Multiplication and Pathogenesis of Cowpea Chlorotic Mottle Virus and Southern Bean Mosaic Virus in Single and Double Infections in Cowpea

C. W. Kuhn and Wm. O. Dawson

Department of Plant Pathology and Plant Genetics, University of Georgia, Athens 30602. Present address of second author: Department of Plant Pathology, University of California, Riverside 92502.
Accepted for publication 25 April 1973.

ABSTRACT

A mixed infection of cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus-cowpea strain (SBMV) caused a synergistic reduction of cowpea growth. Leaf, stem, and root weights; plant height; and yield were reduced more by a double infection than the additive effects of each single infection. The double infection increased seed transmission of SBMV from 12% to 20%. Viral nucleoprotein measurements demonstrated that a simultaneous inoculation of both viruses reduced the yield of SBMV about 50% but had no effect on the yield of CCMV. When either virus became established first and was in the rapid synthesis phase, the synthesis of the challenging virus was significantly retarded. Once past the rapid synthesis phase, synthesis of either virus in challenged plants was similar to synthesis in healthy plants of the same age. Smaller quantities of each virus were produced in new trifoliate leaves, but a 1:3 ratio of CCMV: SBMV nucleoprotein was generally evident, indicating that neither virus became dominant in a double infection.

Additional key words: necrosis symptoms, radioisotope labeling.

Unrelated viruses are capable of infecting and multiplying within the same plant. McWhorter & Price (14) presented evidence that tobacco mosaic and tobacco etch viruses can multiply simultaneously in the same plant cell, and Fujisawa et al. (7) found characteristic inclusion bodies of each of the same two viruses in about 90% of the cells of tobacco plants. Lee & Ross (13) found soybean mosaic virus inclusions and bean pod mottle virus particles in the same cells of soybean plants.

Mixed infections of unrelated viruses are not uncommon in nature. Cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus-cowpea strain (SBMV) were first found in a doubly infected cowpea plant. Other natural mixed infections have been found in cowpea (9) and in other plant species (11). Some viral combinations cause more severe host symptoms than either virus alone (17, 19).

CCMV and SBMV have similar chemical and physical properties. Both have small isometric virions with one nucleoprotein component and a similar quantity of nucleic acid. Biologically, however, CCMV loses infectivity rapidly in vivo (12) and SBMV infectivity is quite stable (C. W. Kuhn, unpublished). Since preliminary studies indicated different rates of synthesis of the two viruses in cowpea, we have studied their synthesis patterns in relation to their effect on the host plant.

MATERIALS AND METHODS.—The two viruses used in this study were CCMV and the cowpea strain of SBMV. CCMV was maintained in Phaseolus vulgaris L. ‘Bountiful’ and SBMV in cowpea, Vigna sinensis (Torner) Savi ‘Early Ramshorn’. The viruses were examined in Early Ramshorn cowpea. Primary leaves, 8 to 10 days after seeding, were inoculated with sap from young, systemically infected, trifoliate leaves. The sap was diluted 1:10 in 0.01 M neutral potassium phosphate buffer. For double inoculations, equal volumes of CCMV and SBMV were mixed; for single inoculations, an equal volume of buffer was substituted for the second virus. One or two plants were grown in each 10- or 15-cm pot containing a soil, sand, vermiculite mixture (2:1:1, v/v), and a complete fertilizer (N, P, K) was added biweekly. The plants were maintained in a greenhouse with a temperature range of 24 to 32 C.

For purification, various concentrations of several buffers were tested to determine which would provide maximum nucleoprotein yields of both CCMV and SBMV. Acetate buffer, 0.1 M, pH 5, was best. Lower molarity of acetate, and other buffers, sometimes caused concentrated (more than 10 mg/ml) virus preparations to precipitate.

Leaves from singly and doubly infected plants were extracted in acetate buffer, chloroform, and n-butanol (1 g of tissue plus 1 ml of each component). Concentration and purification were accomplished by several centrifugations: 10,000 g for 10 min; 100,000 g for 2.5 hr; 10,000 g for 10 min; 220,000 g for 1 hr. Virus nucleoprotein concentration was determined spectrophotometrically; an absorbancy at 260 nm of 6.0 equaled 1 mg/ml for both CCMV and SBMV.

The relative amount of each virus in a mixture was determined by labeling 1 mg of virus on 10 to 40% sucrose columns prepared in 0.1 M acetate buffer, centrifuging 5 hr at 70,000 g, and monitoring a fractionation (ISCO Model D) with an ultraviolet analyzer (ISCO Model UA2). The viruses separated reasonably well (CCMV)S20,w = 885; SBMV)S20,w = 1155) and the peaks were measured with a planimeter.

The rate of 32P incorporation into virus was determined, as described previously (4), with primary leaves of cowpea grown in a controlled-environment chamber at 27 C with a 16-hr photoperiod and an illumination of 9,146 lx (850 ft-c). Twenty plants per treatment were terminated labeled for 24 hr by putting the excised stem of each plant into a tube containing 1.0 ml of 0.02 mc/ml 32P-orthophosphate. The zones of virus from the sucrose density gradients were collected and counted.
in planchets with a Nuclear-Chicago thin-window gas flow counter.

Dry weights of leaves, stems, and roots were determined by drying to a constant weight at 80°C. Seed were dried at 21 to 25°C, and the final weight was determined after weight remained constant for 7 days.

For seed transmission studies, plants were observed for 30 days after planting. Sap from plants with symptoms was used to inoculate Glycine max (L.) Merr. Bragg and Early Ramshorn cowpea, tester plants for CC MV and SB MV, respectively.

RESULTS.—Foliar symptoms.—Typical CC MV and SB MV symptoms developed on systemically infected cowpea leaves with both single and double infections. The bright chlorotic mottle of CC MV and the mosaic and leaf distortion caused by SB MV were observed on all trifoliate leaves of doubly infected plants. Symptoms remained intense until leaf drop at senescence.

On the inoculated primary leaves, SB MV caused diffuse chlorotic spots and CC MV sometimes caused necrotic etchings. Both symptoms were apparent with simultaneous inoculations. However, when a 4- to 6-day-old SB MV infection was challenged with CC MV, the necrosis was intensified and the challenged leaves abscised 4-7 days later. The necrosis usually did not develop on uninoculated leaves, and CC MV infections challenged with SB MV at various intervals after inoculation (2 to 20 days) did not develop any necrosis.

Plant growth.—In general, CC MV reduced plant growth (height and dry wt of leaves, stems, and roots) by 3 to 13% (not statistically significant) and SB MV caused significant reductions of 18 to 32% (Table 1). The double infection caused a synergistic response; overall reductions were 42 to 64% and, in each type of measurement, exceeded the additive effects of the two single infections.

When individual leaves and internodes were considered, the most drastic double infection response was on the intermediate plant growth (Table 1). The first two true leaves, which develop almost simultaneously, usually were not affected by single or double infections. The next three leaves and internodes, however, were reduced 64 and 49%, respectively, as compared to 32 and 37% for the additive responses of single infections. Although subsequent new leaves had typical symptoms, they showed less growth reduction than the intermediate ones.

Yield.—The double infection reduced yield 43% as compared to 0% and 16% reductions for CC MV and SB MV, respectively (Table 2). Yield loss was primarily caused by the production of fewer pods and seeds, but weight per seed was also less for seed from SB MV and double infections. In a second test with two plants/15-cm diam pot, cowpea yields were reduced 0, 11, and 37% by CC MV, SB MV, and double infections, respectively.

Seed characteristics.—All seed (597) produced on 30 doubly infected plants were mottled. SB MV also caused seed coat mottling but only on 38% of the

---

**Table 1. The effect of single and double infections of cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus (cowpea strain) (SB MV) on plant growth of 'Early Ramshorn' cowpea**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant height (cm)</th>
<th>Leaf wt</th>
<th>Stem wt (g)</th>
<th>Root wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Allc</td>
<td>Middeld</td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>30.1x</td>
<td>3.88x</td>
<td>2.36x</td>
<td>22.4x</td>
</tr>
<tr>
<td>CCMV</td>
<td>26.2x</td>
<td>3.55x,y</td>
<td>2.43x</td>
<td>21.7x</td>
</tr>
<tr>
<td>SB MV</td>
<td>23.1y</td>
<td>3.01y</td>
<td>1.60y</td>
<td>18.4y</td>
</tr>
<tr>
<td>CCMV+SB MV</td>
<td>17.4z</td>
<td>2.22z</td>
<td>0.85z</td>
<td>12.3z</td>
</tr>
</tbody>
</table>

a Ten plants/treatment; one plant/15-cm pot; plants were inoculated 10 days after seeding; measurements made 21 days after inoculation.
b Values in each column not followed by the same letter vary significantly at the 1% level. In column six, 0.44 is significantly different from 0.64 at the 5% level.
c The leaf weight was combined for all trifoliate leaves, usually six or seven.
d The leaf weight was combined for the third, fourth, and fifth trifoliate leaves.

---

**Table 2. The effect of single and double infections of cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus (cowpea strain) (SB MV) on seed production of 'Early Ramshorn' cowpea**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pods</td>
</tr>
<tr>
<td>Healthy</td>
<td>6.6x</td>
</tr>
<tr>
<td>CCMV</td>
<td>6.1x,y</td>
</tr>
<tr>
<td>SB MV</td>
<td>5.1y</td>
</tr>
<tr>
<td>CCMV+SB MV</td>
<td>4.3z</td>
</tr>
</tbody>
</table>

a Ten plants/treatment; one plant/15-cm pot; plants were inoculated 10 days after seeding.
b Values in each column not followed by the same letter vary significantly at the 5% level.
c The mean wt/seed was determined by weighing all seed and dividing by the number of seed.

---

**Table 3. Effect of single and double infections of cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus (cowpea strain) (SB MV) on seed mottle and on transmission of the viruses through seed of 'Early Ramshorn' cowpea**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mottled seed (%)</th>
<th>Seed coat color</th>
<th>No. of plants</th>
<th>% with virusb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>7</td>
<td>Normal</td>
<td>124</td>
<td>0</td>
</tr>
<tr>
<td>CCMV</td>
<td>10</td>
<td>Normal</td>
<td>402</td>
<td>0</td>
</tr>
<tr>
<td>SB MV</td>
<td>38</td>
<td>Mottled</td>
<td>307</td>
<td>12</td>
</tr>
<tr>
<td>CCMV+SB MV</td>
<td>100</td>
<td>Mottled</td>
<td>443</td>
<td>20</td>
</tr>
</tbody>
</table>

a Plants were inoculated 10 days after seeding.
b Plants were observed for 4 weeks.
similar mottling occurred on a few seed from healthy and CCMV-infected plants (Table 3). The seed coat mottling apparently was not related to seed transmission. CCMV was not seed transmitted and SBMV was transmitted similarly through normal colored and mottled seed (Table 3). Surprisingly, SBMV was transmitted at a higher rate through seed produced on doubly infected plants than seed from plants singly infected with SBMV (Table 3). In two studies, SBMV transmission was increased from 13.1 to 20.0% and from 12.2 to 19.7%, an average 57% increase caused by the double infection. The mechanism of seed transmission is poorly understood, but the SBMV increase in the CCMV-SBMV double infection is in contrast to an approximate 38% decrease in soybean mosaic virus seed transmission in plants doubly infected with soybean mosaic virus and bean pod mottle virus (16).

**Accumulation of virus nucleoprotein after simultaneous inoculation.**—When cowpea plants were simultaneously inoculated with CCMV and SBMV, the rate of CCMV accumulation was similar in both single and double infections (Fig. 1A). Approximately twice as much CCMV nucleoprotein was produced in the systemically infected trifoliate leaves as in the inoculated primary ones. Similarity in rate of CCMV accumulation in single and double infections was confirmed in numerous greenhouse tests and in three tests at 27°C. The decline in CCMV nucleoprotein concentration (mg of virus/g of plant tissue) in the trifoliate leaves between 4 and 8 days after inoculation was caused by an increase in leaf tissue (growth) and not by an actual loss in virus (Fig. 1A).

On the other hand, SBMV synthesis was more strongly affected by a simultaneous inoculation with CCMV. The accumulation of SBMV nucleoprotein was reduced at all stages of infection between 0 and 22 days after inoculation, and the final maximum SBMV nucleoprotein concentration was about 50% of the concentration in a single infection (Fig. 1B). As with CCMV, the concentration of SBMV was greater in the systemically infected trifoliate leaves than in the inoculated primary leaves in both single and double infections. The maximum SBMV nucleoprotein concentration occurred at about the same time period (15 days after inoculation), whether in single or double infections or in inoculated primary leaves or systemically infected trifoliate leaves.

The time when maximum synthesis occurred varied with the virus. Most of the CCMV was synthesized during the first 6 days after inoculation (Fig. 1A). During the same period in a single infection, about 25% of the total SBMV had been synthesized (Fig. 1B). A double infection obviously delayed SBMV synthesis since only 10% of the maximum nucleoprotein was produced by the 6th day (Fig. 1B). During the peak synthesis period, SBMV nucleoprotein accumulation rates were about 0.125 and 0.215 mg/day in double and single infections, respectively.

Inoculum concentrations did not affect the overall results. Purified virus was used as inoculum at 0.005 and 0.5 mg/ml, which compares to the usual sap inoculum of 0.05 - 0.1 mg/ml. Although both inoculum concentrations caused similar CCMV growth curves in a single infection, the dilute concentration reduced the early (2- to 4-day) CCMV synthesis by about 50% in a double infection. However, the final CCMV nucleoprotein concentration was similar, regardless of inoculum concentrations, at 8 to 10 days. Dilute SBMV inoculum, however, reduced the amount of SBMV synthesized in all combinations, but the general shape of the curves was unchanged.

![Graph](image-url)
Viral multiplication rates after simultaneous inoculation.—The multiplication rate of CCMV and SBMV was determined by measuring the rate of incorporation of $^{32}$P into nucleoprotein particles of each virus (Fig. 2). The multiplication rate of CCMV in the double infection initiated by a simultaneous inoculation with SBMV was almost identical to that of the single infection of CCMV in leaves maintained under identical conditions (4). There was a lag period of less than 1 day, the synthesis rate peaked after 3-4 days, and most of the synthesis occurred over a 5- to 6-day period. The synthesis of SBMV began more slowly. In both the single and double infections of SBMV, the lag period was much longer than that of CCMV. The multiplication of SBMV was faster and peaked earlier in the single infection than in the double infection (Fig. 2). After reaching a maximum rate, the multiplication of SBMV in both the single and the double infections began declining to a low level. The rate of decline, however, was slower for SBMV than CCMV.

The ratio of $^{32}$P incorporated into virus particles to virus nucleoprotein accumulated was much greater for CCMV than for SBMV. When the multiplication rate is integrated, a curve which represents the relative amounts of virus accumulated should result. When this was done, the calculated amount of CCMV in relation to SBMV was 2 to 4 times greater than the actual ratio. The data indicate that CCMV incorporated 2 to 4 times more $^{32}$P per particle than did SBMV.

Biosynthesis after challenge inoculation.—When a 4-day-old SBMV infection was challenged with CCMV, synthesis of CCMV was reduced markedly (Fig. 3). This reduction is in contrast to the lack of inhibition of CCMV synthesis which occurred with a simultaneous inoculation of both viruses (Fig. 1A). When CCMV was synthesizing rapidly (4 days after inoculation), and then the plants were inoculated with SBMV, SBMV synthesis was reduced, as in a simultaneous inoculation (Fig. 4). When plants were inoculated after the rapid synthesis phase for each virus was completed (12 and 20 days after inoculation for CCMV and SBMV, respectively), the synthesis of the challenge virus was similar in both infected and healthy plants of the same age (Fig. 3 & 4). Synthesis of both viruses was less in older plants than in younger ones.

An SBMV infection was challenged with CCMV at 1, 2, and 4 days after inoculation. Even with a 1-day advantage, SBMV synthesis was retarded by CCMV, as in a simultaneous inoculation (Fig. 5). At the 2-day challenge, however, the CCMV infection did not reduce the overall SBMV synthesis.

Biosynthesis in trifoliolate leaves.—Since a double infection did not reduce the number of leaves produced on cowpea plants, the amount of each virus in various trifoliolate leaves was determined. In both single and double infections, the first two trifoliolate leaves had more of each virus than subsequent leaves (Table 4). There was a general trend toward reduced total nucleoprotein of each virus in the newer leaves. The proportion of CCMV was slightly greater in the first leaf than in later ones, but a 1:3 nucleoprotein
ratio of CCMV:SBMV was evident in all leaves past the first one. Thus, there was no evidence that either virus tended to become dominant in its synthesis pattern regardless of the age of infection.

DISCUSSION.—Responses of plants to the synergistic action of two unrelated viruses are well documented (10, 16, 17, 19). It has been generally assumed that the increased severity of symptoms is correlated with an increase in the concentration of at least one of the viruses in the mixture, and this has been the case with two combinations: (i) potato virus Y and potato virus X in tobacco (15), and (ii) soybean mosaic virus and bean pod mottle virus in soybean (13). With CCMV and SBMV, the nucleoprotein concentration of neither virus was increased in doubly infected plants. CCMV concentration was the same in both singly and doubly infected plants, and SBMV nucleoprotein was reduced about 50% in the double infection. The suppression of infectivity, and (by inference) synthesis, of one plant virus by another has been noted (1, 2), but there was no mention of synergistic effects on symptoms or plant growth.

The most detailed mixed infection studies have been made by A. F. Ross and colleagues working with potato viruses X and Y (3, 5, 6, 8, 15, 18). They hypothesized that potato virus X synthesis is enhanced only in cells which are supporting a rapid synthesis of potato virus Y. Apparently the synthesis of potato virus Y provides some factor which encourages potato virus X synthesis. With CCMV and SBMV on the other hand, it appears that rapid synthesis of either virus is detrimental to the synthesis of the other. Although CCMV nucleoprotein concentration was not reduced when plants were simultaneously inoculated with both viruses, CCMV synthesis was retarded, and the final CCMV nucleoprotein concentration was reduced if CCMV inoculation occurred during the period of rapid SBMV synthesis. There was no interference with the synthesis of either virus if plants were inoculated after synthesis of the first virus declined to a low level.

Since the rapid synthesis phase of each virus was antagonistic to the other, CCMV and SBMV may be competing for some cellular factor, such as ribosomes or enzymes. The inhibition of synthesis, however, appears to be more complicated. As the established virus synthesis rate declined, the inhibited synthesis of the challenging virus did not increase accordingly. When the synthesis of the challenging virus was initially inhibited by the rapid synthesis of the established virus, the synthesis of the challenging virus remained inhibited even after the synthesis rate of the established virus had declined to a low level.

It seems unlikely that the viral effects on the plant are directly related to the competitive diversion of metabolites from normal host use to the production of virus particles. The total viral nucleoprotein produced in doubly infected plants was 20 to 25% less than SBMV nucleoprotein in a single infection, but the effect on plant growth was greater. Rather than competition, it appears that the viral infections alter metabolic events which, in turn, have
TABLE 4. Amount of cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus (cowpea strain) (SBMV) nucleoprotein in different-aged trifoliate ‘Early Ramshorn’ cowpea leaves in single and double infectionsa

<table>
<thead>
<tr>
<th>Trifoliolate leaves</th>
<th>Percent total nucleoproteinb</th>
<th>Amount of CCMV (mg/g)c</th>
<th>Amount of SBMV (mg/g)c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCMV</td>
<td>SBMV</td>
<td>Single</td>
</tr>
<tr>
<td>1st &amp; 2nd</td>
<td>30</td>
<td>70</td>
<td>.83</td>
</tr>
<tr>
<td>4th &amp; 5th</td>
<td>23</td>
<td>77</td>
<td>.34</td>
</tr>
<tr>
<td>7th &amp; 8th</td>
<td>24</td>
<td>76</td>
<td>.26</td>
</tr>
</tbody>
</table>

a Primary leaves were inoculated 10 days after seeding; purification and analysis of nucleoprotein concentrations were made 40 days after inoculation. Average of three tests.
b Total nucleoprotein in double infection.
c Mg of virus/g fresh weight of tissue.

detrimental effects on the plant. The necrosis associated with CCMV-SBMV infections and other mixed infections (13, 16, 17, 19) supports this conclusion.

The radioisotope data point out distinct differences between synthesis of CCMV and SBMV. The lag period before production of detectable amounts of virus nucleoprotein was longer for SBMV than for CCMV. After the lag period, the rate of synthesis increased faster for CCMV than SBMV. Following the peak synthesis rate, CCMV synthesis declined rapidly while SBMV synthesis declined more slowly. This may be related to the unusual observation that the ratio of $^{32}$P incorporated into virus particles to virus nucleoprotein accumulated was much greater for CCMV than for SBMV. Some possible explanations are as follows: (i) the assembly rate of SBMV was slower than that of CCMV and a smaller portion of the $^{32}$P-labeled SBMV-RNA was encapsidated during the 24-hr labeling period; (ii) SBMV turned over rapidly and much of the labeled SBMV broke down during the labeling period; or (iii) the two viruses utilize $^{32}$P from different cellular pools.

LITERATURE CITED