Nature of Resistance in Soybean to Cowpea Chlorotic Mottle Virus

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ABSTRACT

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Soybean lines screened to determine their reaction to cowpea chlorotic mottle virus were classified into six disease categories. Lines in three of these categories reacted with local chlorosis and systemic mosaic; they differed on the basis of virus accumulation, symptom severity, and symptom incubation period. Cultivar Davis was susceptible, Jackson was moderately resistant, and plant introduction (PI) 346304 was resistant. Restricted virus replication was responsible for resistance in the latter two soybean lines. Lines in two resistant categories reacted locally with necrotic lesions. Lesions on cultivar Bragg were 3.3 times as large as those on Williams. Small quantities of virus were produced in uninoculated leaves of both hosts. A sixth category was characterized by a complex reaction which included both chlorosis and necrosis in PI 96983; virus accumulation was high in inoculated leaves and very low in uninoculated ones which sometimes recovered from symptoms. Inoculum concentration and time of year strongly influenced virus accumulation; however, neither factor altered the relative rankings of the lines in different disease categories.

Cowpea chlorotic mottle virus (CCMV) is one of seven soybean, *Glycine max* (L.) Merr., viruses that have some economic importance (11). The virus occurs in the southern United States and in Central America in several legumes (3). The soybean strain, CCMV-S, was first isolated and described by Kuhn in 1968 (7). CCMV-S is serologically identical to the type strain (isolated from cowpea) but differs in symptom production in cowpea and levels of virus production (7). The virus can cause significant losses (20–30%) in soybean yield (5). The chemical composition of seeds from infected plants is altered (4), but these changes in chemical composition do not affect their commercial value. Soybean lines were reported to react to CCMV-S in three ways: systemic mosaic, necrotic local lesions, and local and systemic vein necrosis (5). Boerma et al (2) reported that the necrotic local lesion type of resistance to CCMV-S in soybean was controlled by a dominant gene.

The purpose of this investigation was to identify new sources and types of resistance in soybean to CCMV-S. Resistance was categorized on the basis of virus replication and movement, symptomatology, and symptom incubation period. Biological and physicochemical properties of virus isolated from susceptible and resistant lines were compared.

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MATERIALS AND METHODS

Plant growth manipulation. Soybean plants were grown in a greenhouse in 10-cm-diameter plastic pots in a mixture of soil:sand:vermiculite (2:1:1, v/v) which was fumigated with methyl bromide. Greenhouse temperatures were maintained between 20–28 C during November through March. The rest of the year, the temperature ranged from 18–20 C (nighttime) to 24–36 C (daytime). A complete fertilizer (10-10-10, N-P-K) was applied to the soil at weekly intervals.

Virus culture and inoculation. The CCMV isolate used in most experiments was strain S, which was maintained in Davis soybean or California Blackeye cowpea, *Vigna unguiculata* (L.) Walp. subsp. *unguiculata*. Inoculum was partially purified virus (170 μg/ml) or infected tissue ground in a mortar with 0.01 M potassium phosphate buffer (pH 7.0) containing 1% Celite. Plants were inoculated mechanically when the first trifoliolate leaf started to emerge, or approximately 9–13 days after seeding.

Screening tests. Disease reactions were studied with 85 commercial cultivars and 448 lines of plant introductions (PI). The soybean seeds, provided by H. R. Boerma (Department of Agronomy, University of Georgia, Athens), were in maturity groups V-VIII, selected because of their adaptation to the geographical latitudes of Georgia. One pot per line with three to five plants per pot was inoculated with virus and a control pot, placed beside the test pot, was similarly rubbed with buffer and Celite. Observations on disease reactions were made daily for 21 days after inoculation.

Size of local lesions. Bragg and Williams soybean plants, grown in the same pot (two plants of each), were inoculated with 8.6 μg/ml

of CCMV-S 10 days after seeding. Lesion size was measured 6 days after inoculation (six plants of each cultivar, 25 lesions per plant) with a dial caliper.

Virus purification. Infected tissue (1–3 g) was homogenized with a Tekmar tissumizer (model TR-10) for 1 min in a 40-ml tube with 10 ml of 0.2 M acetate buffer (pH 4.5) containing 0.1 M cysteine-hydrochloride, 0.01 M sodium diethyldithiocarbamate, 0.01 M magnesium chloride, and 5 ml each of chloroform and butanol. The homogenate was clarified by centrifugation at 4 C at 10,000 *g* for 10 min, and the aqueous phase was frozen overnight. After thawing, the low-speed centrifugation was repeated and the supernatant was centrifuged for 2 hr at 150,000 *g*. The partially purified virus was resuspended in 0.02 M acetate buffer, pH 5.0. Larger quantities of infected tissue and additional cycles of ultracentrifugation were used to purify virus for antiserum production and studies of virus properties.

Virus quantification. Initial studies to determine virus concentration in different plant lines showed a range of virus from less than 1 μg/g of tissue to more than 1,000 μg/g. Therefore, it was necessary to develop quantitative procedures to measure virus as accurately as possible for the range of virus concentrations anticipated. Virus samples with more than 500 μg (about 170 μg/g of tissue) could be highly purified by two cycles of ultracentrifugation and analyzed spectrophotometrically [$E = 5.8$ $(mg/ml)^{-1}$ cm^{-1} at 260 nm] (1). However, the procedure was unreliable for smaller samples because considerable virus (25–75%) was frequently lost between cycles one and two. Next, highly purified virus samples with 1, 10, 50, 100, 300, and 500 μg/ml were centrifuged on 10–40% sucrose columns for 2 hr at 95,000 *g*. Gradients were analyzed with ultraviolet optics (254 nm), and the area under the virus peaks was correlated with virus quantity.

In the interest of efficiency of time and cost, all virus samples were clarified as described above and concentrated by one cycle of ultracentrifugation. Samples with more than 170 μg/g of tissue were adjusted by subtracting 30 μg/g, an average estimate of ultraviolet-absorbing, normal host constituents which was determined by subjecting healthy soybean tissue to the same procedure. (A similar quantity of ultraviolet-absorbing material was found by density gradient analysis.) Samples with less than 170 μg/g were subjected to density gradient centrifugation and the area of virus peaks was measured with a planimeter.

Virus accumulation studies. Twenty pots of each line (three plants per pot) were randomized on the same greenhouse bench. Inoculated primary leaves were harvested 10 days after inoculation, and uninoculated trifoliolate leaves were harvested at 15 days or later. Approximately 20 leaves or leaflets were harvested at random and combined for one sample with four replications per treatment being analyzed by Duncan's multiple range test.

Serology. Antiserum was prepared by injecting rabbits intravenously (1 mg) and intramuscularly (5 mg, emulsified in Freund's incomplete adjuvant) with CCMV-S at weekly intervals for 4 wk. Immunodiffusion tests were run in 0.8% purified Bacto agar containing 0.2 M acetate buffer (pH 5.0), 0.85% NaCl, and 0.01 M NaN_3. Antiserum (homologous titer = 1/1,024) diluted 1:8 was placed in the center well, and 500 μg/ml of partially purified virus preparations were placed in the outer wells.

Virus properties. RNA was isolated from purified CCMV-S preparations (two cycles of ultracentrifugation) by the pronase-phenol method described previously (14). Gel electrophoresis was performed according to Loening's method (10). The RNA (40–50 μg) was layered on top of 2.6% polyacrylamide gels containing 0.2% sodium dodecyl sulfate. The gels were run at 6 mA per tube for 3–4 hr, then fixed in a mixture of absolute ethanol, glacial acetic acid, and water (40:10:50, v/v) overnight and scanned with ultraviolet optics. Electrophoretic profiles were integrated with a planimeter to determine quantities of RNA molecules. In order to separate virions with different RNAs, isopycnic banding of CCMV-S was conducted by mixing RbCl (1.360 g/ml) and virions and centrifuging at 25 C for 20 hr at 130,000 *g*. Band analysis was performed with ultraviolet optics.

RESULTS

Classification of disease reactions. Three basic criteria were used to evaluate the 533 soybean lines: local symptoms (reaction on inoculated unifoliate leaves), systemic symptoms (reaction on uninoculated trifoliolate leaves), and symptom incubation period (time from inoculation to initial appearance of symptoms). Two distinct reaction categories were evident 2–3 days after inoculation; more than two-thirds of the lines reacted with local necrosis (Table 1). Overall, six disease categories (Table 1) were identified, and each one occurred in all four soybean maturity groups (V, VI, VII, and VIII) which were studied.

Seventy-seven percent of the lines which caused necrosis had small lesions (category N/Ls) that had attained their full size by 2–4 days. Lesions continued to expand on 16% of the lines (category N/Ll) for 7–10 days, sometimes coalescing and causing most of the leaf to be necrotic. With the latter group, a small amount of systemic vein necrosis sometimes occurred on the first two or three trifoliolate leaves. A smaller group (7% of the lines) (category M/N) reacted locally with both necrosis and chlorosis by 3–5 days. Lesions were diffuse and all plants had a severe systemic reaction (vein necrosis, epinasty, and stunt) by 5–7 days, particularly on the first two trifoliolate leaves. Frequently, later-developing leaves were free of necrosis and sometimes symptomless.

The remainder of the lines caused no necrosis. Those in category M/S (44%) developed local chlorosis and systemic mosaic within 5–7 days. Symptoms were pronounced (rugosity, distortion, and stunting) on all trifoliolate leaves. Those in category M/M (52% of the lines) developed local chlorosis in 5–7 days, but systemic mosaic did not occur until the third or fourth trifoliolate leaves developed (14–21 days after inoculation). Mosaic symptoms on new leaves were relatively mild. A very mild reaction occurred on lines (4%) in category M/VM where local chlorosis developed within 5–7 days,

TABLE 1. Classification of 533 soybean lines on the basis of disease reactions to cowpea chlorotic mottle virus

Disease categories			Symptoms[a]		No. of lines	
Major	Subdivisions	Label	Local	Systemic	Cultivars	Plant introductions
Mosaic	Severe	M/S	C	M,R,D,S	10	62
	Moderate	M/M	C	M,R	18	66
	Very mild	M/VM	C	SCP	0	6
Mosaic/Necrosis		M/N	C,DN,[b] VN	M,E,VN,S	6	18
Necrosis	Lesions large, expanding	N/Ll	NL(le)	VN[c]	32	29
	Lesions small, delimited	N/Ls	NL(sd)	None	19	267

[a] C = chlorosis, D = distortion, DN = diffuse necrosis, E = epinasty, le = large expanding, M = mosaic, NL = necrotic lesions, R = rugosity, S = stunt, SCP = scattered chlorotic patches, sd = small delimited, and VN = vein necrosis.
[b] Sometimes the outline of lesions were observed.
[c] Vein necrosis occurred only on a portion (10–50%) of the plants.

and systemic symptoms were small scattered chlorotic patches, sometimes along veins, which occurred first on fourth, fifth, or sixth trifoliolate leaves (14–28 days after inoculation). Many leaflets were free of symptoms.

For critical studies of symptomatology, virus movement, and virus accumulation, representative soybean lines were selected for each reaction category: M/S-Davis; M/M-Jackson; M/VM-PI 346304; M/N-PI 96983; N/Ll, Bragg; and N/Ls, Williams.

Virus in inoculated leaves. Time course assays were conducted to determine virus accumulation in inoculated leaves of the representative lines (except PI 346304) from 3–15 days after inoculation. All lines reached a maximum concentration by 9 days in an August assay (Fig. 1). Thereafter, virus concentration usually declined slowly as has been reported previously for CCMV in cowpea (8).

Virus accumulation at 10 days after inoculation was compared in the six representative lines (Table 2). Although the quantity of virus produced varied from test to test, the relative concentration among the lines was similar. The highest concentration was produced in Davis, although PI 96983 had a similar quantity in one of four tests. PI 346304 had less virus than Davis but more than Jackson ($P = 0.05$). Virus accumulation in inoculated leaves of Jackson, Bragg, and Williams was similar despite the striking difference in disease reaction. Although lesions on Bragg (137 mm in diameter) were larger ($P = 0.05$) than those on Williams (42 mm in diameter), virus concentration was variable, particularly for Bragg. The large area of necrotic tissue on Bragg leaves probably caused degradation and loss of virus particles.

Virus in uninoculated leaves. Virus concentration in uninoculated trifoliolate leaves of Davis was many times greater than in the other five soybean lines (Table 3). When results of several tests were averaged (Tables 2 and 3), Davis had 20% less virus produced in the uninoculated leaves (624 μg/g) than in the inoculated ones (797 μg/g). The reduction was much greater for the other lines. Jackson had an average of 46% less in the uninoculated leaves, and PI 346304 and PI 96983 had strikingly lower virus levels of 89 and 87%, respectively. Bragg and Williams, both with distinct necrotic local lesions, had very small quantities of virus which was clearly detectable in uninoculated leaves. In order to detect the virus, it was extracted, clarified, and concentrated from 100–200 g of plant tissue before centrifugation on sucrose gradients. Bragg plants were divided into two groups: systemically symptomless plants had about 30 ng/g of tissue, and plants with slight vein necrosis had 75 ng/g. Williams plants were systemically symptomless, and they had less than 10 ng/g.

For each soybean line, virus concentration was similar in the first three trifoliolate leaves (Table 3). Among all cultivars, there was a relative correlation between virus concentration and systemic symptomatology. Davis had the most severe symptoms and the most virus. PI 96983, Jackson, and PI 346304 followed in order for both factors. However, there was little correlation for different aged leaves in Davis and Jackson. In Davis virus concentration was similar in the first three true leaves (Table 3), even though the symptoms became progressively more severe with younger leaves. No symptoms occurred on the first two or three leaves of Jackson. Thereafter, mosaic symptoms developed, but these newer leaves had less virus (48 μg/g of tissue) than the older symptomless leaves (84 μg/g) (quantities were determined in a test independent of those reported in Table 3). Symptom severity on PI 96983 varied from experiment to experiment and was poorly related to virus concentration.

Factors affecting disease reactions and virus accumulation. Although these studies were conducted in the greenhouse where temperature was controlled to some extent, the yearly seasonal environment had a major influence on disease reactions and virus accumulation. Symptoms on PI 96983 were intensified greatly during the cooler months (November to April). Necrosis of veins, petioles, and stems was prominent on both inoculated and uninoculated leaves, and plants sometimes died 10–14 days after inoculation. From May to October, less necrosis was observed, and plants survived and produced many trifoliolate leaves with mild

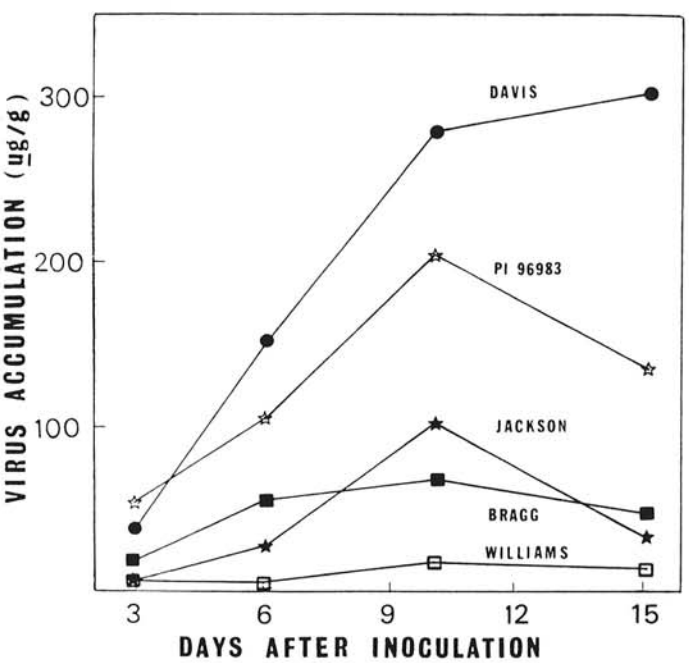

Fig. 1. Accumulation of cowpea chlorotic mottle virus in inoculated leaves of soybean lines Davis, Jackson, PI 96983, Bragg, and Williams. This experiment was conducted during a warm-weather period (July); similar accumulation patterns were observed for an experiment performed in a cool-weather month (December), although virus concentration was 2–3 times higher. Seeds of PI 346304 were not available when the test was conducted.

TABLE 2. Accumulation of cowpea chlorotic mottle virus in inoculated leaves of six soybean lines

Disease category[b]	Soybean lines	Virus accumulation (μg/g)[a] Test number 1	2	3	4	Mean (%)
M/S	Davis	616	266	1354	952	797 (100)
M/M	Jackson	98	16	162	57	83 (10)
M/VM	PI 346304	160	96	542	295	273 (34)
M/N	PI 96983	290	303	799	105	374 (47)
N/Ll	Bragg	25	8	190	67	73 (9)
N/Ls	Williams	31	13	68	57	42 (5)

[a] Infected leaf tissue harvested 10 days after inoculation (μg of virus per gram fresh weight).
[b] Note Table 1 for meaning of symbols.

TABLE 3. Accumulation of cowpea chlorotic mottle virus in uninoculated trifoliolate leaves of six soybean lines

Disease category	Soybean line	Virus accumulation (μg/g) Leaves combined[y] Test 1	Test 2	Leaves[z] First	Second	Third
M/S	Davis	411	213	892 a	763 a	841 a
M/M	Jackson	77	9	21 c	33 c	73 bc
M/VM	PI 346304	11	19	37 c	46 c	32 c
M/N	PI 96983	47	13	78 b	104 b	67 bc
N/Ll	Bragg	<1	<1	<1 d	<1 d	<1 d
N/Ls	Williams	<1	<1	<1 d	<1 d	<1 d

[y] The first, second, and third trifoliolate leaves were combined and homogenized to form a single sample. Each test had two replications per treatment.
[z] Leaves were manipulated separately in this test (the first leaf is oldest and the third leaf is youngest). Treatment means followed by different letters are significantly different according to Duncan's multiple range test, $P = 0.05$; the experiment was run with four replications per treatment.

mosaic symptoms. Lesions on Bragg and Williams were larger in cool months than warm months. Symptoms on Davis, Jackson, and PI 346304 were similar throughout the year.

Time of year also affected the accumulation of virus in soybean. CCMV-S accumulation was 1.5- to 3.5-fold greater in the cooler than in the warmer months (Table 4). Similar relative increases of virus accumulation were noted in all soybean lines tested, regardless of disease category.

Accumulation of CCMV-S also was influenced by inoculum concentration. Quantity of CCMV-S was increased 1.3- to 2.5-fold at day 3 when plants were inoculated with the higher inoculum concentration (Table 5). At day 10 the difference between the two inoculum concentrations was even greater (1.2- to 7.7-fold). PI 96983 was affected most, whereas Williams was only slightly influenced by inoculum concentrations.

Virus properties. Several properties of CCMV-S were compared after virus was purified (10 days after inoculation) from Davis, Jackson, PI 346304, PI 96983, and Bragg. In an immunodiffusion test, a single precipitin line formed when virions from the different soybean lines were compared against CCMV-S antiserum; the lines coalesced and no spurs could be observed. Sedimentation in sucrose and isopycnic centrifugation in RbCl revealed no difference in the virions from the five lines. When the RNAs were isolated from virions from the different hosts, polyacrylamide gel electrophoresis showed a similarity in the number and ratio of RNA molecules. Some RNA breakdown products were observed between RNAs 3 and 4 when virions were isolated from the hosts which reacted with necrosis, Bragg and PI 96983.

Inoculation with strain R. CCMV strain R is known to overcome a type of resistance in cowpea in which the type strain of CCMV remains localized in inoculated leaves and causes no symptoms (13). When five of the representative soybean lines were inoculated with CCMV-R, both local and systemic symptoms and relative virus accumulation in uninoculated leaves were similar to the reactions caused by strain S which was used in this study, except no symptoms occurred on PI 346304 and virus concentration was very low. Therefore, strain R was unable to overcome any type of resistance to CCMV in soybean.

TABLE 4. Influence of time of year on accumulation of cowpea chlorotic mottle virus in inoculated leaves of soybean lines

Disease category	Soybean line	Virus accumulation (μg/g)[a]	
		February	July–August
M/S	Davis	613	309
M/M	Jackson	190	54
M/N	PI 96983	381	220
N/Ll	Bragg	76	50
N/Ls	Williams	53	21

[a] Average of two experiments, each with two replications per treatment.

TABLE 5. The influence of inoculum concentration on accumulation of cowpea chlorotic mottle virus in inoculated leaves of six soybean lines

Disease category	Soybean line	Virus accumulation (μg/g) with inoculum concentrations at:[y]			
		8.6 μg/ml		172 μg/ml	
		3[z]	10	3	10
M/S	Davis	66	952	168	1354
M/M	Jackson	37	54	76	162
M/VM	PI 346304	80	294	103	540
M/N	PI 96983	70	105	171	805
N/Ll	Bragg	34	67	73	190
N/Ls	Williams	52	56	75	65

[y] Data (μg/g) were converted to logarithms and analyzed statistically. Virus accumulation was significantly different ($P = 0.05$) at the two inoculum concentrations, both at the 3- and 10-day harvest times.

[z] Days after inoculation.

DISCUSSION

A survey of 533 soybean lines demonstrated a wide range of disease reactions caused by CCMV-S. The reactions were divided into two broad categories, mosaic and necrosis, and into six specific disease categories (virus-host interactions). No symptomless lines were found. Three distinct interactions occurred within the mosaic category. Cultivar Davis had high virus concentration and severe symptoms and represents a susceptible reaction. Intermediate resistance in Jackson was evidenced by a relatively low virus concentration, a delayed systemic reaction, milder mosaic, and less stunting than in Davis. Although virus concentration in inoculated leaves of PI 346304 was significantly higher than in Jackson, it was similarly low in both lines in uninoculated trifoliolate leaves. Systemic symptoms, however, were milder and delayed longer in PI 346304 than in Jackson, and we consider the plant introduction to be the most resistant of the two lines. The type of resistance in PI 346304 would not have been detected if the commercial cultivars only had been screened.

The resistance in Jackson soybean, as evidenced by reduced virus concentration, appears to be similar to CCMV resistance in some cowpea cultivars (eg, Iron and California Blackeye) (8). In resistant and susceptible lines of both cowpea and soybean, time course assays show a similar time when virus accumulation ceases. (The specific time, 5–10 days after inoculation, can vary with the experiment.) Therefore, the host control mechanism may be regulating the rate of virus replication rather than interrupting the replication cycle or inhibiting replication at an early step.

Two types of necrosis reactions occurred on Bragg and Williams. The latter appeared to be more resistant because less virus was usually produced in its inoculated leaves and the necrotic local lesions did not enlarge beyond 2–4 days after inoculation, thus resulting in less necrotic tissue. Two hypotheses have been suggested to explain localization of virus in necrotic lesions. Kimmin and Wuddah (6) suggest that movement of virus is prevented by the death of cells due to necrosis. Loebenstein (9) suggests that necrogenesis is a separate process which might retard virus multiplication but is not the major factor in localization. Necrosis did not prevent movement of CCMV into uninoculated leaves of necrosis-type soybean hosts Bragg and Williams. However, if virus replication occurred in the uninoculated leaves, it was highly restricted.

In PI 96983, a sixth virus-host interaction was observed which had virus accumulation and symptom characteristics related to both the mosaic and necrosis categories. This complex reaction may be desirable for the study of interactions of virus replication, virus movement, and necrotization.

Virus accumulation was somewhat similar in both the inoculated and uninoculated leaves of the susceptible cultivar Davis. However, in two resistant lines, PI 346304 and PI 96983, there was 6–9 times less virus in the uninoculated leaves. It is not clear from this study if the lower quantity in the uninoculated leaves is due to reduced virus replication or to restriction of either systemic or local movement. Resistance to movement of CCMV (type strain) in cowpea is controlled by a single gene (8,12). A new strain (R) of CCMV, derived in resistant cowpea, could move systemically despite the movement gene (8). Strain R, however, could not overcome the resistance in PI 346304.

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