Effects of Antiviral Compounds on Symptoms and Infectivity of Cowpea Chlorotic Mottle Virus

L. J. C. MANCINO, Graduate Student, and G. N. AGRIOS, Professor, Department of Plant Pathology, University of Massachusetts, Amherst 01003

ABSTRACT

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Symptoms of cowpea plants infected with cowpea chlorotic mottle virus (CCMV) were suppressed when plants were sprayed with ribavirin, amantadine, formycin, and methisazone but not when sprayed with MBC. 2-Thiouracil sprays increased or decreased symptoms, depending on time of application. 2-Thiouracil, formycin, and methisazone also caused phytotoxicity on sprayed plants. Ribavirin reduced and 2-thiouracil increased the infectivity of CCMV in some treatments, whereas the other compounds had little or no effect. Some ribavirin and formycin treatments of soybean plants reduced CCMV local lesion numbers significantly. All other applications of the antiviral compounds caused some but statistically nonsignificant reductions in local lesion formation. Ribavirin and amantadine did not significantly alter detectable viral antigen concentrations in sap from treated, infected cowpea plants as determined by ELISA.

Plant virus chemotherapeutants are needed as tools for plant virus replication studies, for tissue culture therapy, and as alternatives to the preventative means of plant virus control currently in use. 2-Thiouracil (1,3,11,16), ribavirin (4,7,13,14), amantadine (9,15), formycin (8,19), methisazone (18,20), and MBC (6,17) are antiviral compounds that may have potential for use in virus replication studies, and possibly, for control of virus infections in plant crops.

Antiviral compounds inhibit virus infectivity by affecting different stages of the viral infection process. Therefore, plants expressing a systemic infection (cowpea) or the hypersensitive reaction (soybean) were treated with the different compounds at various times before, during, and after inoculation with cowpea chlorotic mottle virus (CCMV) so that any inhibitory effects of the compounds on symptom expression would be more likely to be observed. In addition, purified CCMV possesses good antigenic properties, thereby making it possible to determine the effects of certain antiviral compounds on the presence or relative amounts of CCMV antigen by immunological techniques.

This study was undertaken to determine the effects of 2-thiouracil, ribavirin, amantadine, formycin, methisazone, and MBC on symptom development, virus accumulation, and infectivity of CCMV in hosts known to display systemic symptoms or the hypersensitive response. We sought to gain information on the possibilities of using such compounds as tools in further studies of plant virus replication and as a potential means of controlling plant virus infections.

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MATERIALS AND METHODS

Application of compounds. Each compound was applied to the primary leaves of 11- to 12-day-old cowpea plants (*Vigna unguiculata* (L.) Walp. 'California Blackeye') and soybean plants (*Glycine max* (L.) 'Harosoy') at the highest concentrations determined from preliminary experiments to be nontoxic. In similar preliminary experiments, each compound had been found to have no direct effect on the infectivity of the CCMV inoculum or on the infection process, even at twice the highest nontoxic concentration. 2-Thiouracil and ribavirin (Virazole) were sprayed at 500 mg/L distilled, deionized water. Amantadine was sprayed at 250 mg/L; methisazone (Marboran) was dissolved in 1 N NaOH, which was further diluted 1:10 (v/v) with distilled, deionized water to give a final methisazone concentration of 250 mg/L. Bavistin (BASF., Aktiengesellschaft, West Germany), a commercial preparation containing 50% (w/w) MBC in nonactive filler, was used at 1,000 mg/L.

Plants were sprayed with the antiviral compounds, using DeVilbiss noncorrosive 251 atomizers that delivered a fine mist until droplets began to coalesce to ensure even coverage of the leaves. Plants were watered by direct application to the soil to avoid washing the compounds off the leaves.

Six spray regimes (treatments A–F) were followed for application of the antiviral compounds, which were sprayed as follows: treatment A—2 days and 1 day before inoculation and immediately after inoculation; treatment B—2 days and 1 day before inoculation, immediately after inoculation, and 1 day after inoculation; treatment C—immediately after inoculation; treatment D—immediately after inoculation and 1 day after inoculation; treatment E—immediately after inoculation and 1, 2, 3, and 4 days after inoculation; and treatment F—1, 3, 6, and 8 days after inoculation.

In each experiment, one flat (20 × 12.5 × 9 cm) of plants was used for each treatment. The eight most uniform plants in each flat were selected for treatment. Four replicates of each treatment were performed. Positive controls consisted of plants inoculated with the virus without being sprayed. The negative controls consisted of plants sprayed without being inoculated. Flats receiving the various compounds were randomized within each treatment.

Virus culture and purification. CCMV was maintained in cowpea plants. Systemically infected symptomatic cowpea trifoliolate leaves were ground in a mortar and pestle at 1:10 (w/v) with cold 0.1 M, pH 5.0, sodium acetate buffer, containing 0.01 M sodium diethyl-dithiocarbamate and 0.01 M cysteine hydrochloride. Inoculum was applied to primary cowpea or soybean leaves dusted with 600-mesh Carborundum. Plants were always inoculated in late morning.

Purified CCMV was obtained by differential centrifugation according to a modified procedure of Gay and Kuhn (5), in which the virus was suspended in pH 5.0 sodium acetate buffer.

Virus infectivity and symptom expression assays. Effects of the antiviral compounds on local lesion formation were determined by inoculating sprayed soybean plants with CCMV-infected sap obtained from unsprayed, inoculated cowpea leaf tissue. To determine the effects of the antiviral compounds on the infectivity of CCMV in systemically infected cowpea leaves, soybean plants were inoculated with sap from primary or trifoliolate leaf tissue from cowpea plants receiving the various antiviral treatments. Eight uniform soybean primary half-leaves were inoculated and local lesions were counted after 48 hr. Because of the large number of treatments to be

compared and in order to minimize mistakes and runoff from adjacent treatments, only one half-leaf per plant was inoculated.

Effects of the antiviral compounds on symptom development in cowpea plants were determined by visual observations recorded 10 and 42 days after inoculation. A reduction in symptom development was reported if 50% or more of the plants receiving a given treatment showed decidedly less chlorotic mottling than the inoculated control plants. Effects of the antiviral compounds on the infectivity of CCMV in treated cowpea leaves were determined by infectivity assays performed 10 days after inoculation, using the primary leaves, and 10, 16, and 22 days after inoculation, using the trifoliolate leaves as the inoculum source.

Determination of virus antigen by the enzyme-linked immunosorbent assay (ELISA). A CCMV antiserum with a titer of 1/1,600 was obtained from rabbits immunized with purified CCMV and its γ-globulin was purified. Direct ELISA was performed according to the procedures of Clark and Adams (2). Leaf tissue was homogenized 1:10 (w/v) in 0.02 M, pH 7.4, phosphate buffer containing 0.15 M NaCl, 0.05% (v/v) Tween 20, 2% (w/v) polyvinylpyrrolidone (mol wt 10,000), and 0.2% (w/v) ovalbumin. Alkaline phosphatase-labeled γ-globulin was used at about 1 μg/ml and *p*-nitrophenyl phosphate was used as the enzyme substrate. The optical density of the enzyme-substrate reaction in each well, which at lower virus levels is generally proportional to the viral antigen concentration in the plant sap, was measured spectrophotometrically at 405 nm with a Gilford EIA manual reader. Separate ELISA tests were performed on each of the four replicates of treatment E.

RESULTS

Effects of antiviral compounds on CCMV symptoms on cowpea plants. Several antiviral treatments had marked effects on symptom severity 10 days after inoculation and on plant growth (Table 1). All ribavirin and amantadine treatments almost completely suppressed development of chlorotic mottle, allowing only occasional chlorotic specks. 2-Thiouracil, treatments B–D; methisazone, treatments A–F; and formycin, treatments A–E, reduced the frequency and severity of chlorotic mottling of CCMV-infected cowpea plants observed 10 days after inoculation. MBC had no effect on symptom development. Plants treated with 2-thiouracil, treatments A, E, and F, displayed chlorotic mottling frequency and severity equal to or greater than that of the untreated controls.

Ribavirin, amantadine, and MBC caused no phytotoxicity but the other antiviral compounds caused various degrees of phytotoxicity. 2-Thiouracil caused stunting of plant growth, bleaching of primary and trifoliolate leaves, and puckering of trifoliolate leaf margins. Formycin caused leaf margin necrosis, leaf puckering and curling, and necrotic "shothole" lesion formation on trifoliolate leaves. Methisazone caused striation and narrowing of trifoliolate leaves.

When cowpea plants were observed 42 days after inoculation, symptom frequency and severity had increased somewhat in several treatments that had displayed milder symptoms 10 days after inoculation (Table 1). Such treatments included plants sprayed with 2-thiouracil, treatments B–D; ribavirin, treatments A–D; amantadine, treatments E and F; and formycin, treatment E. Symptoms of phytotoxicity were no longer present on the younger trifoliolate leaves.

Effects of antiviral compounds on local lesions. Five antiviral compounds, ribavirin, amantadine, formycin, methisazone, and MBC, when applied to CCMV-inoculated soybean plants as spray treatment regimes A–D, caused an apparent reduction in numbers of local lesions formed, but lesion numbers were not significantly lower than those on unsprayed, inoculated control plants (Table 2).

The size of individual local lesions on soybean plants was not affected by the antiviral treatments. Local lesions on some of the 2-thiouracil-treated plants coalesced and resembled large, necrotic areas, thereby making it difficult to count individual lesions.

Effects of antiviral compounds on infectivity of CCMV produced in systemically infected cowpea plants. Only 2-thiouracil significantly affected CCMV infectivity in primary leaves of systemically infected cowpea plants within 10 days of inoculation. 2-Thiouracil, treatments E and F, increased virus infectivity in primary leaves (Table 3).

Ribavirin, 2-thiouracil, amantadine, and methisazone were tested further in experiments involving only spray treatments A, C, and E. After treatment and inoculation of the primary leaves, the infectivity of CCMV in trifoliolate leaves from each treatment was tested at 10, 16, and 22 days after inoculation. The infectivity of CCMV in trifoliolate leaves treated with treatment E of ribavirin, amantadine, and methisazone was significantly less at 10 days after inoculation (Table 4). None of the other treatments caused CCMV infectivity to differ significantly from that of unsprayed, inoculated control plants 10, 16, and 22 days after inoculation (Table 4).

Table 1. Effects of antiviral compounds sprayed in different treatment regimes on the frequency and severity of symptoms of cowpea chlorotic mottle virus infected cowpeas 10 and 42 days after inoculation

Antiviral compound	Days after inoculation	Sprayed treatment regime[a]						
		A	B	C	D	E	F	Untreated control
2-Thiouracil	10	+++*	++*	++*	++*	+++*	+++*	+++
	42	+++*	+++*	+++*	+++*	+++*	+++*	+++
Ribavirin	10	±	±	±	±	±	±	+++
	42	±	+	+	+	±	±	+++
Amantadine	10	±	±	±	±	+*	+*	+++
	42	±	±	±	±	++*	++*	+++
Formycin	10	+*	+*	+*	+*	+*	+++*	+++
	42	+*	+*	+*	+*	+++*	+++*	+++
Methisazone	10	+*	++*	++*	+*	++*	++*	+++
	42	+	++	++	+	++	++	+++
MBC	10	+++	+++	+++	+++	+++	+++	+++
	42	+++	+++	+++	+++	+++	+++	+++

[a] ± = A few leaves in < 10% of the plants showing occasional small chlorotic spots; + = a few leaves in < 25% of the plants showing several chlorotic spots or patches; ++ = several leaves in < 50% of the plants showing numerous chlorotic spots on patches; +++ = > 50% of the plants showing general chlorotic mottling; and * = phytotoxic effects were present.

Table 2. Effects of antiviral compound sprays on the number of cowpea chlorotic mottle virus local lesions on soybean plants

Antiviral compound	Spray treatment regime			
	A	B	C	D
2-Thiouracil	102 ± 86[a]	106 ± 79	116 ± 64	115 ± 63
Ribavirin	71 ± 35	46 ± 20*[b]	86 ± 47	83 ± 63
Amantadine	86 ± 66	93 ± 65	82 ± 48	71 ± 45
Formycin	111 ± 39	73 ± 11	67 ± 9	44 ± 6*
MBC	66 ± 11	97 ± 38	69 ± 12	87 ± 1
Methisazone	73 ± 20	88 ± 35	75 ± 20	86 ± 21
Control—no compound	117 ± 49	...	...	...

[a] Each number represents the mean number of local lesions of four replicates ± the standard deviation. Each replicate consisted of eight soybean half-leaves. Local lesion numbers were statistically analyzed by Duncan's new multiple range test ($P = 0.05$).

[b] * = Statistically significant ($P = 0.05$) difference between antiviral compound and control.

Table 3. Effects of antiviral compound sprays on infectivity of cowpea chlorotic mottle virus in cowpea plants assayed 10 days after inoculation

Antiviral compound	Spray treatment regime					
	A	B	C	D	E	F
2-Thiouracil	73 ± 14[a]	59 ± 37	59 ± 11	78 ± 26	139 ± 41*[b]	109 ± 34*[b]
Ribavirin	63 ± 37	68 ± 19	46 ± 26	58 ± 34	64 ± 18	61 ± 15
Amantadine	60 ± 20	67 ± 20	53 ± 19	58 ± 38	77 ± 2	82 ± 9
Formycin	53 ± 32	75 ± 6	79 ± 44	86 ± 55	94 ± 33	93 ± 27
Methisazone	57 ± 40	68 ± 34	76 ± 15	69 ± 32	69 ± 18	87 ± 29
MBC	79 ± 14	69 ± 25	66 ± 11	80 ± 24	78 ± 32	53 ± 37
Control—no compound	80 ± 12	...	...	...	...	...

[a] Each number represents the mean number of local lesions of four replicates ± the standard deviation. Each replicate consisted of eight soybean half-leaves. Local lesion numbers were analyzed by Duncan's new multiple range test ($P = 0.05$).
[b] * = Statistically significant ($P = 0.05$) difference between antiviral compound and control.

Effects of antiviral compounds on viral antigen in CCMV-infected cowpea plants. The relative amounts of CCMV antigen within systemically infected cowpea plants treated with ribavirin and amantadine, treatment E, were determined by ELISA, using primary leaves harvested 2, 4, and 8 days after inoculation and trifoliolate leaves harvested 15 days after inoculation. Control tissues were taken at similar times from healthy plants and unsprayed, inoculated plants. Detectable concentrations of CCMV antigen in leaf tissue from sprayed plants did not differ significantly from those in the unsprayed inoculated controls (Table 5); however, it should be kept in mind that at higher levels of virus, even samples with considerably different virus concentration give small, insignificant differences in A_{405} readings.

Table 4. Effects of antiviral compounds on infectivity of cowpea chlorotic mottle virus in cowpea plants 10, 16, and 22 days after inoculation

Antiviral compound	Spray treatment regime	Days after inoculation		
		10	16	22
2-Thiouracil	A	46 ± 26[a]	75 ± 37	4 ± 5
	C	61 ± 22	66 ± 27	3 ± 3
	E	77 ± 35	83 ± 40	0 ± 0
Ribavirin	A	69 ± 12	85 ± 27	3 ± 2
	C	63 ± 11	60 ± 24	6 ± 4
	E	43 ± 9*[b]	55 ± 19	1 ± 2
Amantadine	A	61 ± 6	67 ± 19	14 ± 16
	C	58 ± 37	58 ± 19	14 ± 7
	E	51 ± 6*	86 ± 23	9 ± 9
Methisazone	A	63 ± 10	82 ± 15	12 ± 9
	C	48 ± 34	51 ± 38	8 ± 3
	E	45 ± 15*	83 ± 16	1 ± 0
Control	No spray	82 ± 15	93 ± 25	5 ± 7

[a] Each number represents the mean number of local lesions of four replicates ± the standard deviation. Each replicate consisted of eight soybean half-leaves. Local lesion numbers were analyzed by Duncan's new multiple range test ($P = 0.05$).
[b] * = Statistically significant ($P = 0.05$) difference between antiviral compound and control.

Table 5. Effects of ribavirin and amantadine on the presence of viral antigen in cowpea chlorotic mottle virus-infected cowpea primary leaves detected by the enzyme-linked immunosorbent assay (ELISA) 2, 4, and 8 days after inoculation and in trifoliolate leaves 15 days after inoculation

Treatment	Days after inoculation			
	2	4	8	15
Healthy control[a]	0.00 ± 0.00[b]	0.00 ± 0.00	0.07 ± 0.01	0.05 ± 0.05
Ribavirin	0.32 ± 0.05	0.71 ± 0.38	1.05 ± 0.21	0.21 ± 0.08
Amantadine	0.39 ± 0.17	1.02 ± 0.21	1.11 ± 0.34	0.28 ± 0.09
Inoculated control	0.36 ± 0.12	0.86 ± 0.17	1.27 ± 0.14	0.16 ± 0.02

[a] Statistically significant ($P = 0.05$) differences were detected only between healthy control and other treatments at each time of ELISA.
[b] Each number represents the mean light absorption reading at 405 nm (A_{405}) of ELISA wells ± the standard deviation. The A_{405} readings were analyzed by Duncan's new multiple range test ($P = 0.05$).

DISCUSSION

Several antiviral compounds, particularly ribavirin and amantadine, were very effective in suppressing development of CCMV symptoms on inoculated cowpea plants (Table 1). However, statistical analysis showed that only a few treatments resulted in CCMV infectivity significantly different from that in inoculated control plants. This was probably due, at least in part, to the fact that local lesions were counted on separate plants rather than opposite half-leaves so the local lesion numbers obtained for each experimental treatment showed great variability, which in turn, resulted in large standard deviations among the treatment means. However, it was clear from the results that none of the antiviral compound treatments, even those with ribavirin and amantadine that almost completely inhibited symptom development, completely inhibited or greatly suppressed multiplication and infectivity of CCMV in treated cowpea plants. Transformation of local lesion numbers according to Kleczkowski (10) before statistical analysis revealed no additional treatments that resulted in CCMV infectivity significantly different from that of the controls.

With the exceptions of 2-thiouracil and ribavirin, application of antiviral compounds onto inoculated cowpea plants failed to significantly affect the infectivity of CCMV 10 days after inoculation (Tables 3 and 4). The increased infectivity of CCMV in plants sprayed with 2-thiouracil in treatments E and F (Table 3) paralleled an increase in development of systemic symptoms. These increases, along with the decrease in development of systemic symptoms on plants sprayed with 2-thiouracil in treatments B–D, indicate that 2-thiouracil may inhibit or enhance virus infectivity, depending on the time of application. Steele and Black (16) observed that poliovirus was inhibited by 2-thiouracil early in the infection process because of an inhibition of viral absorption. In contrast, Dawson and Kuhn (3) observed that when 2-thiouracil was applied daily after inoculation, infectivity of CCMV in cowpea plants increased.

The reduction in CCMV infectivity in inoculated cowpea plants sprayed with treatment E of ribavirin, amantadine, and methisazone (Table 4) coincided with variable suppression of symptom development on these plants (Table 1). Because the reduction in CCMV infectivity was only apparent in younger trifoliolate leaves, CCMV multiplication and/or infectivity in trifoliolate leaves may have been more sensitive to the inhibitory effects of these compounds. Increased inhibition of virus multiplication by ribavirin in upper leaves of plants as the distance from the lower inoculated leaves increased has been reported (13). On the other hand, Kuhn et al (12) showed that

symptomatology of CCMV, based on intensity of chlorosis, was unrelated to virus accumulation. The ELISA results indicate that in tissue of sprayed plants that did not display systemic symptoms, viral antigen concentrations were comparable to those in inoculated control plants (Table 5).

MBC had no effect either on CCMV infectivity in primary leaves of cowpea plants (Table 3) or on symptom development on CCMV-inoculated cowpea plants (Table 1).

None of the antiviral compounds significantly reduced CCMV infectivity or further suppressed symptom development after the 10th day and up to 42 days after inoculation in relation to the controls (Table 4). This indicates that virus stability was not affected by the antiviral compounds. The antiviral compound effects were not always permanent because plants that showed suppression of symptom development 10 days after inoculation showed increased symptom development 42 days after inoculation.

The toxicity of repeated applications of 2-thiouracil, formycin, and methisazone to the virus-infected host plant may render them unsuitable as plant-virus chemotherapeutants. The lack of toxicity of ribavirin and amantadine to the virus-infected host plants along with the ability of both antiviral compounds to suppress viral symptoms indicate that these (and probably other chemicals) may have potential for use as plant virus symptom suppressants, but their value as chemotherapeutants or for studying virus replication will have to be studied for each individual virus.

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