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Effects of certain antiviral compounds on symptoms and infectivity of cowpea chlorotic mottle virus in cowpea and soybean plants.

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EFFECTS OF CERTAIN ANTIVIRAL COMPOUNDS ON SYMPTOMS

AND INFECTIVITY OF COWPEA CHLOROTIC MOTTLE

VIRUS IN COWPEA AND SOYBEAN PLANTS

A Thesis Presented

By

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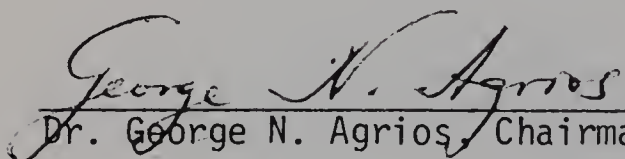EFFECTS OF CERTAIN ANTIVRIAL COMPOUNDS ON SYMPTOMS

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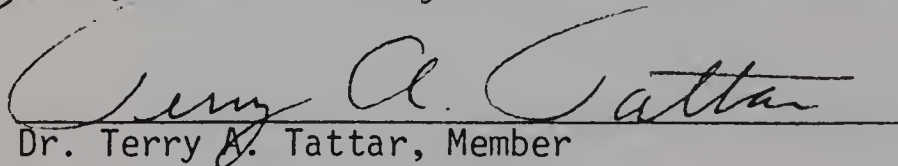
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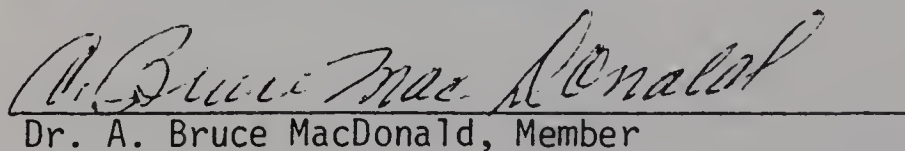
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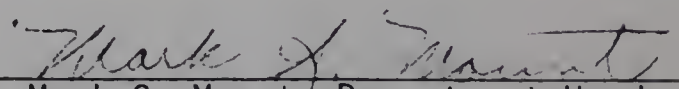
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CHAPTER I

INTRODUCTION

A large number of antiviral compounds are currently being studied for possible use against viral infections of humans and animals. Very little is known about the effects of antiviral compounds on viral infections of plants.

Plant virus chemotherapeutants are needed as an alternative to the preventative means of plant virus control currently used to avoid crop losses. Ideally, a viral chemotherapeutant would selectively inhibit the viral infection process without having toxic effects on the host cell metabolism. The compound would also have to move systemically throughout the plant while retaining its activity for a reasonable period of time to be effective.

Antiviral compounds inhibit virus infectivity by affecting different stages of the viral infection process. Therefore, plants were treated with the different compounds at various times before, during, and after inoculation, so that their inhibitory effects would be more likely to be expressed.

To best observe the effects of each antiviral compound on systemic and hypersensitive virus infection, it was necessary to work with a virus-host system in which systemic symptom development and the hypersensitive response could be easily observed. Such a system is provided by the cowpea chlorotic mottle virus (CCMV) which causes systemic infection in cowpea plants, _Vigna unguiculata_, variety "California

Blackeye," and hypersensitive response on soybean plants, _Glycine max_, variety "Harasoy." In addition, purified CCMV possesses good antigenic properties thereby making it possible to determine the effects of certain antivirval compounds on the presence of CCMV antigen by immunological techniques.

This study was undertaken to determine the effects of certain antiviral compounds on the presence and infectivity of CCMV in hosts known to exhibit systemic symptoms or the hypersensitive response, and thus to gain information on the possibilities for use of such compounds as a means of control against plant virus infections.

C H A P T E R   II

LITERATURE REVIEW

Crop losses due to plant virus diseases generally occur in three situations: 1) where perennial crops develop viral symptoms after much time and land have been invested; 2) where annual seed crops become infected due to cultural or environmental conditions which favor virus infection; and 3) where mild but widespread infections of vegetatively propagated plants occur leading to small losses every year (Matthews, 1970). Currently, only evasive control measures are available for the prevention of crop losses due to plant virus diseases. These measures include removal of virus-infected sources, control of vectors, use of virus-free seed and vegetative stocks, modifications of cultivation procedures, and ideally, use of resistant crop varieties (Agrios, 1978).

So far no antivrial compounds have been found which would directly protect or cure a crop from virus infection. However, certain antiviral compounds are known to inhibit virus infections in human, animal, and plant hosts (Maugh, 1976). Any one of these compounds may have potential for use in the prevention or cure of virus infections in plant crops.

2-Thiouracil is a pyrimidine nucleoside analog which has been shown to have several different effects on virus infectivity. According to Steele and Black (1967), 2-thiouracil inhibits the absorption of poliovirus onto rhesus kidney cells by inactivating functional capsid sulfhydryl groups. When 2-thiouracil (10^{-3} M) was added to poliovirus-

infected rhesus kidney monolayers, virus titre (PFU/ml) was less than 10% of the titre of the control cultures containing no 2-thiouracil.

Multiplication of tobacco mosaic virus (TMV), potato virus X (PVX), potato virus Y (PVY), and henbane mosaic virus was reduced in leaves of tobacco, Nicotiana tabacum, variety "White Burley," floated in 2-thiouracil (Bawden and Kassanis, 1954). ^{35}S-labelled 2-thiouracil was incorporated into both host and viral RNA of TMV infected tobacco leaves. The ^{35}S-labelled 2-thiouracil suppressed the incorporation of ^{32}P into viral RNA with little effect on ^{32}P incorporation into host RNA (Ralph, et al., 1965). Therefore, it was thought that 2-thiouracil may decrease TMV infectivity by incorporating itself into viral RNA, thereby interfering with virus replication (Kado and Agrawal, 1972).

Contrary to these findings, Dawson and Kuhn (1972) observed an enhancement of cowpea chlorotic mottle virus (CCMV) infectivity by 2-thiouracil. Sizes of local lesions on soybean plants, Glycine max, variety "Bragg," increased up to 13 times after primary leaves were inoculated and floated in a solution of 2-thiouracil (0.005 M). Sap infectivity and ^{32}P incorporation into viral RNA also increased when primary leaves of cowpea plants, Vigna unguiculata, variety "Early Ramshorn," were sprayed daily with 2-thiouracil.

Addition of uracil counteracts both the inhibitory effect of 2-thiouracil on TMV multiplication (Commoner and Mercer, 1951) and the enhancement effect of 2-thiouracil on CCMV multiplication (Kuhn, 1971). Plants sprayed with 2-thiouracil exhibit symptoms of toxicity such as leaf yellowing, curling, and growth inhibition (Matthews, 1970).

Addition of uracil had no effect upon alleviation of these toxic symptoms (Bawden and Kassanis, 1954). Due to the toxic nature of 2-thiouracil, its use in the control of virus diseases has been avoided.

Ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a nucleoside analog active against a variety of RNA and DNA viruses (DeClercq and Luczak, 1975; Maugh, 1976). The multiplication of several plant viruses is also known to be inhibited by ribavirin. Lerch (1977) reported that the multiplication of PVX in 4-week-old tobacco plants, Nicotiana tabacum, variety "Xanthi NC," was inhibited when roots were placed in a nutrient solution containing ribavirin (5 ug/ml). Inhibition was measured by performing infectivity assays in which sap from PVX-infected tobacco leaves was inoculated onto detached leaves of Gomphrena globosa. Inhibition ranged from 23 to 78%, increasing in upper tobacco leaves as the distance from the lowest inoculated leaves increased. Inoculated tobacco plants treated with ribavirin remained symptomless and grew more vigorously than the control plants.

A 40% reduction in the development of systemic symptoms was reported when tomato plants, Lycopersicon esculentum, were sprayed with ribavirin (500 mg/l) immediately after inoculation with tomato white necrosis virus (deFazio, et al., 1978). Virus replication, as measured by infectivity assays on Chenopodium amaranticolor, was reduced by 89% in inoculated, ribavirin-treated plants.

Multiplication of apple chlorotic leafspot virus in Chenopodium quinoa was completedly inhibited when ribavirin was applied between two days before and eight hours after inoculation (Hansen, 1979).

Infectivity was measured by inoculating sap from ribavirin-treated C. quinoa onto fresh C. quinoa. Applications of ribavirin (250 ppm) by leaf spray, soil drench, and brief sand culture were equally effective. Concentrations of ribavirin in excess of 500 ppm were phytotoxic, causing marginal waviness and twisting of young leaves.

Shepard (1977) found that when calli derived from PVX-infected protoplasts of tobacco, Nicotiana tabacum variety "Xanthi NC," were exposed to ribavirin (10 mg/l) in a medium that promoted the onset of shoot morphogenesis, the regenerated tobacco plants showed an almost complete absence of PVX infection. Exposure of calli to ribavirin concentrations of 100 mg/l resulted in morphological abnormalities including leaf narrowing and underdevelopment and a weakening of stem and horizontal growth.

The mechanisms of action of ribavirin was investigated by Streeter, et al. (1978). Ribavirin is converted to its 5'-phosphate derivative within the host cell. It then becomes a potent competitive inhibitor of inosinate dehydrogenase which is involved in the conversion of inosinate to xanthylate, the immediate precursor of guanylate and guanosine 5'-monophosphate. This inhibition directly interferes with the synthesis of viral RNA or DNA. The use of ribavirin as a chemotherapeutant has so far been limited due to its toxic effects which include teratogenicity when pregnant mammals are exposed to it (Oxford, 1979; Maugh, 1976).

Amantadine (1-adamantanamine hydrochloride), a tricyclic amine, is the only compound currently used in the prevention of viral infections

of the respiratory tract caused by influenza A virus (Whitley and Alford, 1978). Amantadine appears to interfere with a virus-cell membrane interaction such as penetration, uncoating, or release (Hermans, 1977; Maugh, 1979). Kato and Eggers (1969) observed an inhibition of the uncoating of fowl plaque virus in chick embryo cell cultures when amantadine (25 ug/ml) was present 1 hour before or at the time of inoculation. Inhibition was characterized by a delay in the hemagglutination of chicken red blood cells and appearance of infective virus (PFU/ml) and a decrease in virus yield by 80 to 90%.

Oxford (1979) observed that amantadine (50-100 ug/ml) had no effect on the absorption, penetration, uncoating, or RNA transcripase activity of the amantadine-sensitive influenza A virus. Instead, decreases in polypeptide synthesis were seen using polyacrylamide gel electrophoresis with ^{35}S-methionine labelled polypeptides. Influenza A recombinants derived from resistant and suceptible parents showed that gene 7, which codes for the virion matrix protein, also carries the property of amantadine resistance. Matrix protein has been shown to affect late uncoating and the subsequent transcription of influenza A virus RNA necessary for cell infection. Therefore, amantadine may inhibit late uncoating (or some unknown event accompanying this) through its effects on matrix protein.

The molecular basis for the mechanism of action of amantadine is still unknown (Oxford, 1979; Prusoff and Ward, 1976). Amantadine has not yet been tested for its effects on plant viruses.

Methisazone (N-methylisatin-B-thiosemicarbazone) is a potent

inhibitor of the pox viruses (Maugh, 1976; Whitley and Alford, 1978; Hermans, 1977). However, the virtual eradication of smallpox has eliminated the need for methisazone. Methisazone is thought to inhibit an intracellular event necessary for virus replication (Prusoff and Ward, 1976). Woodson and Jolik (1965) observed a reduction in the yield (PFU/ml) of vaccinia virus grown on HeLa cell cultures in the presence of methisazone (3.3 ug/ml). Methisazone appears to reduce the amount of "late" viral messenger RNA (present from 3 hours after infection on) thereby resulting in a reduction of 80 to 90% in the synthesis of viral protein and mature viral progeny (Woodson and Jolik, 1965; Whitley and Alford, 1978). It has been speculated that methisazone may also inhibit viral replication by inhibiting the RNA-dependent-RNA polymerase necessary for the replication of RNA viruses (Oxford, 1979). The RNA-dependent-RNA polymerase of influenza virus may be a zinc-activated or zinc metallo-enzyme (Oxford and Perrin, 1974). Certain thiosemicarbazones are chelators of heavy metal ions and have been shown to inhibit RNA-dependent-RNA polymerases (Gingras, et al., 1962). The precise mechanism of action of methisazone is still unknown (Whitley and Alford, 1978) and it has yet to be tested for its effects on plant viruses.

Many benzimidazole compounds are known to inhibit virus multipli-cation (Matthews and Smith, 1955). Tamm and Eggers (1963) reported that 2-hydroxybenzyl-benzimidazole selectively inhibits the RNA poly-merases of certain RNA animal viruses. Methyl benzimidazol-2yl-carbamate (MBC) is the fungitoxic principle of benomyl, a systemic

fungicide widely used in agriculture (Clemons and Sisler, 1969). MBC (2 grams per plant) was shown to suppress symptom expression of TMV in tobacco plants, <u>Nicotiana tabacum</u> variety "White Burley," and beet western yellows virus in lettuce, variety "Cobham Green" (Tomlinson, <u>et al.</u>, 1976). MBC may affect the host metabolism by preventing the degeneration and loss of chlorophyll which is commonly caused by TMV infection (Waygood, 1965).

Fraser and Whenham (1978) observed that TMV multiplication in tobacco plants, <u>Nicotiana tabacum</u>, varieties "White Burley" and Samsun," was inhibited when MBC (0.5 grams per plant) was applied as a solution to the roots, MBC reduced the accumulation of TMV RNA (ug TMV RNA/ gram fresh weight) in upper, systemically infected leaves by 98% of that of the control plants, as seen by absorbance of purified extracts at 265 nm. MBC was shown to decrease TMV infectivity and eliminate the inhibition of leaf growth resulting from TMV infection. The inhibition of TMV infectivity and stimulation of leaf growth caused by cyto-kinins (Fletcher, <u>et al.</u>, 1968) suggests that MBC may indirectly sup-press TMV RNA accumulation, infectivity, and symptom expression by its known cytokinin activity (Thomas, 1974). MBC is known to be system-ically translocated throughout the plant and is stable after absorption at concentrations which are nontoxic thereby making it a potentially useful plant virus chemotherapeutant.

Huang, <u>et al.</u> (1966) reported that formycin, a base analog of adenosine, caused an inhibition of TMV multiplication and symptom development without toxicity to the host plants, <u>Nicotiana tabacum</u>

variety "Bright Yellow," and <u>Phaseolus</u> <u>vulgaris</u> variety "Pinto." Leaf disks of <u>N</u>. <u>tabacum</u> variety "Bright Yellow," inoculated with TMV and floated on a medium containing formycin (100 ppm), exhibited a complete absence of systemic symptoms and an 80% inhibition of TMV multiplication. Complete inhibition of local lesion formation was observed on <u>P</u>. <u>vulgaris</u> variety "Pinto, " when formycin (100 ppm) was sprayed within 12 hours before and 12 hours after TMV inoculation.

Wawrosh and Sarkar (1974) observed an inhibition of TMV biosynthesis in tobacco plants, <u>Nicotiana</u> <u>tabacum</u> variety "Samsun," by formycin (50 ug/ml). Although, specific infectivity of TMV and TMV RNA were reduced in the presence of formycin, no incorporation of formycin into TMV or host RNA could be detected. The mechanism of action by which formycin inhibits TMV biosynthesis is still unknown.

Several methods are commonly used for the application of antiviral compounds onto plant tissue. Floating leaves or leaf disks on a solution containing the compound has the advantage of uniformly exposing the leaf tissue to a precise amount of the compound (Huang, <u>et</u> <u>al</u>., 1966; Franki, 1962; Kuhn, 1971). However, this method does not permit observations on the effects of a compound on plant growth, virus multiplication, and the development of systemic symptoms over a long period of time. In addition, leaves removed from a host plant exhibit metabolic changes which may influence virus multiplication and response to the antiviral compound.

Leaf sprays (Dawson and Kuhn, 1972; Bawden and Kassanis, 1954) and soil drench (Fraser and Whenham, 1978; Hansen, 1979) are two

methods of application which are useful for screening tests in addition to having potential for practical use. Both methods permit the assessment of the effects of a compound on plant growth, virus multiplication, and the development of systemic symptoms throughout the life of the plant. Leaf sprays have an additional advantage of requiring less compound than soil drench when small plants or small numbers of plants are used.

Cowpea chlorotic mottle virus (CCMV) was discovered by Kuhn in 1964. Since then, its structure and activity have been studied extensively. CCMV is an icosohedral RNA virus with a particle diameter of approximately 25 nm and a molecular weight of 4.6×10^6 daltons (Maramorosch, 1977). The virus primarily infects legumes and is transmitted by inoculation with infected sap onto healthy host plants. Symptoms include the development of a bright yellow mottle in systemically infected cowpea plants (Vigna sp.) approximately two weeks after inoculation and the formation of dark brown local lesions on the hypersensitive host soybean, Glycine max, from 24 to 48 hours after inoculation.

Purified CCMV nucleoprotein can be obtained in relatively high concentrations of approximately 0.4 mg of virus per gram of infected cowpea leaf tissue (Verduin, 1978). CCMV is most stable at a pH of 4.7. Therefore, CCMV purification is usually carried out in pH 4.7 acetate buffer by the use of chloroform-butanol extraction and differential ultracentrifugation (Bancroft, 1971).

The hypersensitive response of local lesion formation on soybean provides a useful system for assaying the viral infectivity of plant sap

from infected hosts. In addition, CCMV is a good antigen (Maramorosch, 1977), making it possible to use immunological assays for the detection of viral antigen. Immunofluorescence was used to detect the presence of CCMV in the cytoplasm of protoplasts isolated from tobacco plants, Nicotiana tabacum variety "White Burley" (Motoyoshi, et al., 1973).

The enzyme-linked immunosorbent assay (ELISA) has been recently used for the highly sensitive detection of plant viruses. The use of ELISA avoids many of the limitations previously imposed by the use of serological techniques and infectivity assays (Clark and Adams, 1977). ELISA enables results to be obtained quickly and quantitatively when a colorimeter is used for the spectrophotometric detection of hydrolyzed substrate. Low virus concentrations can be detected and virus inactivators commonly present in plant extracts do not affect the detection of viral antigen by ELISA. ELISA was found to be 100 times more sensitive than the infectivity assay for detecting maize dwarf mosaic virus in extracts of infected maize leaves (Sum, et al., 1979). ELISA was found to be the only reliable method for the year round detection of chrysanthemum B virus in Chrysanthemum indicum, when compared with other plant virus assays (Koenig, et al., 1979).

Since ELISA does not indicate the infectivity of the virus particles, it is necessary to use both the infectivity assay and ELISA to provide a system for determining infectivity and concentration of the virus. Both of these methods have been useful for studying the effects of antivrial compounds on CCMV.

C H A P T E R   III

MATERIALS AND METHODS

The Plants

Cowpea (<u>Vigna</u> <u>unguiculata</u>, variety "California Blackeye") and soybean (<u>Glycine</u> <u>max</u>, variety "Harasoy") plants were grown in the greenhouse in sterilized soil consisting of a 1:1:1 mixture of soil, sand and peat moss. A solution of soluble fertilizer (16-32-16) was applied to the soil once a week. Plants were watered daily by direct application of water to the soil.

When the primary leaves were fully expanded (9-11 days after planting), the plants were inoculated with the appropriate dilution of virus-infected cowpea sap. The plants and their response to various treatments were then observed and recorded.

In each experiment, one flat of plants was used for each treatment. Although 12 seeds per flat were planted, the 8 most uniform plants in each flat were selected for treatment. Four replications of each treatment were performed.

The Virus

<u>Maintenance</u>. Cowpea chlorotic mottle virus (CCMV) was maintained in cowpea plants for use in subsequent inoculations of cowpea and soybean plants. Plant sap inoculum was prepared by grinding with a mortar and pestle a 1:10 weight/volume ratio of systemically infected cowpea trifoliate leaves and cold 0.1 M, pH 5.0, sodium acetate buffer, containing 0.01 M

sodium diethyldithiocarbamate (NaDIECA) and 0.01 M cysteine hydrochloride. The inoculum was strained through several layers of cheesecloth and then stored on ice at 0°C while inoculations were performed.

Inoculation was carried out by first dusting the primary leaves of plants with 600-mesh carborundum. The inoculum was applied by gently rubbing a bent glass rod dipped in inoculum once over the surface of each primary leaf. Inoculated leaves were then rinsed with tap water. Chlorotic mottling appeared on the trifoliate leaves of cowpea plants within 10-14 days after inoculation. Local lesions appeared on the primary leaves of soybean plants within 24-48 hours after inoculation and were counted on half leaves using a hand lense.

Purification. Purified CCMV was obtained by differential centifugation according to a modified procedure of Gay and Kuhn (1968). Approximately 200 grams of systemically infected trifoliate leaves were harvested from cowpea plants 10-14 days after the plants were inoculated. The plant tissue was homogenized for 3 minutes in a Waring blendor with 2.0 M, pH 5.0 sodium acetate buffer (containing 0.01 M NaDIECA and 0.01 M cysteine hydrochloride), chloroform, and butanol (1.0 ml of each per gram of tissue). All solutions were chilled prior to purification and the preparation was kept on ice in order to maintain it between 0° and 4°C. After straining the homogenate through 8 layers of cheesecloth, it was centrifuged for 10 minutes at 1600 g in a Sorvall RC-2B refrigerated centrifuge. The supernatant was removed and centrifuged for 10 minutes at 25,000 g. The supernatant was once again removed and centrifuged for 1 hour at 98,000 g in a Beckman L2-65B ultracentrifuge.

Each pellet was suspended in 1 ml of cold 0.1 M, pH 5.0, sodium acetate buffer, containing 0.01 M $MgCl_2$ (Dawson and Kuhn, 1972), using a glass homogenizer. The homogenate was then centrigured for 10 minutes at 20,000 g. The supernatant was removed and centrifuged for 1 hour at 138,000 g. Each pellet was suspended in 0.1 M, pH 5.0, sodium acetate buffer, containing 0.01 M $MgCl_2$. The homogenate was centrifuged at 20,000 g. The supernatant was removed and centrifuged for 1 hour at 138,000 g. Each pellet was again suspended in 0.01 M, pH 5.0, sodium acetate buffer, containing 0.01 M $MgCl_2$. The homogenate was centrifuged once more at 20,000 g. The supernatant contained purified CCMV in buffer. The amount of virus nucleoprotein was estimated by measuring the optical density at 260 nm using a Beckman DB-G grating spectro-photometer. A virus nucleoprotein concentration of 1 mg/ml has an optical density of 6.0 (Dawson and Kuhn, 1972). Yields of purified CCMV ranged from 0.2 to 0.6 mg per gram infected plant tissue. The purified CCMV preparation was stored at 4°C until used.

The Antiserum

Production. Purified CCMV was suspended in 0.01 M, pH 7.0, phosphate buffer, containing 0.85% NaCl, (PBS) for injection into rabbits. A preparation of purified CCMV nucleoprotein, suspended in acetate buffer, was centrifuged for 1 hour at 138,000 g. The pellets were suspended and homogenized in PBS. The homogenate was centrifuged at 20,000 g. The supernatant contained purified CCMV nucleoprotein suitable for use as antigen in the production of CCMV antisera. Two rabbits were immunized

and bled according to the procedure of Otsuki and Takebe (1969). Each rabbit was injected intravenously with 10 mg of purified CCMV antigen in 1 ml PBS. Four days later, the rabbits received intramuscular injections of 50 mg CCMV in 1.2 ml PBS emulsified with an equal volume of Freund's complete adjuvant. After 1 month a final intravenous injection of 10 mg CCMV in 0.8 ml PBS was injected into each rabbit. The rabbits were bled by cardiac puncture using a 50 cc sterile plastic syringe 10 days after the last immunization. Food was withheld from the rabbits for 16 hours before bleeding to reduce serum lipids.

The antiserum was prepared according to the procedure of Williams and Chase (1967). The blood was allowed to clot for 1 hour at 37°C in sterile 50 ml plastic centrifuge tubes. The tubes were rimmed with a wooden applicator stick and refrigerated overnight at 4°C for clot contraction. The clot was removed and the serum was centrifuged for 20 minutes at 300 g in a Sorvall RC-2B refrigerated centrifuge to remove blood cells. Complement was inactivated by incubating the serum at 56°C for 20 minutes. The serum was stored in 3 ml serum vials and frozen until used.

Titer and specificity. The antiserum from each rabbit was titered by using the microprecipitin test according to a modified procedure of Ball (1974). Grid patterns were drawn on microprecipitin plates so that serial dilutions of CCMV-antiserum (from each rabbit) or normal rabbit serum and antigen (purified CCMV) could be placed in each square. Normal rabbit serum was included to be certain that a nonspecific precipitin reaction was not occurring between CCMV antigen and rabbit

serum proteins. Each serum was serially diluted in 0.01 M, pH 5.0, sodium acetate buffer. One ul drop of each serial dilution of anti-serum and CCMV antigen was placed in each square on a microprecipitin plate. The drops of antiserum and antigen in each square were gently mixed with wooden applicator sticks. The same procedure was carried out with normal rabbit serum and CCMV antigen. The plates were incubated overnight at 4°C in a moisture chamber. They were observed the following day under a light microscope for the presence of a cloudy, white precipitate. The greatest antiserum dilution at which a precipitate was seen represented the titer of the antiserum. The antiserum titers for both rabbits were between 1/800 and 1/1600.

Specificity of the antisera for CCMV antigen, as opposed to normal plant tissue antigens, was demonstrated by the Ouchterlony gel double diffusion test and the viral neutralization test.

Ouchterlony plates were prepared by dissolving 0.9 g Agarose agar in 100 ml 0.01 M, pH 7.0, PBS. The agar was autoclaved for 18 minutes and preserved by the addition of 0.025% sodium azide. The agar was poured into sterile disposable plastic petri plates. After the agar had solidified, 7 wells were cut using a size 5 cork borer. The center well contained CCMV antiserum diluted 1:10 in PBS. The six surround-ing outer wells contained either purified CCMV antigen (100 ug/ml in sodium acetate buffer), CCMV-infected plant sap, or healthy plant sap (both diluted 1:10 in sodium acetate buffer). The plates were incu-bated in a moisture chamber for one week at 4°C. Precipitin bands formed only between purified CCMV antigen and CCMV antiserum and

between CCMV-infected plant sap and CCMV antiserum but never between healthy plant sap and CCMV antiserum.

For virus neutralization, CCMV antiserum was serially diluted with PBS. One ml of each serial dilution was incubated with 1 ml purified CCMV antigen (100 ug/ml) for 30 minutes. Each mixture was then inoculated onto the primary leaves of soybean plants (6 leaves/treatment). After 48 hours, the presence of antiserum (diluted up to 1:1600) in the inoculum was seen to reduce local lesion numbers. Neutralization of virus infectivity suggested that the antiserum contained antibodies specific for CCMV.

The Antiviral Compounds

Preparation of antiviral compounds. Each compound was applied onto cowpea and soybean plants at the highest concentration determined to be nontoxic. 2-Thiouracil (supplied by Sigma Chemical Co., St. Louis, Missouri) and ribavirin, trademark named Virazole, (supplied by ICN Pharmaceuticals, Irvine, California) were suspended at a concentration of 500 mg per liter distilled, deionized water. Amantadine (supplied by Pfaltz and Bauer, Inc., Stamford, Connecticut) was suspended at a concentration of 250 mg per liter distilled, deionized water. Methisazone, trademark named Marboran, (supplied by Burroughs Wellcome Co., Research Triangle Park, North Carolina) was suspended in 1 N NaOH which was further diluted 1:10 with distilled, deionized water to give a final methisazone concentration of 250 mg per liter. Formycin, trademark named Formycin B, (supplied by Sigma Chemical Co., St. Louis,

Missouri) was suspended at a concentration of 25 mg per liter distilled, deionized water. Bavistin (supplied by B.A.S.F., Aktiengesellschaft, West Germany), a commercial preparation containing 50% (w/w) methyl benzimidazol-2yl-carbamate in non-active filler, was suspended at a concentration of 1,000 mg per liter distilled, deionized water.

Method of application. Both cowpea and soybean plants were sprayed with the antiviral compounds using DeVilbiss Non-Corrosive 251 Atomizers. The plants were sprayed with a fine mist until droplets began to coalesce to assure even coverage of the leaves.

Timing of compound application. Six spray regimes (Treatments A-F) were followed for the application of the antiviral compounds. The compounds were sprayed as follows:

> Treatment A--2 days and 1 day before inoculation and at the time of inoculation.
>
> Treatment B--2 days and 1 day before inoculation, at the time of inoculation, and 1 day after inoculation.
>
> Treatment C--At the time of inoculation.
>
> Treatment D--At the time of inoculation and 1 day after inoculation.
>
> Treatment E--At the time of inoculation and 1 day, 2 days, 3 days, and 4 days after inoculation.
>
> Treatment F--1 day, 3 days, 6 days, and 8 days after inoculation.

Plants were always inoculated in the morning. When plants were sprayed on the day of inoculation, spraying took place immediately after inoculation.

Experimental Design

Direct in vitro effects of antiviral compounds on CCMV infectivity. The
direct or in vitro effects of the antiviral compounds were tested to
determine whether any of the compounds directly influenced the infec-
tivity of the CCMV inoculum or the infection process. Systemically
infected tissue from cowpea plants was homogenized in 0.1 M, pH 5.0,
sodium acetate buffer containing 0.01 M NaDIECA and 0.01 M cysteine
hydrochloride to give a 1:5 plant sap dilution. Double strength spray
solutions were made for each of the six compounds. Three ml of plant
sap were mixed with 3 ml of each spray solution. The mixtures were
allowed to incubate for 1 hour at 4°C. The pH of each mixture was
determined using a pH meter. Each mixture was inoculated onto 8 soybean
primary leaves. Control plants were inoculated with a mixture contain-
ing 3 ml of plant sap and 3 ml of distilled, deionized water. Local
lesions were counted after 48 hours.

Effects of antiviral compounds on local lesion formation on soybean
plants. Preliminary experiments were done to examine the effects of
2-thiouracil, ribavirin, amantadine, methyl benzimidazol-2yl-carbamate,
methisazone, and formycin on local lesion formation on soybean plants.
The six compounds were applied using spray treatments A, B, C, and D.
The control consisted of plants which were inoculated without being
sprayed. Eight soybean primary leaves were inoculated and local lesions
were counted after 48 hours.

Further testing of ribavirin, amantadine, formycin, and methisazone

was accomplished by spraying the soybean plants three times on the day of inoculation. Plants were sprayed at 8:00 A.M., 12:00 A.M., and 5:00 P.M. while inoculation was carried out at approximately 11:00 A.M. A spreader-sticker was added to each spray solution at a concentration of 50 ul in 30 ml of solution. Controls consisted of inoculated soybean plants which were unsprayed, sprayed with distilled water, or sprayed with distilled water containing spreader-sticker. Inoculation of counting of local lesions were done according to the procedures pre-viously described.

Effects of antiviral compounds on systemic CCMV infection of cowpea plants. Preliminary experiments were done to determine what effects, if any, the six antiviral compounds exhibited on systemic infection of cowpea plants. The six compounds were applied using spray treatments, A, B, C, D, E, and F. The positive control consisted of plants which were inoculated with CCMV without being sprayed. The negative control consisted of plants which were sprayed without being inoculated. To determine the effects of the antiviral compounds on the infectivity of CCMV within the systemically infected plants, infectivity assays were performed on soybean plants using cowpea tissue from each treatment as an inoculum source. To prepare the inoculum, six primary leaves were randomly selected from cowpea plants receiving a given treatment. The six leaves were shredded into small 3 mm pieces with a razor blade. Using a mortar and pestle, 0.5 g of shredded leaf tissue was homogenized with 4.5 ml of pH 5.0, 0.1 M., sodium acetate buffer containing 0.01 M NaDIECA and 0.01 M cysteine hydrochloride. Eight soybean primary leaves

were inoculated and local lesions were counted according to the proce-
dures previously described. These assays were performed 10 days after
the cowpea plants were inoculated. Visual observations were recorded
10 days and 42 days after inoculation to determine the effects of each
compound on symptom development and plant growth.

A second set of experiments were done to further test four of the
antiviral compounds which appeared to have an effect on systemic CCMV
infection of cowpea plants in the preliminary experiments. 2-Thiouracil,
ribavirin, amantadine, and methisazone were applied using spray treat-
ments A, C, and E. The control plants were inoculated without being
sprayed. Infectivity assays were performed according to the procedure
described in the preliminary experiment. Two infectivity assays were
performed 10 days after inoculation using either the primary leaves or
the trifoliate leaves as an inoculum source. Two additional infectivity
assays were performed 16 and 22 days after inoculation using trifoliate
leaves as an inoculum source.

A final set of experiments was done to determine the effects of two
of the antiviral compounds on the presence of CCMV antigen within
systemically infected cowpea plants. Ribavirin and amantadine were
applied using spray treatment E. The controls consisted of healthy
plants which were not inoculated or sprayed and inoculated plants which
were not inoculated or sprayed and inoculated plants which were not
sprayed. The enzyme-linked immunosorbent assay (ELISA) was performed
2, 4, 8, and 15 days after inoculation. Randomly selected primary
leaves (at 2, 4, and 8 days after inoculation) and trifoliate leaves (at

15 days after inoculation) from each treatment were homogenized in the ELISA virus (conjugate) buffer (see below) using a mortar and pestle to give 1:10 (w/v) dilutions of plant sap in which the presence of viral antigen could be detected. Separate ELISA tests were performed on each of the 4 replications of each antiviral compound spray treatment.

The Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was performed in accordance with the procedures of Clark and Adams (1977).

Preparation of buffers and glassware. Several buffers and solutions were prepared for ELISA. Coating buffer was prepared by adjusting the pH of 0.05 M sodium carbonate buffer to 9.6 with HCl. PBS-Tween washing solution was prepared by adding 0.15 M NaCl and 0.05% (v/v) Tween 20 to 0.02 M, pH 7.4, phosphate buffer. Conjugate (virus) buffer was prepared by adding 2.0% (w/v) polyvinylpyrrolidone (MW 10,000) and 0.2% (w/v) ovalbumin to PBS-Tween. Substrate buffer was prepared by adjusting the pH of 10% (v/v) diethanolamine to pH 9.8 with HCl. A solution of 3 M NaOH was prepared to stop enzyme activity before the ELISA plates were read. Sodium azide (0.02% w/v) was added to all buffers to prevent contamination.

Any glassware which contained antibodies was siliconized with Sigmacote to prevent loss of protein by absorption.

Immunoglobulin G purification. One ml samples of CCMV antiserum were diluted with 9 ml distilled, deionized water. Ten ml of saturated

ammonium sulphate solution were added slowly while stirring to 10 ml

of diluted antiserum. After the mixture was allowed to stand for 1

hour at room temperature, it was centrifuged for 5 minutes at 4,000 g to

collect the precipitate. The precipitate was dissolved into 2 ml of

half-strength PBS. The sample was then dialyzed overnight at 4°C against

500 ml of half-strength PBS with 2 changes of the buffer during the

dialysis.

Further purification of IgG was achieved by passing the sample

through a short chromatographic column which was packed to a height of

5 cm with DE 22 cellulose (Whatman, Inc.). DE 22 cellulose was pre-

equilibrated in half-strength PBS and prepared for use according to the

manufacturer's instructions. Malachite green dye was passed through the

packed column to make certain the column was properly packed before the

IgG sample was loaded onto the column. Once a sample was loaded onto

the column, the column was washed with half-strength PBS until purified

IgG protein was eluted. Two ml fractions were collected and monitored

spectrophotometrically at 280 nm. The fraction containing purified

IgG (unabsorbed protein) was eluted soon after the void volume. The

peak fraction had an optical density of 2.84 at 280 nm. The presence

of purified IgG was confirmed by a positive precipitin reaction with

purified CCMV antigen. The purified IgG was diluted in half-strength

PBS until an optical density of 1.4 was obtained to give a concentration

of approximately 1 mg/ml. It was then stored in siliconized glass

tubes at 4°C.

Conjugation of enzyme with IgG. A liquid preparation containing 2 mg of

Sigma No. P-4502 Type VII alkaline phosphatase in 0.001 M $MgCl_2$ and 0.0001 M $ZnCl_2$ was centrifuged at 4,000 g for 5 minutes. The supernatant was discarded and the precipitate was dissolved by direct addition of 1 ml (=1 mg) of purified IgG to the centrifuge tube. The preparation was dialized against 500 ml PBS overnight at 4°C with 2 changes of the buffer during dialysis. After dialysis, 25 % glutaraldehyde solution was mixed with the IgG preparation to give a final glutaraldehyde concentration of 0.05% (v/v). The mixture was incubated for 4 hours at room temperature after which time a very faint sandy brown color developed. The conjugate preparation was dialzyed overnight at 4°C against 500 ml PBS with 0.01% sodium azide with 2 buffer changes during the dialysis. After the addition of 5 mg/ml bovine serum albumin, the conjugate was stored at 4°C in a siliconized glass test tube.

ELISA procedure. Purified IgG was diluted 1:10,000 in coating buffer to give an optimum concentration of 0.1 ug/ml coating IgG. The wells of the ELISA plates (Gilford Instruments, Inc.) were each coated with 200 ul of coating IgG. Outer wells which were not used were filled with distilled water throughout this procedure. The plate was covered with Saran Wrap and incubated in a moisture chamber for 4 hours at 37°C or overnight at 4°C. The wells were washed by flooding them with PBS-Tween and allowing them to sit for 3 minutes before emptying the plate. After the wells were washed 3 times, test samples diluted in virus (conjugate) buffer and filtered through 2 layers of Miracloth were added in 200 ul aliquots to the coated wells. The plate was incubated and washed as described above. Alkaline phosphatase conjugated IgG was

diluted 1:1600 in conjugate buffer to give an optimum concentration of conjugated IgG. The conjugate was added in 200 ul aliquots to each well. The plate was incubated and washed as described above. P-nitrophenyl phosphate tablets were dissolved into substrate buffer to give a final substrate concentration of 1 mg/ml. Substrate was added in 300 ul aliquots to each well. After a 1-hour incubation at room temperature, the reaction was stopped by the addition of 50 ul 3 M NaOH to each well. The results were determined within a half hour by quantitating the absorbance of the hydrolyzed substrate at 405 nm using a Gilford EIA processor/reader.

Determination of optimum coating IgG and enzyme-conjugated IgG concen-trations. The ELISA procedures described above were used to determine optimum concentrations of coating and enzyme-conjugated IgG. The wells of an ELISA plate were coated with serial dilutions of coating IgG (10, 1.0, and 0.1 ug/ml) in coating buffer. Three replicates of each coating IgG concentration were used. Each of the three replicates re-ceived either a 1:200, a 1:800, or a 1:3200 dilution of enzyme conju-gated IgG. The following test samples were used: 1:10, 1:100, 1:1,000 (w/v) dilutions of virus-infected tissue in virus (conjugate) buffer: 1:10 and 1:100 (w/v) dilutions of healthy tissue in virus (conjugate) buffer; and virus conjugate buffer. Optimum concentra-tions were determined spectrophotometrically as those concentrations of coating and enzyme-conjugated IgG which produced A_{405} readings between 0 and 2.0.

Statistical Analysis

Statistical analysis of local lesion numbers and ELISA A_{405} readings was done by a one-way analysis of variance for the variable experimental treatment (Zar, 1974). For analysis of local lesion numbers, the mean values of 8 local lesion numbers (from 8 primary half leaves of soybean plants) were used to represent each experimental treatment. Since 4 replications of each experimental treatment were performed in all experiments, the group mean of the 4 replications for each experimental treatment were determined. Analysis of ELISA A_{405} readings was accomplished by using the group mean values from 4 replications of each experimental treatment. The Duncan new multiple range test was used for a comparison of the group means to determine which experimental treatments produced significantly different effects on CCMV infectivity at the 0.05 level of significance (Zar, 1974).

Since local lesion numbers deviate greatly from a normal frequency distribution, analysis of variance by the Duncan new multiple range test was also performed on local lesion numbers which were transformed according to the procedure of Kleczkowski (1955). The transformation $z = \log_{10}(x+c)$ was used when local lesion numbers were greater than 10, where x=number of lesions and c=5. Numbers less than 10 were transformed according to the formula $z = \log_{10} \frac{1}{2}(x+c+\sqrt{x^2+2cx})$. However, transformed local lesion numbers are only presented when they exhibit differences among experimental treatments which were not evident from the analysis of untransformed local lesion numbers.

C H A P T E R   IV

RESULTS

Effects of Antiviral Compounds on the Development
of Viral Symptoms and Plant Growth

Six antiviral compounds namely 2-thiouracil, ribavirin, amantadine,
formycin, methisazine, and methyl benzimidazol-2yl-carbamate, were
applied on cowpea leaves according to spray treatment regimes A, B,
C, D, E, or F (described in Materials and Methods). Their effects
on CCMV symptom expression and their toxicity to plant growth were det-
ermined by visual observations recorded 10 and 42 days after the inocula-
tion of cowpea plants. Symptom development and plant growth were com-
pared to that of unsprayed, inoculated and sprayed, uninoculated control
plants.

Cowpea plants inoculated with CCMV and sprayed with 2-thiouracil,
treatments A, E, and F, exhibited severe CCMV symptoms consisting of
chlorotic mottling 10 days after inoculation, while plants sprayed with
2-thiouracil, treatments B, C, and D, exhibited slight chlorotic mottl-
ing when compared with the unsprayed, inoculated control plants (Fig. 1
and Fig. 2). Inoculated and uninoculated plants sprayed according to
any of the treatment regimes (A through F) exhibited symptoms of toxi-
city which included stunting of plant growth, bleaching of primary and
trifoliate leaves, and puckering of trifoliate leaf margins (Fig. 2).
Forty-two days after inoculation, sprayed cowpea plants still exhibited
puckering of young, newly expanded, trifoliate leaf margins. Symptoms

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Fig. 1. Chlorotic mottling on trifoliate leaves of cowpea plants systemically infected with cowpea chlorotic mottle virus observed ten days after inoculation.

Fig. 2. Reduction (left) and increase (right) in symptom development of cowpea chlorotic mottle virus-infected cowpea plants caused by different 2-thiouracil treatments within ten days after inoculation. Stunting, bleaching, and puckering of trifoliate leaves were toxic effects caused by 2-thiouracil application.

of CCMV continued to remain severe with treatments A, E, and F while
with treatments B and D symptoms became severe on young trifoliate leaves
of plants observed 42 days after inoculation. Symptoms developed only
slightly on plants sprayed according to treatment C and observed 42
days after inoculation.

Application of ribavirin, treatments A through F, caused cowpea
plants to remain almost completely free of CCMV symptoms during the 10
days after inoculation. No toxic effects were exhibited on the plants
when sprayed according to any of the six treatment regimes (Fig. 3).
When the cowpea plants were observed 42 days after inoculation, they
still did not exhibit any symptoms of toxicity. In addition, young tri-
foliate leaves of plants sprayed with treatments E and F were completely
free of CCMV symptoms 42 days after inoculation while those sprayed with
treatment A exhibited only very faint symptoms. Young trifoliates of
plants sprayed according to treatments B, C, and D exhibited more severe
symptoms at 42 days after inoculation than did trifoliates at 10 days
after inoculation but the symptoms were milder than those exhibited 42
days after inoculation by unsprayed, control plants.

Amantadine, applied according to treatments A through F, reduced
the chlorotic mottling symptoms of CCMV-infected cowpea plants observed
10 days after inoculation. Treatments A, B, C, and D resulted in almost
complete suppression of CCMV symptoms, while treatments E and F allowed
mild symptom development during the first 10 days after inoculation.
Trifoliate leaves showed minimal margin necrosis when amantadine was
applied according to treatments E and F on both sprayed inoculated and

Fig. 3. Inhibition of cowpea chlorotic mottle virus symptom development and absence of toxic effects observed ten days after inoculation when cowpea plants were sprayed with ribavirin.

sprayed, uninoculated control plants (Fig. 4) when observed 10 days after inoculation. Forty-two days after inoculation, some leaf margin necrosis was still observed on trifoliate leaves of cowpea plants sprayed according to treatments E and F. Young trifoliate leaves on plants sprayed according to treatments A, B, C, and D were almost completely free of CCMV symptoms when observed 42 days after inoculation. Treatments E and F allowed the development of some symptoms on young trifoliate leaves 42 days after inoculation but these were much less severe than the symptoms of unsprayed, inoculated control plants.

Formycin, although nontoxic to the directly sprayed primary leaves of cowpea plants, was highly toxic to young, newly expanding, trifoliate leaves of sprayed, inoculated plants and sprayed, uninoculated control plants. The toxic effects included leaf margin necrosis, leaf puckering and curling, and the formation of necrotic 'shot-hole' lesions on trifoliate leaves. The development of CCMV symptoms was inhibited by formycin treatments A, B, C, D, and E when evaluated 10 days after inoculation (Fig. 5). At 42 days after inoculation, the toxic effects caused by formycin were less severe than at 10 days after inoculation. CCMV symptoms in young trifoliate leaves observed 42 days after inoculation were reduced by treatments A, B, C, and D just as they were 10 days after inoculation; however, treatment E was no longer as effective in reducing symptom development. Young trifoliates of plants sprayed according to treatments E and F exhibited CCMV symptoms as severe as the symptoms on unsprayed, inoculated control plants 42 days after inoculation.

Fig. 4. Amantadine induced reduction in cowpea chlorotic mottle virus symptom development of cowpea plants within 10 days after inoculation.

Fig. 5. Severe phytotoxicity but reduction in cowpea chlorotic mottle virus symptom development observed on cowpea plants ten days after inoculation as a result of formycin application.

Application of methisazone treatments A through F inhibited CCMV symptom development of cowpea plants observed 10 days after inoculation. All methisazone treatments induced severe toxic effects consisting of striation and narrowing of trifoliate leaves in both sprayed,inoculated and sprayed,uninoculated control plants observed 10 days after inoculation (Fig. 6). Forty-two days after inoculation, these toxic effects were no longer observed on trifoliate leaves. CCMV symptoms were absent in young trifoliates of plants sprayed with treatments A through F and observed 42 days after inoculation.

Cowpea plants sprayed with methyl benzimidazol-2yl-carbamate according to treatments A through F, exhibited CCMV symptoms that were comparable to the symptoms on unsprayed,inoculated control plants with no evidence of any toxic effects throughout the 42 days of observation (Fig. 7).

Direct In Vitro Effects of Antiviral
Compounds on CCMV Infectivity

Mixtures of each antiviral compound and plant sap from CCMV-infected cowpea leaves were incubated for 1 hour at 4°C to determine the direct effects, if any, of each compound on the infectivity of CCMV. The in vitro effects were determined by performing infectivity assays using the antiviral compound-CCMV infected sap mixtures as the inoculum. When local lesion numbers were compared only methisazone directly reduced CCMV infectivity significantly. This was apparently due to the elevation of the pH of the inoculum when mixed with methisazone. When transformed local lesion numbers were compared, treatment of CCMV in sap

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Fig. 6. Formation of narrow, striated trifoliate leaves and re-
duced cowpea chlorotic mottle virus symptom development observed on
methisazone-treated cowpea plants observed ten days after inoculation.

Fig. 7. Absence of toxic effects and presence of normal cowpea
chlorotic mottle virus symptoms on trifoliate leaves of cowpea plants
sprayed with methyl benzimidazol-2yl-carbamate and observed ten days
after inoculation.

with either methisazone or ribavirin reduced CCMV infectivity signifi-
cantly (Table 1).

Effects of Antiviral Compound Sprays on Formation
of Local Lesions on Soybean Plants

The six antiviral compounds namely 2-thiouracil, ribavirin, amanta-
dine, formycin, methisazone, and methyl benzimidazol-2yl-carbamate were
sprayed on soybean plants according to treatment regimes A through D
and the soybean primary leaves were inoculated with CCMV-containing
sap from systemically infected cowpea leaves. The number of local
lesions produced on the soybean primary leaves 48 hours after inocula-
tion was taken as a measure of the effect of each antiviral compound on
local lesion formation of CCMV. Although each of the compounds, with
all treatment regimes, reduced the number of local lesions formed, the
lesion numbers were not significantly lower than those on unsprayed, in-
oculated control soybean plants (Table 2). Transformed local lesion
numbers yielded no further statistically significant differences. Local
lesion size remained the same in the control plants and in plants re-
ceiving the various treatments.

Effects of Antiviral Compounds on Formation
of Local Lesions on Soybean Plants Receiving
Three Spray Applications on Day of Inoculation

Ribavirin, amantadine, formycin, and methisazone were applied on
soybean plants three times (at 8:00 A.M., 12:00 A.M., and 5:00 P.M.) on
the day of their inoculation with CCMV in order to test further the
effect of these compounds on local lesion formation. Each compound was

TABLE 1

Effect of Antiviral Compounds on pH and Infectivity of Cowpea Chlorotic Mottle Virus Inoculum

Antiviral Compound	Inoculum pH	Inoculum Infectivity[a]	Local Lesion Transformation[b]
2-Thiouracil	5.7	73.00 ± 66.03	1.73
Ribavirin	5.7	48.97 ± 46.96	1.58*
Amantadine	5.2	65.38 ± 44.60	1.77
Formycin	5.3	104.94 ± 17.09	2.04
Methisazone	11.0	0.03*± 0.07	0.41*
Methyl benzimidazol-2yl-carbamate	6.3	85.47 ± 28.57	1.94
Control--No Compound	5.5	85.72 ± 29.50	1.94

[a] Each number represents the mean number of local lesions of 4 replications ± the standard deviation. Each replication consists of 8 soybean leaves.

[b] Each number represents a logarithmic transformation of the mean number of local lesions (Kleczkowski, 1955).

*Statistically significant difference in infectivity between antiviral compound and control when analyzed by the Duncan new multiple range test (P=0.05).

TABLE 2

Effect 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	102.19 $\pm$ 86.91[a]	106.59 $\pm$ 79.87	115.97 $\pm$ 64.29	115.41 $\pm$ 63.80
Ribavirin	71.63 $\pm$ 35.47	46.16 $\pm$ 20.21	86.63 $\pm$ 47.64	83.87 $\pm$ 63.91
Amantadine	86.22 $\pm$ 66.12	93.38 $\pm$ 65.22	82.91 $\pm$ 48.90	71.72 $\pm$ 45.22
Formycin	111.61 $\pm$ 39.15	73.00 $\pm$ 11.30	67.81 $\pm$ 9.15	44.00 $\pm$ 6.90
Methyl benzimidazol-2yl-carbamate	66.69 $\pm$ 11.13	97.53 $\pm$ 38.08	69.60 $\pm$ 12.62	87.41 $\pm$ 1.73
Methisazone	73.13 $\pm$ 20.64	88.13 $\pm$ 35.50	75.94 $\pm$ 20.77	86.75 $\pm$ 21.19
Control--No Compound	117.41 $\pm$ 49.30			

[a]Each number represents the mean number of local lesions of 4 replications $\pm$ the standard devia-
tion. Each replication consists of 8 soybean half leaves. The local lesions numbers were stat-
istically analyzed by the Duncan new multiple range test (P=0.05).

mixed with a spreader-sticker before it was sprayed on the plants. The numbers of local lesions produced on plants sprayed with the four antiviral compounds were significantly lower than those produced on inoculated control plants that were left unsprayed, sprayed with distilled water, or sprayed with distilled water containing spreader-sticker. Furthermore, the number of local lesions produced on methisazone-treated plants was significantly lower than the number of local lesions produced on plants treated with any of the other antiviral compounds. Transformed local lesion numbers yielded no further significant differences. Local lesion numbers were also lower on inoculated control plants sprayed with a mixture of distilled water and spreader-sticker, but these numbers were not significantly lower than the number of local lesions produced on unsprayed plants (Table 3). None of the antiviral treatments affected the size of individual local lesions.

Effects of Antiviral Compound Sprays on Infectivity of CCMV in Cowpea Plants

The antiviral compounds 2-thiouracil, ribavirin, amantadine, formycin, methisazone, and methyl benzimidazol-2yl-carbamate were sprayed on CCMV-infected cowpea plants according to spray treatment regimes A, B, C, D, E, and F. The treated plants were assayed for virus infectivity, as determined by the number of local lesions produced on soybean plants (Fig. 8), by harvesting the primary leaves of the treated cowpea plants 10 days after inoculation and using them as a source of inoculum for the inoculation of soybean plants.

The treatments were evaluated for their effects on the infectivity of

TABLE 3

Effect of Antiviral Compound Sprays, Applied 3 Times on the
Day of Inoculation, on the Number of Cowpea Chlorotic Mottle Virus
Local Lesions on Soybean Plants

Spray Treatment	Number of Local Lesions*
Methisazone	$3.07^a \pm 1.42$
Ribavirin	$6.57^b \pm 2.61$
Amantadine	$8.38^b \pm 1.81$
Formycin	$9.03^b \pm 4.43$
Control--Distilled H_2O and Spreader-Sticker	$16.72^c \pm 2.54$
Control--Distilled H_2O	$27.51^c \pm 2.26$
Control--No Spray	$29.38^c \pm 2.86$

*Each number represents the mean number of local lesions of 4 replications $\pm$ the standard deviation. Each replication consists of 8 soybean half leaves. Means followed by a common letter are not significantly different. The local lesion numbers were statistically analyzed by the Duncan new multiple range test (P=0.05).

Fig. 8. Local lesions on primary leaves of soybean plants observed
24 to 48 hours after inoculation.

CCMV in cowpea plants by comparing the infectivity of CCMV in sap from sprayed plants to that of unsprayed, inoculated control plants (Table 4). Although many of the treatments caused some decrease in CCMV infectivity, none of these were significantly lower than the infectivity of sap from unsprayed, inoculated control plants. The six compounds reduced CCMV infectivity in cowpea plants when applied according to the following spray treatment regimes: 2-thiouracil, A, B, C, and D; ribavirin, A, B, C, D, E, and F; amantadine, A, B, C, D, and E; formycin, A, B, and C; methisazone, A, B, C, D, and E; methyl benzimidazol-2yl-carbamate, A, B, C, E, and F. Eight of the treatments caused some increase in CCMV infectivity. These were: E and F for 2-thiouracil; F for amantadine; D, E, and F for formycin; F for methisazone; and D for methyl benzimidazol-2yl-carbamate. 2-Thiouracil treatments E and F increased infectivity significantly (Table 4). Transformed local lesion numbers yielded no further significant differences.

Effects of Antiviral Compounds on Infectivity of CCMV in Cowpea Plants at 10, 16, and 22 Days After Inoculation

Four of the antiviral compounds namely 2-thiouracil, ribavirin, amantadine, and methisazone which appeared to have the greatest effects on CCMV infectivity and on symptom development 10 days after inoculation, were further tested for their effects on CCMV infectivity in cowpea plants. Infectivity assays were performed 10 days after inoculation using as inoculum either the inoculated primary leaves or the first trifoliate leaves. The purpose of this experiment was to determine whether CCMV infectivity in the younger trifoliates was more or less sensitive

TABLE 4

Effect 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	73.16[a] +14.14	58.72 +37.23	59.72 +11.65	78.50 +26.93	139.94* +41.18	109.00* +34.24
Ribavirin	62.72 +37.14	68.00 +19.07	46.50 +26.46	58.35 +34.76	64.53 +18.03	61.00 +15.93
Amantadine	60.03 +20.08	67.41 +20.13	52.94 +19.39	58.88 +38.89	77.66 +2.67	82.56 +9.21
Formycin	52.56 +32.41	75.47 +5.93	79.53 +44.11	86.25 +55.06	94.25 +33.62	93.10 +27.51
Methisazone	56.72 +40.55	68.60 +34.71	75.91 +15.06	69.25 +32.42	69.41 +18.54	87.91 +29.34
Methyl benzimidazol-2yl-carbamate	78.75 +13.64	69.06 +24.80	65.88 +10.70	80.47 +24.96	78.78 +32.78	53.10 +37.27
Control--No Compound	79.66 +12.72					

[a]Each number represents the mean number of local lesions of 4 replications + the standard deviation. Each replication consists of 8 soybean half leaves. The local lesion numbers were analyzed by the Duncan new multiple range test (P=0.05).
*Statistically significant difference between antiviral compound and control.

to the effects of the antiviral compounds than CCMV infectivity in the inoculated primary leaves. Trifoliate leaves were also used as inoculum when infectivity assays were performed at 16 and 22 days after inoculation to determine whether the antiviral compounds become more or less effective against the virus at later stages of infection. The compounds were applied according to spray treatment regimes A, C, and E. The treated cowpea plants were assayed for virus infectivity (as determined by the number of local lesions produced on soybean plants) by using tissue from treated plants as inoculum for inoculation of soybean plants. Local lesion numbers produced by sap from sprayed plants were compared to those produced by sap from unsprayed, inoculated control plants (Table 5). Transformation of local lesion numbers yielded no additional significant differences.

When the primary leaves of treated cowpea plants were assayed, ribavirin, amantadine, and methisazone reduced virus infectivity but these reductions were not statistically significant (Table 5). 2-Thiouracil decreased virus infectivity when applied according to treatment A, but when it was applied according to treatments C and E, 2-thiouracil increased virus infectivity. 2-Thiouracil treatment E increased virus infectivity considerably over the virus infectivity in unsprayed control plants (Table 5).

When infectivity of virus in trifoliate leaves was assayed 10 days after primary leaf inoculation, all of the antiviral compound treatments reduced CCMV infectivity in the sprayed cowpea plants. However, only plants sprayed with ribavirin treatment E had significantly lower virus

TABLE 5

Effect of Antiviral Compounds on Infectivity of Cowpea Chlorotic
Mottle Virus in Cowpea Plants at 10, 16, and 22 Days After Inoculation

Antiviral Compound	Treatment	Days After Inoculation--Tissue Assayed[a]			
		10 - Pri.	10 - Tri.	16 - Tri.	22 - Tri.
2-Thiouracil	A	43.13 ± 16.40[b]	46.06 ± 26.06	75.16 ± 37.88	4.03 ± 5.09
	C	63.66 ± 21.69	61.81 ± 22.26	66.60 ± 27.18	3.28 ± 3.91
	E	71.53 ± 23.04	77.66 ± 35.43	83.03 ± 40.67	0.35 ± 0.45
Ribavirin	A	59.22 ± 17.74	69.19 ± 12.64	85.13 ± 27.70	3.32 ± 2.86
	C	59.19 ± 23.06	63.35 ± 11.23	60.63 ± 24.72	6.75 ± 4.95
	E	37.69 ± 7.73	43.41 ± 9.96*	55.32 ± 19.63	1.81 ± 2.21
Amantadine	A	50.03 ± 9.03	61.57 ± 6.49	67.29 ± 19.74	14.00 ± 16.08
	C	54.78 ± 17.15	58.22 ± 37.49	58.91 ± 19.53	14.22 ± 7.24
	E	45.72 ± 17.47	51.72 ± 6.72	86.38 ± 23.91	9.75 ± 9.12
Methisazone	A	48.35 ± 18.58	63.38 ± 10.16	82.94 ± 15.91	12.97 ± 9.24
	C	45.28 ± 21.77	48.50 ± 34.84	51.96 ± 38.85	8.85 ± 3.34
	E	44.35 ± 18.34	45.94 ± 15.21	83.47 ± 16.71	1.00 ± 0.67
Control	No Spray	62.39 ± 20.54	82.07 ± 15.70	93.13 ± 25.03	5.04 ± 7.24

[a]Primary leaf tissue (Pri.) and trifoliate leaf tissue (Tri.) were assayed.

[b]Each number represents the mean number of local lesions of 4 replications ± the standard devia-
tion. Each replication consists of 8 soybean half leaves. The local lesion numbers were analyzed
by the Duncan new multiple range test (P=0.05).

*Statistically significant difference between antiviral compound and control.

infectivity than unsprayed, inoculated control plants (Table 5).

When infectivity of virus in trifoliate leaves was assayed 10 days after primary leaf inoculation, all of the antiviral compound treatments continued to reduce CCMV infectivity in the sprayed cowpea plants. However, none of these reductions were statistically significant when compared to the infectivity of inoculated control plants (Table 5).

Infectivity assays performed 22 days after inoculation showed that CCMV infectivity was reduced in unsprayed, inoculated control plants as well as in plants sprayed with each of the antiviral compound treatments. Although CCMV-infected plants sprayed with 2-thiouracil, treatments A, C, and E; ribavirin, treatments A, and E; and methisazone, treatment E, had less infectivity than unsprayed, inoculated control plants, plants sprayed with ribavirin, treatment C; amantadine, treatments A, C, and E; and methisazone, treatments A and C had greater infectivity than inoculated, unsprayed control plants. The infectivity in the plants sprayed with these treatments, however, was not significantly different from that of inoculated, unsprayed control plants (Table 5).

Effects of Ribavirin and Amantadine on the Presence of Viral Antigen in CCMV-Infected Cowpea Plants

The presence of viral antigen was detected by the enzyme-linked immunosorbent assay (ELISA) in CCMV-infected cowpea plants sprayed with ribavirin or amantadine according to spray treatment E. The ELISA tests were carried out at 2, 4, 8, and 15 days after inoculation. The absorbance of the ELISA reaction at 405 nm indicated the relative amount

of viral antigen present. In all ELISA tests, sap from healthy control plants gave significantly lower or zero absorbance readings due to background absorbance as a result of nonspecific reactions (Table 6).

At 2 days after inoculation, sap from inoculated cowpea plants sprayed with ribavirin contained slightly less viral antigen than un-sprayed, inoculated control plant sap while sap from plants sprayed with amantadine contained slightly more viral antigen than unsprayed, inoculated control plant sap. However, neither one of the viral antigen concentrations were significantly different from the viral antigen concentration in unsprayed, inoculated control plants (Table 6).

At 4 days after inoculation, sap from inoculated cowpea plants sprayed with ribavirin contained slightly less viral antigen than sap from unsprayed, inoculated control plants while sap from plants sprayed with amantadine contained slightly more viral antigen than sap from un-sprayed, inoculated control plants. However, these differences in viral antigen concentration were also not statistically significant (Table 6).

Sap from inoculated cowpea plants sprayed with ribavirin and aman-tadine and assayed by ELISA 8 days after inoculation, contained less but not significantly lower viral antigen than inoculated control plants.

When cowpea sap was assayed 15 days after inoculation, sap from plants treated with ribavirin and amantadine contained more viral anti-gen than inoculated control plants, but these differences were not statistically significant either (Table 6).

TABLE 6

Effect of Ribavirin and Amantadine on the Presence of Viral Antigen in Cowpea Chlorotic
Mottle Virus-Infected Cowpea Plants detected by the Enzyme-Linked Immunosorbent
Assay (ELISA) at 2, 4, 8, and 15 Days After Inoculation

Treatment	Days After Inoculation			
	2	4	8	15
Healthy Control[b]	0.000 ± 0.000[a]	0.000 ± 0.000	0.068 ± 0.015	0.055 ± 0.051
Ribavirin	0.319 ± 0.055	0.706 ± 0.383	1.055 ± 0.212	0.206 ± 0.085
Amantadine	0.388 ± 0.175	1.017 ± 0.215	1.114 ± 0.344	0.278 ± 0.088
Inoculated Control	0.361 ± 0.116	0.859 ± 0.175	1.269 ± 0.140	0.165 ± 0.023

[a]Each number represents the mean light absorption reading at 405 nm (A_{405}) of 4 ELISA wells ± the
standard deviation. The A_{405} readings were analyzed by the Duncan new multiple range test (P=
0.05).

[b]Statistically significant differences were detected only between healthy control and other treat-
ments at each time of ELISA.

CHAPTER V

DISCUSSION

Many of the antiviral compounds were very effective in suppressing
the development of CCMV symptoms on inoculated cowpea plants. However,
statistical analysis of the infectivities of CCMV in plants receiving
the various antiviral compound treatments and in inoculated control
plants seldom revealed statistically significant differences between
the effects of the treatments and the untreated inoculated controls.
This was probably due to the fact that the local lesion numbers obtained
for each experimental treatment exhibited great variability which re-
sulted in large standard deviations among the treatment means. Trans-
formation of local lesion numbers according to Kleczkowski (1955) before
statistical analysis also resulted in large standard deivations. In
spite of the lack of statistically significant differences, certain con-
sistent trends were observed in the effects of the antiviral compounds
on CCMV infectivity.

The antiviral compounds appeared capable of altering CCMV infecti-
vity depending on the times at which compounds were applied and at
which virus assays were performed in relation to the time of plant inoc-
ulation. Changes in CCMV infectivity in soybean plants sprayed with
treatment regimes A, B, C, and D, prior to or immediately after inocula-
tion, may indicate a compound effect on an early stage of the viral in-
fection process (absorption, penetration, uncoating, or de novo RNA
synthesis) since these effects were apparent within 48 hours after

inoculation (at about the time local lesions appeared)(Table 2).

Similar conclusions may perhaps be drawn from experiments in which

CCMV-inoculated soybean plants were sprayed with the antiviral compounds

3 times on the day of inoculation (Table 3). Changes in CCMV infectiv-

ity that become evident in virus assays (Tables 4 and 5) or from obser-

vations of virus symptom development within 10 days after inoculation

(when peak CCMV infectivity is normally reached) probably indicate that

the antiviral compound affects a later stage of the viral infection

process (replication, maturation, or long distance movement). Changes

in CCMV infectivity that become evident after 15 or more days from inoc-

ulation of sprayed plants probably indicate that virus breakdown or

stability was affected by the antiviral compound. Such changes were

monitored by infectivity assays performed at 16 and 22 days after inocu-

lation (Table 5) and observation of symptoms at 42 days after inocula-

tion. In addition, since ribavirin and amantadine were most successful

at suppressing virus symptom development without causing phytotoxicity,

their effects on viral antigen concentration in sprayed cowpea plants

were quantitatively determined by ELISA performed at 2, 4, 8 and 15

days after inoculation to ascertain whether the compounds may have

affected early or late stages of the viral infection process or virus

breakdown.

Of the six antiviral compounds tested for their effects on CCMV

infectivity, only methisazone and ribavirin may have directly affected

the infectivity of CCMV in the inoculum (Table 1). Methisazone appears

to have directly inactivated CCMV by elevating the pH of the inoculum

(Table 1) since the CCMV virion becomes unstable and noninfectious when exposed to a pH above 7.0 (Verduin, 1978). Although the pH of the inoculum containing methyl benzimidazol-2yl-carbamate was somewhat elevated at 6.3 (Table 1), there was no inhibitory effect on the infectivity of CCMV in the inoculum. The direct effect of ribavirin on the infectivity of CCMV could not be a pH effect since the addition of ribavirin did not change the pH of the inoculum to one in which CCMV would be unstable. It is possible that it is not the CCMV in the inoculum that is directly affected by ribavirin but rather the infectivity of CCMV in the plant possibly due to the entrance of ribavirin into the soybean plant during inoculation and subsequent interference of ribavirin with the CCMV infection process. Of the six antiviral compounds tested for their direct effects on CCMV infectivity, 2-thiouracil, ribavirin, and amantadine were previously determined by others to have no direct effects on the infectivity of certain viruses (Dawson and Kuhn, 1972; Lerch, 1977; Kato and Eggers, 1969), while no such information was available for formycin, methisazone, and methyl benzimidazol-2yl-carbamate.

The early infection processes of CCMV in soybean plants may have been affected by all six antiviral compounds when applied prior to or immediately after inoculation according to spray treatment regimes A, B, C, and D (Table 2), and by methisazone, ribavirin, amantadine, and formycin when applied 3 times on the day of inoculation (Table 3). Although local lesion numbers on soybean plants were reduced by application of antiviral compounds in both experiments (Tables 2 and 3),

statistically significant reductions in local lesion numbers were only
observed when soybean plants were sprayed 3 times on the day of inocula-
tion with antiviral compounds (Table 3). The significant reductions in
local lesion numbers shown in Table 3 are probably the result of in-
creased uptake of the antiviral compounds due to greater amounts of com-
pound on the leaf surface from 3 applications on the day of inoculation,
abrasion of the leaf surface during inoculation, and the presence of a
spreader-sticker. Although a spreader-sticker may possess detergent
activity causing solubilization of viral protein (Matthews, 1970),
significant reductions in local lesion numbers due to the presence of
spreader-sticker were not observed. Spreader-sticker was not used for
the application of antiviral compounds onto cowpea plants because it
caused primary leaves sprayed with many of the compounds to exhibit
toxic effects possibly resulting from increased compound absorption.
Besides, the absence of leaf hairs on cowpea plants assured better spray
coverage by the antiviral compounds than was possible on the primary
leaves of soybean plants which are covered with leaf hairs.

Although the differences in local lesion numbers were not statis-
tically significant, local lesion numbers on inoculated soybean plants
were consistently greater on plants sprayed with 2-thiouracil (all
treatment regimes) than with any of the other antiviral compounds and
similar to the lesion numbers of control plants (Table 2). Local le-
sions on some of the 2-thiouracil-treated primary leaves were so numer-
ous that they coalesced and gave the appearance of large necrotic areas,
thereby making it difficult to count individual local lesions. Numbers

of individual local lesions on 2-thiouracil-treated plants may there-
fore have been even greater than what was actually counted. On the
other hand, Kuhn (1971) observed significant increases in local lesion
size when CCMV-inoculated soybean plants were sprayed with 2-thiouracil
within 24 hours after inoculation. It is possible that some of the
increases in local lesion size observed by Kuhn (1971) may have been
due to local lesions becoming so numerous that they could no longer
be distinguished from each other and were observed as single large
local lesions. Francki (1962) and Kuhn (1971) observed that 2-
thiouracil affects virus synthesis and not the host response to infec-
tion. Therefore, the results presented here along with the results of
others suggest that 2-thiouracil may affect an early stage of the virus
infection process.

The early effects of ribavirin on the CCMV infection process may
be attributed to the inhibition of general RNA synthesis by ribavirin
through its ability to competitively inhibit an enzyme necessary for
the synthesis of guanine ribonucleotides in virus-infected cells
(Browne, 1979; Byhan, et al., 1978; Goswami, et al., 1979). A slight
but non-significant decrease in viral antigen concentration in the pri-
mary leaves of cowpea plants sprayed with ribavirin according to treat-
ment regime E was detected 2 days after inoculation by ELISA (Table 6);
this again suggests that ribavirin may inhibit an early stage of the
viral infection process or possibly early viral protein synthesis. The
inhibitory effects of ribavirin are thought to occur during the early
synthesis of viral nucleic acid (DeClercq and Luczak, 1975; Simon, et al.,

1973) regardless of whether ribavirin is applied before or immediately after virus inoculation.

The early effects of amantadine on the CCMV infection process may be attributed to the inhibition by amantadine of viral uncoating (Kato and Eggers, 1969) or penetration into the host cell (Grunert, et al., 1965). The slight increase in viral antigen concentration in the primary leaves of cowpea plants sprayed with amantadine according to the treatment regime E and detected by ELISA at 2 days after inoculation (Table 6), if significant, may represent an increase in viral coat protein rather than complete virions. However, amantadine has also been reported to reduce the synthesis of viral polypeptides (Oxford, 1979).

Formycin may inhibit the virus infection process by exhibiting an early effect on viral RNA synthesis when applied immediately after inoculation (Wawrosch and Sarkar, 1975; Huang, et al., 1966). White and Dawson (1979) found that cordycepin, which is an adenosine derivative like formycin, inhibited in vitro formation of CCMV-RNA by CCMV replicases.

Methisazone was reported to be an effective inhibitor of RNA viruses (Woodson and Jolik, 1965; Pearson and Zimmerman, 1969) possibly through its effect on the RNA-dependent RNA polymerase responsible for transcription of the viral genome early in the infection process (Tonew, et al., 1974). The significant reduction of local lesion numbers on soybean plants sprayed with methisazone 3 times on the day of inoculation, when compared to the local lesion numbers on control plants as well as

those on plants receiving the other antiviral treatments, may be parti-
ally due to an elevation of the pH at the leaf surface from repeated
methisazone application (Table 3).

Although the reductions in local lesion numbers on plants sprayed
with methyl benzimidazol-2yl-carbamate prior to or immediately after
inoculation were not significant (Table 2), the results were in accord-
ance with those of Fraser and Whenham (1978) who found that early appli-
cation of methyl benzimidazol-2yl-carbamate onto tobacco plants (up to
30 days before inoculation) was most effective for inhibiting TMV infec-
tivity. It is uncertain whether methyl benzimidazol-2yl-carbamate
affects early viral RNA synthesis (Fraser and Whenham, 1978) or indi-
rectly affects virus synthesis through an early effect on host RNA syn-
thesis (Tamm and Eggers, 1963).

With the exceptions of 2-thiouracil (Tables 4 and 5) and ribavirin
(Table 5), application of the antiviral compounds onto inoculated cowpea
plants failed to significantly affect the infectivity of CCMV within 10
days after inoculation. This suggests that later stages of the viral
infection process occurring within 10 days after inoculation are not
directly affected by these chemicals. The systemic infection in CCMV-
inoculated cowpea plants may have been less sensitive to the effects of
the antiviral compounds than the hypersensitive response of local lesion
formation on CCMV-inoculated soybean plants. Since the systemic infec-
tion process in virus-infected plants occurs over a much longer period
of time than local lesion formation, differences in the infectivity of
CCMV in systemically infected cowpea plants may be more subtle than the

differences in local lesion numbers produced on soybean plants. This is because virus replication in cowpea plants may have a chance to re- cover from the effects of the antiviral compounds within the 10 days after inoculation and then proceed at an almost normal rate. Therefore, the same effects that were induced by the antiviral compounds early in the infection process of soybean plants may also have occurred in inocu- lated cowpea plants treated with the antiviral compounds. However, the effects on systemically infected cowpea plants were not as evident either because of the lesser susceptibility of the systemic viral infec- tion process to the effects of the antiviral compounds or because of recovery of viral replication within 10 days after inoculation. Similarly, expression of systemic symptoms in CCMV-inoculated cowpea plants appeared to be more sensitive to the effects of the antiviral compounds than was CCMV infectivity in inoculated cowpea plants since many of the antiviral compounds suppressed viral symptoms in inoculated cowpea plants while infectious virus was still present.

2-Thiouracil may inhibit or enhance the CCMV infection process de- pending on the time of its application. CCMV infectivity in inoculated cowpea plants sprayed prior to or immediately after inoculation (treat- ment regimes B, C, and D) (Table 4) was slightly but nonsignificantly reduced. The reduction in infectivity of CCMV in inoculated cowpea plants sprayed with 2-thiouracil prior to or immediately after inocula- tion coincides with a decrease in the development of systemic symptoms on plants sprayed with treatment regimes B, C, and D (Fig. 2); this again suggests that 2-thiouracil may inhibit an early stage of the virus

infection process. In contrast, the infectivity of CCMV was significantly increased when inoculated cowpea plants were sprayed with 2-thiouracil after inoculation (treatment regimes E and F) (Tables 4 and 5) and the increase in CCMV infectivity paralleled an increase in the development of systemic symptoms (Fig. 2). Holmes (1955) and Francki (1962) observed that 2-thiouracil exhibited a preventative effect against TMV multiplication in tobacco when applied before or immediately after inoculation. Steele and Black (1967) observed that poliovirus was inhibited by 2-thiouracil early in the infection process due to an inhibition of viral absorption. In contrast, Dawson and Kuhn (1972) observed that when 2-thiouracil was applied daily from inoculation for up to 40 days after inoculation infectivity of CCMV from cowpea plants increased beginning 4 to 5 days after inoculation. Therefore, 2-thiouracil may have two different sites of action: one responsible for virus inhibition early in the infection process and another responsible for virus enhancement which occurs later in the infection process. Both the inhibitory and enhancing effects of 2-thiouracil on virus infectivity can be counteracted by uracil (Dawson and Kuhn, 1972; Bawden and Kassanis, 1954) which suggests that 2-thiouracil probably affects virus replication by interfering with the incorporation of uracil into viral RNA.

The slight reductions in the infectivity of CCMV in inoculated cowpea plants sprayed with all ribavirin treatment regimes evident within 10 days after inoculation (Tables 4 and 5) and the reduction of viral antigen from 4 to 8 days after inoculation (Table 6) coincide

with an almost complete absence of viral symptoms on ribavirin-treated cowpea plants (Fig. 3) and the early inhibitory effects on local lesion formation on inoculated soybean plants (Tables 2 and 3). In addition to the early inhibitory effects of ribavirin on viral RNA synthesis ribavirin may also indirectly affect virus synthesis through possible effects on the resistance of the host (deFazio, et al., 1980). Riba-. virin may induce increased tolerance or host resistance of cowpea plants to CCMV infection since ribavirin-treated plants are almost free of viral symptoms although they still contain infectious CCMV after 10 days from inoculation (Tables 4, 5, and 6). Since a significant reduction in the infectivity of CCMV from trifoliate leaves of cowpea plants sprayed with treatment regime E was observed within 10 days after inoculation (Table 5), CCMV in younger trifoliate leaves may have been more sensitive to the inhibitory effects of ribavirin regardless of whether ribavirin affected early viral RNA synthesis or host resistance to viral infection.

Application of amantadine also did not significantly reduce the infectivity of CCMV in inoculated cowpea plants within 10 days after inoculation. However, the slight reductions in the infectivity of CCMV in plants sprayed with treatment regimes A through E (Tables 4 and 5) and the slight reduction of viral antigen at 8 days after inoculation (Table 6) coincide with an almost complete absence of viral symptoms in cowpea plants sprayed with treatment regimes A through E (Fig. 4). Treatment regime F which did not begin until 1 day after inoculation was not effective in reducing CCMV infectivity (Table 4) which again

suggests, along with the early inhibitory effects on local lesion formation on soybean plants (Tables 2 and 3), that amantadine inhibits virus infectivity early in the infection process (absorption or uncoating) when applied prior to or immediately after inoculation.

Although formycin, methisazone, and methyl benzimidazol-2yl-carbamate failed to significantly reduce the infectivity of CCMV in inoculated cowpea plants within 10 days after inoculation (Tables 4 and 5) formycin prevented the development of viral symptoms when applied according to treatment regimes A through E (Fig. 5) and methisazone prevented the development of viral symptoms when applied according to treatment regimes A through F (Fig. 6). These reductions in symptom development coincide with the reductions in local lesion numbers on inoculated soybean plants (Tables 2 and 3) and may be due to possible early effects of formycin and methisazone on viral RNA synthesis. This again suggests that symptom development of inoculated cowpea plants may be more sensitive to the effects of the antiviral compounds than the infectivity of CCMV in the inoculated cowpea plants or that symptom development could be affected if the antiviral compound increases host resistance to virus infection rather than exhibiting a direct effect on the virus. Methyl benzimidazol-2yl-carbamate, in addition to having little effect on CCMV infectivity in primary leaves of cowpea plants (Table 4), did not affect symptom development on CCMV-inoculated cowpea plants (Fig. 7). Since virus in the younger, upper leaves of the plant may be more sensitive to the effects of methyl benzimidazol-2yl-carbamate (Fraser and Whenham, 1978), it may be necessary to assay the

younger trifoliate leaves of inoculated cowpea plants to detect possible effects of methyl benzimidazol-2yl-carbamate on CCMV infectivity.

None of the antiviral compounds significantly reduced CCMV infectivity when compared to the infectivity of CCMV in inoculated control plants after 15 or more days from inoculation of sprayed cowpea plants suggesting that virus breakdown or stability was not affected by any of the antiviral compounds (Tables 5 and 6). The reduced infectivity of CCMV in all inoculated cowpea plants at 22 days after inoculation when compared to the infectivity of CCMV in inoculated cowpea plants at 10 and 16 days after inoculation and the extreme variability in infectivity of CCMV 22 days after inoculation in all inoculated cowpea plants (Table 5) may be attributed to the fall in virus concentration frequently observed with increasing age of the infected leaves (Matthews, 1970). Therefore, further comparisons of the effects of the antiviral compounds on the infectivity of CCMV at 22 days after inoculation may be meaningless. However, the reduced infectivity of CCMV observed 22 days after inoculation in cowpea plants sprayed with 2-thiouracil treatment regime E when compared to the infectivity of CCMV in control plants and plants treated with all other antiviral treatments (Table 5) may be due to increased breakdown of CCMV since Dawson and Kuhn (1972) observed an increased breakdown of CCMV in 2-thiouracil-treated cowpea plants beginning 14 days after inoculation. The infectivity of CCMV remained considerably greater in amantadine and some methisazone-treated cowpea plants assayed 22 days after inoculation (Table 5) than in control plants or in plants treated with the other antiviral compounds. This suggests

that amantadine and perhaps methisazone may help to prevent virus break-

down and to prolong its infectivity.

No further reductions in the development of symptoms on CCMV-

inoculated cowpea plants treated with any of the antiviral compounds

occurred after the 10th and up to the 42nd day after inoculation. This

indicates that virus stability was not affected by the antiviral com-

pounds after relatively long periods of time from inoculation. Moreover,

the young trifoliate leaves of inoculated cowpea plants sprayed with

2-thiouracil treatment regimes B and D; ribavirin treatment regimes A,

B, C, and D; amantadine treatment regimes E and F; and formycin treat-

ment regime E, which exhibited suppression of symptoms within 10 days

after inoculation, showed increased symptom development 42 days after

inoculation indicating that the antiviral effects are not always perman-

ent.

The toxicity of 2-thiouracil, formycin, and methisazone to the

virus-infected host plant (Fig. 2, 5, and 6) may render them useless as

plant virus chemotherapeutants. It is not likely that methyl

benzimidazol-2yl-carbamate would be useful as a plant virus chemothera-

peutant due to its inability to suppress symptom development on the

virus-infected host plant (Fig. 7). The lack of toxicity of ribavirin

and the presence of only mild toxicity of amantadine to the virus-

infected host plants along with the ability of both antiviral compounds

to suppress viral symptoms (Fig. 3 and 4) suggest that these two chem-

icals may have potential for use as plant virus chemotherapeutants.

Although the local lesion assay generally results in considerable

variability and consistently yields a large standard deviation when lo-
cal lesion numbers are statistically analyzed, it is so far the most
accurate, if not the only, assay for detecting the infectivity of
plant viruses. The eight replications per treatment used here is the
normal number of replications employed in experiments of this type.
However, due to the extreme variability in the system perhaps a consid-
erably greater number of treatment replications would have resulted in a
more normal distribution of the local lesion numbers and would have
lowered the standard deviations among treatment means so that signifi-
cant differences would become apparent (Zar, 1974). For these reasons,
only general trends in the effects of these antiviral compounds on the
infectivity of CCMV can be reported. These trends in infectivity are
also generally supported by visual observations of the reduction in
symptoms caused by the antiviral compounds. Alternative methods of
compound application or more frequent or prolonged applications of these
compounds on the plant might also have made the effects of these anti-
viral compounds on CCMV infectivity more evident. Finally, although
the virus-host system of CCMV in cowpea and soybean plants provided a
rapid means for screening the antiviral compounds, it may not have been
the most suitable system for studying the effectiveness of these chemi-
cals as antiviral agents. It is possible that other viruses with dif-
ferent nucleoprotein composition and replication may be more sensitive
to these compounds than the isometric, single-stranded RNA virion of
CCMV.

C H A P T E R   VI

SUMMARY

Six compounds all previously shown to inhibit certain animal or
other plant viruses, were sprayed on the primary leaves of cowpea and
soybean plants to determine their effects on symptom development on the
virus-infected plant and on the infectivity of the virus, cowpea chloro-
tic mottle virus (CCMV). The compounds sprayed were as follows: 2-
thiouracil, ribavirin, amantadine, formycin, methisazone, and methyl
benzimidazol-2yl-carbamate.

Inoculated cowpea plants sprayed with ribavirin and amantadine
exhibited almost complete suppression of symptom development, but only
slight decreases in CCMV infectivity, and little or no toxic effects.
Inoculated cowpea plants sprayed with 2-thiouracil exhibited somewhat
increased symptom development and increased CCMV infectivity, along with
severe phytotoxicity. Inoculated cowpea plants sprayed with formycin
exhibited a slight reduction in the development of viral symptoms, little
or no decreases in CCMV infectivity, and severe phytotoxicity.
Methisazone suppressed symptom development on inoculated cowpea plants
and caused slight reductions in CCMV infectivity, along with a hormonal-
like phytotoxic effect on the plants. Methyl benzimidazol-2yl-carbamate
had no toxic effects on inoculated cowpea plants but symptom expression
and CCMV infectivity remained comparable to that of the inoculated
control plants.

All six antiviral compounds caused some but statistically non-

significant reductions in local lesion formation on CCMV-inoculated soybean plants. However, when ribavirin, amantadine, formycin, and methisazone were sprayed on soybean plants three times on the day of inoculation, they all reduced local lesion numbers significantly.

Quantitative determination of viral antigen by ELISA showed that ribavirin and amantadine caused slight, but statistically nonsignificant reductions in viral antigen in sap from infected cowpea plants compared to the inoculated control plants.

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