and SMV inclusions were found in most tissues of infected plants, but occurred more commonly in trichomes, epidermal, and

mesophyll cells. SMV- and doubly infected cells contained SMV inclusions of pinwheels, bundles, and circular shapes (Fig. 1, 2). Three weeks after SMV inoculation, greater numbers of inclusion bodies and SMV particles were observed in doubly infected and singly infected plants than were observed 2 weeks after inoculation. This increase in virus structures coincided with symptom severity. Particles of BPMV were indistinguishable from ribosomes except when the particles formed either crystalline arrays or were very concentrated (Fig. 4). Both SMV inclusions and BPMV particles were found in the same cell (Fig. 2). Cells infected with either or both viruses contained more peripheral vesicles (Fig. 3) and fewer starch granules of smaller size than did noninfected cells.

Except in association with necrotic tissue, cells in doubly and singly infected plants appeared similar. As doubly infected cells became necrotic, starch granules became smaller and fewer in number, osmiophilic globules appeared and grew in size, grana and lamellae disappeared, and chloroplast membranes ruptured (Fig. 4, 5). Cell membranes protruded into the cytoplasm more frequently in doubly infected than in singly infected plants (Fig. 7), and densely stained cytoplasm mixed with long endoplasmic reticulum appeared in the periphery of necrotic cells (Fig. 6). Cellular contents of necrotic leaf and stem tissues became electron dense and indistinguishable 3 weeks after SMV inoculation, but cell walls maintained their identity, and SMV particles and pinwheels became less electron dense.

Light microscopy studies.—Tissue from the same areas used in EM studies were fixed in formalin-acetic acid, dehydrated in a t-butanol series, embedded in Tissuemat, sectioned on a rotary microtome, and stained in safranin O and fast green. Inclusion bodies were studied in epidermal tissue stripped from petioles and stained with phloxine B. Sections 1 μ thick, of young leaves and stem terminals embedded in Epon 812, were cut with a glass knife on the ultramicrotome and stained with periodic acid-Schiff reagent.

Diseased and healthy tissues appeared similar except in necrotic areas of doubly infected plants. These areas first appeared 3 weeks after SMV inoculation as heavily red-stained cells in the upper and lower epidermis of young leaves and in young stem tissue; these tissues subsequently collapsed. Necrotic areas also appeared in cortical, pith, and xylem parenchyma tissues in the stem. Amorphous inclusion bodies were found in petioles of young leaves of singly and doubly infected plants 1 week after SMV inoculation. SMV inclusion bodies also developed in plants inoculated with BPMV 1 week prior to SMV. Nuclei of cells in doubly infected plants during onset of necrosis were up to 2.5 times larger than normal. Progeny of the cross Dare (SMV-susceptible) X Ogden (SMV-resistant) that become necrotic when inoculated with SMV alone also developed these areas with enlarged nuclei. Fewer starch granules were observed in chloroplasts of singly and doubly infected plants than in those of healthy plants. The number of starch granules in singly infected and doubly infected plants appeared similar.

DISCUSSION.—Top necrosis of soybean doubly infected with SMV and BPMV depends on several factors: age of plant, host genotype, order of inoculation, temperature, and inoculum titer. Since the over-all interaction of these factors probably determines the appearance of top necrosis, development of the latter probably depends on a balance among them. Although greenhouse tests showed that younger plants appear more susceptible and sensitive to the necrotic reaction, previous field observations have shown that old plants may become necrotic when doubly inoculated by vectors.

The development of top necrosis by certain progeny of Dare X Ogden infected by SMV alone indicates that SMV plays a key role in the necrotic and severe symptoms of doubly infected plants. The following results obtained in these studies also support this theory: (i) the direct relationship between SMV inoculum concentration and incidence of top necrosis of doubly infected plants; (ii) the increase in SMV titers in doubly infected plants compared to those in singly infected plants as measured by particle counts and serology during the 3rd and 4th week after inoculation; (iii) the fact that temperatures favoring SMV symptoms (27-30 C) also favor necrosis of doubly infected plants; (iv) the lack of response of both SMV-infected and doubly infected plants to applications of GA, suggesting that the severe stunting of doubly infected plants results from a severe reaction to SMV rather than a unique reaction to both viruses. The lack of response to GA by SMV-infected plants is similar to that of GA derivative-treated beans infected with Mexican severe bean mosaic virus (14).

The discrepancy found between SMV concentrations as measured by particle counts and those measured by infectivity dilution end points 3 weeks after SMV inoculation indicates that the infectivity per particle of SMV from doubly infected plants may be less than that from singly infected plants. SMV infectivity dilution end points of sap from singly and doubly infected plants 3 weeks after inoculation were similar and agreed with results of Ross (10) and Tu & Ford (13). Titers of BPMV in doubly infected plants may also increase above those in singly infected plants (10); however, investigations have since shown that this phenomenon is not consistently associated with top necrosis in doubly infected plants (J. P. Ross, *unpublished data*).

Throughout these experiments, the incidence of top necrosis was greatest in greenhouse-grown, doubly infected plants during April and October, and least during midwinter and summer months. These observations indicate that day-length may also affect the necrotic reaction of doubly infected plants.

Since both viruses move out of inoculated leaves at about the same rate, and previous work (10) has shown that necrosis usually develops only when plants become infected with SMV before BPMV, necrosis is apparently favored by systemic spread of SMV before that of BPMV.

Pinwheel, circular, and bundle inclusions of SMV are similar to those previously described in hosts infected with viruses of the PVY group (1). These structures may be unimportant in development of necrotic symptoms, since SMV inclusions appeared morphologically similar in singly and doubly infected plants. Occurrence of BPMV particles and SMV inclusions in the same cell, even though BPMV was inoculated 1 week after SMV, implies that there is no antagonism between SMV and BPMV. These results confirm the previous suggestions of McWhorter & Price (8) and Honda et al. (5) that two unrelated viruses can coexist in the same cell, but differs from the results of Fujisawa et al. (4).

The occurrence of chloroplasts with fewer starch grains and larger, more numerous osmiophilic globules in the necrotic areas of doubly infected plants than occurred in chloroplasts in nonnecrotic tissue indicates an interference with the starch synthesizing system and/or a stimulation of the starch degrading metabolism systems. These results are similar to those of Carroll & Kosuge (2), who found large osmiophilic globules instead of grana and lamella in cells near necrotic tobacco tissue infected with tobacco mosaic virus. In contrast to soybeans, however, the tobacco tissue contained both osmiophilic globules and many starch granules. An increased development of osmiophilic globules has been associated with degradation of grana (3).

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