Effect of injected antiviral compounds on apple mosaic and other diseases of apple trees.

EFFECT OF INJECTED ANTIVIRAL COMPOUNDS ON APPLE MOSAIC AND OTHER DISEASES OF APPLE TREES

A Thesis Presented

By

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CHAPTER I

INTRODUCTION

The successful use of systemic fungicides in controlling plant pathogenic fungi as well as the use of antibiotics against bacterial and mycoplasma diseases in plants has led to increased efforts in the search for chemical substances effective against viral diseases in plants. So far, however, these efforts have met with limited success.

Recent medical advances in the chemotherapy of viruses has helped to stimulate research in the agricultural field. Medical breakthroughs include such clinical successes as the prophylactic effectiveness of amantadine hydrochloride against influenza, prevention of smallpox with methisazone (n-methyl isatin B-thiosemicarbazone), the use of ara-A (adenosine arabinoside) against herpes simplex virus, and the action of ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) in combatting influenza.

Work of virus inhibition in plants by chemical methods has so far been carried out by spraying antiviral compounds on virus-infected hosts. However, several tree diseases caused by cellular pathogens can be controlled quite effectively with chemicals that are distributed through the plant systemically. For example, tetracyclines have been used successfully against certain mycoplasma and bacterial diseases. In addition, the systemic benzimidazole fungicides are commonly used against fungal disorders such as Dutch elm disease.

The development of systemic chemicals effective against plant diseases has made pressure injection a very attractive method of applying these chemicals to affected trees. Benomyl injections are the current method of control in trees affected by Dutch elm disease. Likewise, mycoplasma diseases can be controlled through a single tetracycline injection. The fact that injections under pressure can result in translocation of certain chemicals through the plant via its transpiration stream, makes the possibility of controlling viral diseases in trees with antiviral compounds an appealing approach to the problem.

Viruses are known to reduce growth and depress yields in fruit trees and to affect the overall quality of the fruit. Virus diseases in orchards are controlled today through the use of virus-free stock. However, virus infections often become apparent in previously healthy-looking, established orchard trees due to the presence of latent viruses in the nursery stock or by natural infections in the orchard. When this occurs, the only control measure available is to remove the infected trees, which can often be an expensive procedure. Consequently, there is a need to develop practical and effective alternatives to the present method of controlling virus diseases in deciduous fruit trees.

Apple mosaic is a viral disease of apples which causes striking symptoms on the foliage. Apple mosaic is used in these investigations as a model disease in the determination of the effect of certain antiviral compounds applied by injection on the expression of foliar symptoms caused by a plant virus.

Scar skin and dapple apple diseases are apple disorders presumed to be of viral etiology primarily because of symptomatology and because the diseases are transmissible only through grafting. However, no virus or other pathogen has ever been observed in association with the diseased trees. Therefore, the effects of known antiviral compounds upon the development and expression of fruit symptoms in scar skin and dapple apple-infected apple trees were studied in order to provide additional information concerning the nature of these two tree disorders, and, if they are of viral etiology, to determine the effect of the antiviral compounds on the expression of the fruit symptoms caused by these disorders.

CHAPTER II

LITERATURE REVIEW

There has been relatively little work done to date in controlling plant viruses through chemical means. Several extensive reviews deal with the successes of systemic fungicides and of antibiotics for controlling certain fungal, bacterial, and mycoplasma plant infections (Bove and Duplan, 1974; Erwin, 1973; Lewis and Hickey, 1972; Marsh, 1977). Likewise, research concerned with the chemotherapy of animal viruses has progressed much further and at a much more rapid rate than its counterpart in plant virology (Carter, 1975; Galasso et al, 1979; Herrman, 1979).

One of the earliest synthetic compounds known to exhibit significant antiviral activity against plant viruses is the nucleoside analogue 2-thiouracil. This compound was shown to inhibit the synthesis of tobacco mosaic virus (TMV) in infected tobacco leaf discs (Commoner and Mercer, 1951) and to delay the spread of systemic infections when it was sprayed onto tobacco plants infected with TMV (Bawden and Kassanis, 1954). However, when cowpea chlorotic mottle virus (CCMV)-infected cowpea plants were sprayed daily with 2-thiouracil infectivity and synthesis of the virus were enhanced (Dawson and Kuhn, 1972).

The exact mechanism responsible for the virus inhibiting and/or enhancing effects of 2-thiouracil still remains obscure. It was initially thought that the inhibitory action of 2-thiouracil was due to its incorporation into the viral RNA as 2-thiouridylic acid. This incorporation of 2-thiouracil into viral RNA resulted in a 10% replacement of the RNA base uracil (Jeener and Rosseels, 1953). 2-Thiouracil inhibited turnip yellow mosaic virus (TYMV) multiplication to the same extent as it inhibited the multiplacation of TMV, however, the compound was not incorporated into the TYMV RNA (Francki and Matthews, 1962). Subsequent work with TYMV showed virus synthesis to be inhibited and the synthesis of empty viral protein shells to be increased in infected cabbage leaf discs treated with 2-thiouracil. These results were used to support the suggestion that 2-thiouracil affects virus synthesis by inhibiting the biosynthesis of uridylic acid (Ralph, 1976).

The mechanism by which 2-thiouracil enhances virus infectivity appears to be unrelated to that which causes inhibition. Whereas the inhibitory effects of the compound are immediate, indicating little dalay in cellular incorporation of the compound, the signs of enhanced infectivity are delayed for about five days after initiation of treatment (Dawson and Kuhn, 1972).

Unfortunately, 2-thiouracil holds little promise of becoming a practical chemotherapeutant for plant virus control due to its severe phytotoxicity (Bawden and Kassanis, 1954; Francki, 1962).

Other synthetic base analogues are also known to have inhibitory effects against plant virus multiplication. These include such compounds as 8-azaguanine and 5-fluorouracil. 8-azaguanine has been reported to inhibit a number of plant viruses in a variety of hosts. It significantly reduced virus concentration and delayed the systemic spread of alfalfa mosaic virus in tobacco plants when administered around the time of virus inoculation. The analogue was not effective if applied after systemic movement of the virus had begun (Matthews, 1955). TMV-infected Physalis floridana plants treated with 8-azaguanine resulted in the production of separate fractions of TMV, one with high infectivity and one with low infectivity (Lindner et al, 1960). TMV RNA, after exposure to 5-fluorouracil, was shown to be more sensitive to inactivation by ultraviolet light than untreated viral RNA (Lozeron and Gordon, 1964).

MBC (methyl-benzimidazol-2yl-carbamate), the water decomposition product of the widely used systemic fungicide benomyl, has also exhibited antiviral properties.

Soil drenches of MBC, supplied as an aqueous suspension of Bavistin, suppressed the expression of virus symptoms in TMV infected tobacco plants and also in lettuce plants infected with beet western yellows virus (Tomlinson et al, 1976, Fraser and Whenham, 1976). Benomyl sprays have been reported to decrease mosaic symptoms on the foliage of apple mosaic virus-infected "Jonathan" apple trees (Minoiu, 1976). On the other hand, MBC had no effect upon the severity of virus symptoms in tobacco and cucumber plants infected with lettuce mosaic virus (Tomlinson, 1977). Also, when TMV infected tomato leaf discs and cucumber mosaic virus (CMV) infected cucumber cotyledon discs were floated on solutions of benomyl, the virus content within the leaf discs was increased. However, soil drenches of Benlate, a benomyl fungicide, decreased the amount of virus in CMV infected seedlings (Bailiss et al, 1977). Benomyl had no effect upon lettuce big vein agent (LBVA) in infected lettuce plants. This disease is believed to be of a viral nature. However, benomyl did kill the fungal vector of this disease, _Olpidium brassicae_, which was present within the root cells of the lettuce plants (Campbell, 1980).

Although the exact mechanism of action of MBC as an antiviral compound is still obscure, it has been postulated that its symptom-suppressing effects may be due to cytokinin-like properties, specifically an ability to prevent the destruction of the host plant chloroplasts (Tomlinson, 1976, 1977). Additional research has demonstrated the ability of MBC to inhibit viral RNA accumulation in leaf cells and this had also been attributed to the cytokinin, anti-senescent properties of the compound. It was suggested that MBC may inhibit the virus by maintaining the host cell in a condition unfavorable to multiplication of the virus (Fraser and Whenham, 1978).

One of the most promising and more recent antiviral compounds is the synthetic nucleoside analogue ribavirin (1,B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide). It has a broad spectrum of activity against both DNA and RNA viruses, making it an extremely appealing addition into the area of virus chemotherapy (Sidwell, 1977). Due to its apparently selective antiviral action, ribavirin has received considerable attention as a potential virus chemotherapeutant. Consequently, it is one of the more extensively researched antiviral compounds (Sidwell et al, 1979). In fact, it has already been used in successful clinical trials against influenza virus (Salido-Rengell et al, 1977; Zertuche, 1977).

Considerably less research has been done concerning the effectiveness of this compound against plant viruses.

However, much of the work that has been done shows ribavirin to be a very effective inhibitor of plant virus multiplication.

Ribavirin reduced virus concentration when it was sprayed onto tomato plants infected with tomato white necrosis virus. In addition, only 30% of the ribavirin-treated plants showed virus symptoms whereas, symptom expression was 100% in the group of control plants not treated with ribavirin (DeFazio, 1978). Ribavirin was also effective in inhibiting the multiplication of apple chlorotic leaf spot virus in *Chenopodium quinoa* plants when applied as either a soil drench or foliar spray. Rapid absorption and good translocation of the compound by roots and foliage of *C. quinoa* was reported as well as residual effectiveness of the compound in the soil and plants for at least four days (Hansen, 1979). Furthermore, when potato virus X infected tobacco plants were supplied with ribavirin through their roots, the plants remained symptomless, while untreated control plants exhibited good mosaic symptoms. There also appeared to be a delay in the systemic spread of the virus through the host based on the observation that virus concentration decreased as distance from the inoculated leaves was increased (Lerch. 1977).

Rose ring pattern, a component of the rose mosaic disease complex, is believed to be of viral etiology due to observed symptoms, graft transmissibility, inactivation by heat, and lack of association with microorganisms. Gravity-flow injections of ribavirin into rose ring pattern infected Burr"multiflora" plants resulted in symptom remission and virorous growth of the new symptomless shoots. The ribavirin-treated plants were symptomless whereas the control plants produced typical disease symptoms. However, ribavirin appeared to have virustatic as opposed to viricidal effects since indexing of symptomless shoots from ribavirin-treated plants onto symptomless plants, resulted in transmission of the causal agent and subsequent development of rose ring pattern symptoms (Secor and Nyland, 1978).

Foliar sprays of ribavirin at concentrations which were phytotoxic resulted in significant reductions in potato virus X (PVX) concentration in infected tobacco plants (Schuster, 1976). Typical signs of ribavirin photoxicity included a narrowing of the leaf blade and chlorotic spotting of the leaves. However, when abscissic acid was used in combination with ribavirin, phytodamage was reduced. There was a widening of the leaf and an increased dark green leaf colour of tobacco plants treated with both ribavirin and abscissic acid. In addition, the reduction in

PVX concentration of ribavirin-treated tobacco plants was significantly greater than the reduction in virus concentration seen in plants treated with ribavirin alone. This synergistic antiviral effect was also produced by combined treatments of ribavirin with other plant hormones namely IAA (B-indolyle acetic acid), kinetin, ethylene, and gibberellic acid (Schuster, 1979).

Big vein agent-infected lettuce seedlings treated with ribavirin administered as bi-weekly soil drenches, showed significant reductions in the severity of foliar symptoms. There was also a decrease in LBVA titer in the _Olpidium_ _brassicae_ vector found within the lettuce roots though ribavirin had no obvious effect upon the fungal vector itself. However, ribavirin did not appear to eradicate the virus from the lettuce plants since ten weeks after the final ribavirin treatment, normal big vein symptoms began to appear in many of the treated plants (Campbell, 1980).

Ribavirin sprays effectively suppressed symptom development in cowpea plants systemically infected with cowpea chlorotic mottle virus (CCMV), however, virus infectivity was not significantly reduced. In addition, foliar sprays of ribavirin significantly reduced local lesion formation on CCMV infected soybean plants when plants were sprayed with the chemical three times on the day of inoculation

(Cassel, 1981).

The mode of action of ribavirin in animal virus systems has been studied extensively. Ribavirin is not viricidal. It does not induce production of interferon and it does not affect the virus at the sites of attachment to or penetration into the host cell. Initial studies demonstrated that the molecular basis for ribavirin's antiviral activity came from an intracellular metabolite of the drug, ribavirin-5'-monophosphate, which is a competitive inhibitor of inosine monophosphate dehydrogenase, an enzyme essential in the guanosine biosynthetic pathway. However, subsequent studies now support the possibility of an additional specific antiviral effect of ribavirin. These studies demonstrate that inhibition of influenza virus multiplication depends upon the selective inhibition of RNA polymerase by ribavirin-5'monophosphate (Sidwell et al, 1979).

The antibiotic formycin B is another nucleoside analogue that has demonstrated antiviral activity against plant viruses. Foliar sprays of formycin B resulted in a reduction in CCMV symptom development in infected cowpea plants however, the compound had no effect upon virus infectivity (Cassel, 1981). Formycin B also inhibited TMV synthesis in detached tobacco leaves when it was administered immediately after inoculation, suggesting that the compound affects some early stage of TMV multiplication (Wawrosch and Sarkar, 1974). It is believed that formycin B inhibition is selective for the synthesis of low molecular weight RNAs and has no effect upon the high molecular weight nucleic acids of the host (Abelson and Penman, 1979).

Tetracycline antibiotics are known to be effective inhibitors of many bacterial and mycoplasma related plant diseases. However, there have been limited reports of their possible antiviral activity. Tetracycline and oxytetracycline sprays were said to reduce foliar symptoms in chlorotic leaf spot virus infected apple trees and sharka virus infected plum trees. In addition, soil treatments with tetracyclines inhibited sharka virus symptoms in plum trees as well as the symptoms of chlorotic leaf spot virus and rubbery wood mycoplasma in apple trees (Minoiu, 1978).

Gravity-flow injections of dimethyl sulfoxide into three year old peach trees infected with either peach mosaic virus or necrotic ringspot virus, suppressed development of virus symptoms for one year. These injections were effective when carried out between bud swell and full leaf but ineffective when done during dormancy. A similar series of injections using dimethyl sulfoxide as a solvent for 2-thiouracil, 8-azaguanine, and benzimidazole also resulted in suppression of peach mosaic and necrotic ringspot virus symptoms on infected peach trees (Pine, 1964).

Some of the compounds shown to be effective against plant virus diseases were initially developed for use against animal viruses. However, quite a few of the better known antiviral drugs are just beginning to be considered as possible plant virus inhibitors. Included among these compounds are methisazone (1-methylisatin-B-thiosemicarbazone) and amantadine hydrochloride (1-adamantanamine HCl).

Methisazone is one of a group of synthetic compounds known as the thiosemicarbazones which are active against a wide spectrum of DNA and RNA animal viruses (Bauer, 1972). In clinical research this compound has been therapeutically effective against vaccinia virus (McClean, 1977). It has also been approved for limited clinical use against smallpox virus as a prophylactic agent (Bauer, 1965; Maugh, 1976). Initial research with plant viruses has shown that methisazone suppresses symptom development in cowpea plants infected with CCMV (Cassel, 1981).

Although its mode of action is still not completely understood, methisazone along with the other thiosemicarbazones are believed to affect the late viral messenger RNA in a way that inhibits the formation of certain viral proteins (Magee and Bach, 1965). Investigations with vaccina virus has shown that methisazone brings about the dissolution of the polyribosome late messenger RNA complex (Woodson and Joklik, 1965).

Amantadine HCl and the other adamantanamines collectively exhibit a wide spectrum of antiviral activity. Amantadine HCl has been proven to be prophylactically effective against influenza A virus. The compound effectively suppressed CCMV symptom development in inoculated cowpea plants but had only a slight inhibitory effect upon virus infectivity. There are at least two proposed explanations for the antiviral activity of this compound. Many of the mode of action studies report that amantadine HCl blocks virus penetration into the host cell (Cochran et al, 1965). However, amantadine HCl was also shown to inhibit uncoating of the virus particle in the cell (Kato and Eggers, 1967).

The effectiveness with which a certain agricultural chemical will combat a certain plant disease largely depends upon the method of application. Foliar sprays of many systemic fungicides and antibiotics have been shown to offer successful control of various fungal and bacterial diseases. However, the efficiency and economics of control have occasionally been improved by injection techniques. Foliar sprays of oxytetracycline to control bacterial spot caused by Xanthamonas pruni on infected apricot and peach trees, although successful, were too expensive to be considered as a practical control possibility. Satisfactory control of X. pruni with oxytetracycline through foliar spraying was computed to require eight to ten times more antibiotic per tree than the amount required for successful control via trunk injections (Keil, 1979; Keil and Civerolo, 1979).

Foliar sprays of oxytetracycline solutions did not control X-disease symptoms on infected peach trees whereas trunk injections of oxytetracycline during autumn give symptom remission for one year (Rosenberger and Jones, 1977).

Foliar sprays and soil drenches of oxytetracycline solutions were ineffective in suppressing expression of symptoms of the lethal yellowing disease on coconut palms. Trunk injections and petiole injections of oxytetracycline, however, resulted in acceptable levels of symptom remission in coconut palms infected with the mycoplasma-like-organism (McCoy, 1976; 1977).

Probably the most extensive investigation of trunk injection techniques has come from research involving Dutch elm disease. Therapeutic and protective results with pressure injection of carbendazim solutions into infected elm trees have done much to support and promote the entire idea of systemic injection (Smalley, 1977).

Unfortunately, systemic injections are subject to several significant disadvantages: Injections require the formation of one or more sizeable wounds. Tree growth and physiology limit effective applications to certain months of the year. Injected systemic chemicals rely on the transpiration stream of the xylem for their distribution. This can result in movement of a compound through the xylem tissue so rapidly that it becomes ineffective. Injections quite often result in the erratic distribution of a chemical through the tree, especially in the terminal twigs.

Trunk injections of MBC into pear trees during autumn resulted in rapid distribution of the fungicide before leaf drop. The following spring, MBC was easily taken up by the emerging leaves. There was a lag period between injection and accumulation of the fungicide in newly emerging leaves, however, when MBC injections were performed in the spring. MBC injections into pear trees during dormancy resulted in poor distribution of the compound (Shabi et al, 1974).

Pressure injection of thiabendazole into apple trees resulted in the accumulation of the compound in and around the point of application. It was believed that the fungicide precipitated there due to a rise in pH of the fungicide formulation from 3.4 to 6.7 (Pinkas et al, 1973).

September injections of oxytetracycline were suggested to be the most desirable for providing remission of X-disease symptoms in peach trees. October and November treatments produced toxic levels of oxytetracycline in newly emerging leaves the following spring. Two possible reasons were offered to explain the toxicity of these late autumn treatments. Tetracyclines are inhibitors of protein synthesis. Therefore, it is possible that tetracyclines injected into the trees during late autumn are stored in the tree and move into new growth the following spring at concentrations harmful to the synthesis and development of young leaves. It is also proposed that late autumn treatments performed after leaf drop may result in accumulation of the oxytetracycline in dormant buds at concentrations that damage the proplastids (Rosenberger and Jones, 1977).

Benzimidazole fungicides are used routinely in many orchards to control various fungal diseases on fruit and foliage. Pear scab caused by the fungus Venturia pyrina is effectively controlled by foliar sprays of benzimidazole fungicides. Pressure injections of carbendazim HCl into pear trees during autumn failed to provide adequate control of pear scab the following summer. Control of the disease requires that the young leaves and fruit be protected from fungal infection early in the spring. It was shown that the concentrations of carbendazim present throughout the tree as a result of pressure injection, were insufficient in controlling the fungus (Shabi et al, 1979).

The rapid vegetative propagation and extensive distribution of plant material by apple tree nurserymen and growers have increased the importance of viruses which infect apple trees. There are presently no practical treatments for curing virus-infected trees once they are set out in an orchard. The production and distribution of virus-free propagating material is the primary means of controlling apple virus diseases. Propagation of virus-free meristems and heat treatments are the major techniques in use today, for obtaining virus-free material from virus-infected plants (Hollings, 1965).

Apple mosaic virus can cause striking mosaic symptoms on the foliage of susceptible infected apple trees. Symptoms tend to be most noticeable on leaves that emerge in the spring and early summer, whereas leaves that develop during periods of very high temperatures quite often are free of visible symptoms. There has been no vector associated with transmission of the virus and its spread in commercial orchards is very slow. Root grafts have been reported as the cause of natural spread of the virus among nursery stock. Oftentimes, apple mosaic virus does not become fully systemic in the tree and virus-free material can be obtained through the propagation of these actively growing shoot tips (Posnette, 1963).

Scar skin is an apple disorder which, at present, is presumed to be of a viral nature due to symptomatology, graft transmission, and lack of association with other microorganisms. However, no virus has yet been observed in the infected trees. Scar skin produces symptoms only on the fruit of infected apple trees. Symptom expression begins with numerous, tiny water-soaked blotches around the stem end with subsequent scar tissue development which radiates out and down the sides of the fruit. By harvest time, fifty percent of the fruit surface may be covered with this corky, scarred tissue. The fruit remain small and ripening is noticeably delayed (Millikan, 1963).

Dapple apple also produces symptoms only on the fruit of infected trees. Spotting of the fruit first becomes evident around mid-July as small pale circles form near the calyx end. The spots tend to expand and coalesce as the season progresses. The spots remain yellowish-green as the apple develops a red colour and consequently, at fruit maturity the dappled effect becomes very pronounced (McCrum, 1963).

CHAPTER III

MATERIALS AND METHODS

The viruses used in this research were obtained from infected apple trees growing in the University Fruit Research orchard in Belchertown, Massachusetts. Apple mosaic virus was transmitted from infected MacIntosh apple trees, scar skin disease was transmitted from infected Red Delicious apple trees, and dapple apple disease was transmitted from infected Hyslop crab apple trees, all of which were growing on East Malling VII rootstocks.

Trees used in the field experiments were growing in the University orchard and consisted of eight-year-old MacIntosh, Red Delicious, and Hyslop crab apple trees grafted on East Malling VII rootstocks. MacIntosh trees were bud-inoculated with apple mosaic virus in the summer of 1979. At the same time, Red Delicious trees were inoculated with scar skin disease and Hyslop crab apple trees were inoculated with dapple apple disease. Field experiments also included three-year-old MacIntosh trees grafted on East Malling IX rootstocks.

For greenhouse experiments, scion wood of Golden Delicious was grafted onto seedling rootstocks and grown in 15 cm pots. Scion wood was obtained from healthy apple trees growing in the University orchard.

The synthetic antiviral compounds used in this research included ribavirin (Virazole, ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio), 2-thiouracil (Sigma Chemical Co., St. Louis, MO), formycin B (Sigma Chemical Co., St. Louis, MO), oxytetracycline hydrochloride (Terramycin, Tree Injection Formula, Pfizer, Chemical Division, New York, N.Y.), amantadine hydrochloride (Pfaltz and Bauer, Inc., Stamford, Conn), and methisazone (Marboran, Burroughs Wellcome Co., Greenville, N.C.).

Field Experiments

Treatments were carried out on individual branches of virus-infected eight-year old apple trees. Each branch had an approximate diameter of 4 cm and was 150 cm in length. Injection holes were drilled with a 3mm drill bit to a depth of 15mm. Two holes were drilled per branch with the lowermost hole about 10 cm from the main trunk and the second hole about 3 cm above and opposite the first hole. 1 ml of an aqueous chemical solution per injection hole was pressure-injected into each branch using the injection apparatus developed by Sterrett and Creager (1977) Each branch, therefore, received two injections for a total of 2 ml of chemical solution with a one day interval between the two injections. Preliminary injections to help determine dosage, concentration and volume of antiviral solutions plus size of treated branches for optimum results were done in June, 1979.

Injections were carried out according to the schedule outlined below. From October 20 to 25, 1979, branches of apple mosaic-infected MacIntosh trees scar skin-infected Red Delicious trees and dapple apple-infected Hyslop Crab apple trees were injected with 2 ml aqueous solutions of either ribavirin or 2-thiouracil at concentrations of 4.0, 16.0, and 24.0 mg/ml.

From May 15 to 20, 1980 branches of apple mosaic-infected MacIntosh trees and scar skin-infected Red Delicious trees were injected with 2 ml aqueous solutions of ribavirin at 4.0, 16.0, and 24.0 mg/ml, 2-thiouracil at 4.0, 16.0, and 24.0 mg/ml, formycin B at 0.5, 2.5, and 5.0 mg/ml, amantadine HCl at 5.0, 12.5, and 25 mg/ml, and methisazone at 1.0, 2.0, and 5.0 mg/ml.

From October 30 to November 2, 1980 branches of apple mosaic-infected MacIntosh apple trees and scar skin-infected Red Delicious apple trees were injected with 2 ml aqueous solutions of ribavirin at 8.0 and 12.0 mg/ml, or formycin B at 0.5 and 1.0 mg/ml, or terramycin at 12.0 and 20.0 mg/ml. There were six replications per treatment.

From May 5 to 10, 1981 branches of apple mosaic-infected MacIntosh apple trees and scar skin-infected Red Delicious apple trees were injected with 2 ml aqueous solutions of ribavirin at 8.0 and 12.0 ml/mg, formycin B at 0.5 and 1.0 mg/ml, or terramycin at 12.0 and 20.0 mg/ml.

Control treatments for the above experiments consisted of inoculated but uninjected branches. Periodic observations of individual treatments were made from May through August. In August, leaves from apple mosaic infected branches were collected, counted, and the degree of symptom severity was visually rated. Fruit from scar skin-infected branches were collected in September, counted and visually rated for symptom severity.

Symptom ratings for foliage and fruit were determined as follows. Virus symptoms covering less than 25% of the leaf or fruit surface were rated as light and given a numerical value of one. Virus symptoms covering less than 50% of the leaf or fruit surface were rated as moderate and given a numerical value of two. Virus symptoms covering over 50% of the leaf or fruit surface were rated as severe and given a numerical value of three. For each treatment, a disease severity index was calculated by multiplying the percentage of leaves or fruit with light, moderate, and severe symptoms times their respective numerical value, adding them up and then dividing by 100.

In July 1979, another set of field experiments were carried out on two-year old healthy MacIntosh trees. Each tree was injected with 2 ml of aqueous solutions of ribavirin at 4 mg/ml, 2-thiouracil at 4.0 mg/ml, or terramycin at 5.0 mg/ml. One week later, the trees were inoculated with two apple mosaic virus-infected buds per tree. The trees were observed for subsequent development of virus symptoms and phytotoxicity during the following two growing seasons.

Greenhouse Experiments

Young Golden Delicious grafts growing in 15cm plastic pots were each inoculated with two apple mosaic virus-infected buds. After virus symptoms began to appear in the leaves, the foliage was cut back and one week later the rootstocks were injected with the antiviral compound. One hole per tree was drilled with a 1 mm drill bit to a depth of 6mm about 2.5 cm above the soil line. The trees were pressure-injected with 0.5 ml of aqueous solutions of the antiviral compounds using the pressure injection apparatus mentioned previously (Sterrett and Creager, 1979). The chemical treatments consisted of aqueous solutions of ribavirin at 0.2, 0.3, and 0.5 mg/tree, terramycin at 0.3, 0.5, and 1.0 mg/tree, and formycin B at 0.005, 0.01, and 0.05 mg/tree. Control treatments consisted of inoculated, uninjected trees. Trees were observed on a weekly basis over a four month period for symptom development any phytotoxicity.

In a separate set of similar injections, Golden Delicious grafts injected in October 1980 with ribavirin at 0.1, 0.3, and 0.5 mg/tree, formycin B at 0.005, 0.01, and 0.05 mg/tree and terramycin at 0.3, 0.5, and 1.0 mg/tree were overwintered and observed for symptom development and phytotoxicity the following spring and summer.

Indexing

Apple mosaic virus-infected branches from orchard trees and apple mosaic virus-infected Golden Delicious grafts injected with ribavirin and showing no virus symptoms in the summer of 1981, were indexed onto healthy Golden Delicious grafts in the greenhouse. Three grafts with two buds per graft were indexed per treated branch or young tree.

Bioassay

An attempt was made to assay for the presence of ribavirin in the leaf and bark tissue of injected trees. Ribavirin was reported to exhibit moderate inhibitory activity against a limited number of bacteria. One of the most ribavirin-sensitive bacteria listed was Pseudomonas aeruginosa, which was selected for use in this bioassay (Sidwell et al, 1979). The bacteria were overlaid onto nutrient agar plates (0.1 ml P. aeruginosa/3 ml of 0.5% soft agar overlay) and allowed to dry for one hour before filter discs impregnated with the test compounds were applied. Aqueous solutions of ribavirin at various concentrations were used to test the sensitivity of the P. aeruginosa culture to ribavirin. Assay paper discs impregnated with ribavirin at 8.0, 4.0, 2.0, 1.0, 0.5, 0.1, 0.05, 0.01, and 0.005 mg/disc were plated, one disc per plate, onto petri dishes inoculated one hour earlier with P. aeruginosa.

Golden Delicious grafts were injected with 0.5, 0.3, or 0.1 mg of ribavirin. At one, three, and five hours following injection, trees were cut with pruning shears and cross-sections of the stem were plated onto the petri dishes in which the bacteria had been inoculated one hour before. The cross-sections were taken at 5mm and 10mm above the injection site, and at the base of the current season's growth. Control plates consisted of stem cross-sections from uninjected grafts. Leaves of injected Golden Delicious grafts were bioassayed twenty-four hours after injection. Four leaves were randomly selected from each tree, ground, and filtered leaf sap was collected from each set of leaves. Sterile assay paper discs were soaked in distilled water and leaf sap from uninjected trees. All assay plates were incubated for eighteen to twenty hours at $28^{\circ}C$.

CHAPTER IV

RESULTS

Results from Autumn 1979 and Spring 1980 Injections of Antiviral Compounds on Apple Mosaic, Scar Skin, and Dapple Apple Symptoms

Many of the MacIntosh trees inoculated with apple mosaic virus in the summer of 1979 and injected with antiviral compounds in autumn 1979 and spring 1980, showed few, poorly distributed mosaic symptoms on the foliage by the summer of 1980. The leaves on a number of tree branches, including some inoculated but uninjected control branches, were completely free of apparent virus symptoms. A similar situation occurred in Red Delicious trees inoculated with scar skin disease during the summer of 1979 and in Hyslop crab apple trees inoculated with dapple apple disease during the summer of 1979, where many tree branches showed no apparent virus symptoms on the fruit in the summer of 1980. The data from these series of experiments are presented here (Tables 1,2,3,4, and 5) only to illustrate the situation and, perhaps, to provide support for experiments performed subsequently, but no attempt is made to draw any conclusions solely from the results presented in these tables.

TABLE 1

Effect of Autumn Injections (1979) of Antiviral Compounds into Apple Mosaic Infected MacIntosh Branches on Foliar Symptom Frequency and Severity

Treatment		Number of branches			Percent of leaves with symptoms per branch[a,b]		Disease Severity Index[c,d]	
Chemical	Amount (mg/branch)	Treated	With symptoms					
		1	1980	1981	1980	1981	1980	1981
Ribavirin	8 mg	6	2	2	10	10	0.10	0.30
	16 mg	6	3	3	6	11	0.11	0.17
	24 mg	6	4	4	10	32	0.11	0.41
2-Thiouracil	8 mg	6	6	6	28	71	0.38	1.01
	16 mg	6	2	4	11	36	0.13	0.53
	24 mg	6	4	4	16	32	0.18	0.48
Control		6	2	4	5	37	0.07	0.51

[a] Each number is the average of 6 replications.

[b] Percent of leaves with symptoms = number of leaves with symptoms per total number of leaves on branch.

[c] Disease severity index = (% leaves with light symptoms x 1) + (% leaves with moderate symptoms x 2) + (percent leaves with severe symptoms x 3) ÷ 100.

TABLE 2

Effect of Spring Injections (1980) of Antiviral Compounds into Apple Mosaic Infected MacIntosh Branches on Foliar Symptom Frequency and Severity

Treatment		Number of branches			Percent of leaves with symptoms per branch[a,b]		Disease severity index[a,c]	
		Treated	With symptoms					
Chemical	Amount (mg/branch)		1980	1981	1980	1981	1980	1981
Ribavirin	8 mg	4	3	4	24	25	0.28	0.29
	16 mg	4	4	4	11	12	0.14	0.16
	24 mg	4	2	2	22	6	0.29	0.07
2-Thiouracil	8 mg	4	2	4	23	56	0.24	0.79
	16 mg	4	0	4	0	47	0.00	0.70
	24 mg	4	3	4	11	57	0.12	0.91
Formycin B	1 mg	4	4	4	44	65	0.55	0.83
	2 mg	3	3	3	37	56	0.45	0.66
	5 mg[d]	4	-	-	--	--	----	----
Amantadine HCl	10 mg	4	3	-	22	--	0.25	----
	25 mg	4	2	-	50	--	0.53	----
	50 mg	4	2	-	13	--	0.15	----
Methisazone	5 mg	3	3	-	26	--	0.29	----
	10 mg	3	3	-	60	--	0.65	----
	25 mg	3	3	-	68	--	0.75	----
Control		4	4	4	30	38	0.35	0.32

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[a] Each number is the average of 4 replications except the Formycin B 2 mg treatment and all Methisazone treatments which are an average of 3 replications.

[b] Percent of leaves with symptoms = number of leaves with symptoms per total number of branch leaves.

[c] Disease severity index = (% leaves with light symptoms x 1) + (% leaves with moderate symptoms x 2) + (% leaves with severe symptoms x 3) ÷ 100.

[d] All 4 branches were dead in August 1980.

TABLE 3

Effect of Autumn Injections (1979) of Antiviral Compounds on Fruit Symptom Expression in Scar Skin Infected Red Delicious Branches

Treatment		Percent of fruit with symptoms per branch[a,b]		Disease Severity Index[b,c]	
Chemical	Amount (mg/branch)	1980	1981	1980	1981
Ribavirin	8 mg	80	100	0.82	1.84
	16 mg	79	97	0.95	1.34
	24 mg	49	79	0.49	1.16
2-Thiouracil	8 mg	87	100	1.00	1.99
	16 mg	69	100	0.99	1.09
	24 mg	52	100	0.52	1.66
Control		61	197	0.87	1.77

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[a] Each number is the average of 6 replications.

[b] Percent of fruit with symptoms = number of fruit with symptoms per total number of fruit on branch.

[c] Disease severity index = (% fruit with light symptoms x 1) + (% fruit with moderate symptoms x 2) + (% fruit with severe symptoms x 3) ÷ 100.

TABLE 4

Effects of Spring Injections (1980) of Antiviral Compounds on Fruit Symptom Expression in Scar Skin Infected Red Delicious Branches

Treatment		Percent of fruit with symptoms per branch[a,b]		Disease Severity Index[a,c]	
Chemical	Amount (mg/branch)	1980	1981	1980	1981
Ribavirin	8 mg	100	100	1.17	2.26
	16 mg	100	100	1.05	1.44
	24 mg	58	100	0.58	2.21
2-Thiouracil	8 mg	52	100	0.52	1.62
	16 mg	28	87	0.28	1.55
	24 mg	0	100	0.00	1.83
Formycin B	1 mg	49	100	0.82	1.00
	2 mg	0	100	0.00	1.50
	5 mg	50	100	0.66	1.00
Amantadine HCl	10 mg	50	---	0.50	----
	25 mg	22	---	0.22	----
	50 mg	72	---	0.74	----
Methisazone	5 mg	75	---	0.37	----
	10 mg	25	---	1.25	----
	25 mg	21	---	0.21	----
Control		50	100	0.50	2.27

[a] Each number is the average of 4 replications.

[b] Percent of fruit with symptoms = number of fruir with sumptoms per total number of fruit.

[c] Disease severity index = (% fruit with light symptoms x 1) + (% fruit with moderate symptoms x 2) + (% fruit with severe symptoms x 3) ÷ 100.

TABLE 5

Effect of Autumn Injections (1979) of Antiviral Compounds in Fruit Symptom Expression in Dapple Apple Infected Hyslop Crab Apple Branches

Treatment		Percent of fruit with symptoms per branch[a,b]	Disease severity index[b,c]
Chemical	Amount (mg/branch)	1980	1981
Ribavirin	8 mg	93	1.61
	16 mg	80	1.47
	24 mg	85	1.09
2-Thiouracil	8 mg	100	1.88
	16 mg	100	1.92
	24 mg	100	1.65
Control		86	1.51

[a] Each number is the average of 6 replications.

[b] Percent of fruit with symptoms = number of fruit with symptoms per total number of fruit on branch.

[c] Disease severity index = (% fruit with light symptoms x 1) + (% fruit with moderate symptoms x 2) + (% fruit with severe symptoms x 3) ÷ 100.

It appears that ribavirin may have some inhibitory effect upon foliar symptom development. In comparison with 2-thiouracil treatments, the subsequent increase in percent of leaves with foliar symptoms and disease severity two years after chemical injection (August 1981) was less in ribavirin treatments than in 2-thiouracil and control treatments (Tables 1 and 2). In fact, spring 1980 injections of ribavirin at 24 mg per branch resulted in a noticeable decrease in foliar symptoms and symptom severity in 1981 (Table 2).

The percent of leaves with symptoms on branches injected with amantadine HCl were comparable to the levels of symptoms on 2-thiouracil and ribavirin treated branches during August 1980. However, injections of methisazone at 10 mg and 25 mg per branch showed the greatest percentage of leaves with symptoms at 60 and 68% respectively (Table 2).

Injections of antiviral compounds into scar skin-infected Red Delicious branches and dapple apple-infected Hyslop crab apple branches during autumn and spring appeared to have no noticeable effect upon virus symptom development in the fruit (Tables 3,4, and 5).

Effects of Autumn Injections of Antiviral Compounds into Apple Mosaic Infected MacIntosh Tree Branches on Foliar Symptoms

In October 1980, antiviral compounds were injected into apple mosaic infected MacIntosh tree branches that had shown typical mosaic symptoms in August 1980. The results of this series of injections are summarized in Table 6. Four out of six branches injected with 16 mg of ribavirin were completely free of virus symptoms at the end of August 1981. Similarly, four out of six branches injected with 24 mg of ribavirin were also symptomless in August 1981. Indexing of the symptomless branches on Golden Delicious grafts in the greenhouse showed that apple mosaic virus was present in the symptomless branches. Furthermore, the ribavirin injected branches that did show symptoms had much fewer leaves with symptoms and the symptoms were much milder than in any of the other injected or control branches.

Formycin B injections at 1 mg and 2 mg per branch resulted in levels of foliar symptoms that were not significantly different from the levels of foliar symptoms on control branches. However, terramycin injections of 40 mg per branch resulted in significantly higher percentages of foliar symptoms per branch than control branches.

TABLE 6

Effect of Autumn Injections (1980) of Antiviral Compounds into Apple Mosaic Infected MacIntosh Branches on Foliar Symptom Frequency and Severity

Treatment		Number of branches		Percent of leaves with symptoms per branch[a,b]	Disease severity index[a,c]
Chemical	Amount (mg/branch)	Treated	With symptoms		
Ribavirin	16 mg	6	2[d]	4*± 7.6	0.05*±0.09
	24 mg	6	2[d]	8*±13.4	0.10*±0.17
Formycin B	1 mg	6	6	50 ±13.6	0.72 ±0.19
	2 mg	6	6	47 ±15.5	0.64 ±0.23
Terramycin	24 mg	6	6	61 ±10.7	1.07*±0.31
	40 mg	6	6	70*±15.7	1.08*±0.27
Control		6	6	53 ± 9.4	0.72 ±0.35

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[a] Each number is the average of 6 replications ± standard deviation.

[b] Percent of leaves with symptoms = number of leaves with symptoms per total number of branch leaves.

[c] Disease severity index = (% leaves with light symptoms x 1) + (% leaves with moderate symptoms x 2) + (% leaves with severe symptoms x 3) ÷ 100.

[d] Indexing results showed apple mosaic virus present in the four symptomless trees (August 1981).

[*] Treatment is significantly different from respective control (P = 0.05).

Moreover, the symptoms on the terramycin treated branches at both 24 and 40 mg were significantly more severe than symptoms on control branches (Table 6).

Effects of Spring Injections of Antiviral Compounds into Apple Mosaic Infected MacIntosh Tree Branches on Foliar Symptoms

The results of the May 1981 injections into apple mosaic infected MacIntosh branches that had shown typical apple mosaic symptoms in August 1979 are summarized in Table 2.

In August 1981, branches injected with 8, 16, and 24 mg of ribavirin had a significantly lower percentage of foliar symptoms compared to the control branches. The severity of the symptoms was also significantly lower than that of untreated control branches. Unlike the October 1980 injections, spring injections of ribavirin did not completely inhibit mosaic symptom development in any of the branches.

Branches treated with 1 mg and 2 mg of formycin B had just as many leaves showing symptoms as did untreated branches. However, branches injected with 1 mg of formycin B had more severe symptoms than untreated control branches (Table 7).

TABLE 7

Effect of Spring Injections (1981) of Antiviral Compounds into Apple Mosaic Infected MacIntosh Branches on Foliar Symptom Frequency and Severity .

Treatment		Number of branches		Percent of leaves with symptoms per branch[a,b]	Disease severity index[a,c]
Chemical	Amount (mg/branch)	Treated	With symptoms		
Ribavirin	16 mg	4	4	11*+ 6.4	0.13+0.08*
	24 mg	4	4	27 ±32.1	0.24±0.35
Formycin B	1 mg	4	4	51 +30.7	0.62+0.38
	2 mg	4	4	31 ±12.5	0.36±0.18
Terramycin	24 mg	4	4	46 +26.9	0.51+0.28
	40 mg	4	4	22 ±10.0	0.24±0.14
Control		4	4	49 ±22.2	0.55±0.20

[a] Each number is the average of 4 replications ± standard deviation.

[b] Percent of leaves with symptoms = number of leaves with symptoms per total number of branch leaves.

[c] Disease severity - (% leaves with light foliar symptoms x 1) + (% leaves with moderage foliar symptoms x 2) + (% leaves with severe foliar symptoms x 3) ÷ 100.

[*] Treatment is significantly different from respective control (P = 0.05).

Terramycin injected branches at 24 mg/branch resulted in percentage and severity of foliar symptoms similar to the control group. However, branches injected with 40 mg of terramycin showed a lower percentage of leaves with symptoms (22) and corresponding severity index (0.24) than any of the other treatment groups excluding ribavirin at 16 mg/branch.

Phytotoxic Effects of Injected Antiviral Compounds on the Apple Trees

Many branches injected with an antiviral compound during either autumn or spring showed circular, depressed necrotic areas of bark around the injection sites. Often, this necrosis extended up to 3 cm above and below the injection site. At some concentrations of the compounds, small lateral branches above the injection site appeared completely necrotic. The signs of phytotoxicity usually became apparent after the first of June. On branches injected with ribavirin, occasional phytotoxicity became noticeable on newly emerging leaves which appeared narrower than normal and slightly chlorotic. Formycin B injections of 2 mg per branch appeared to be the most toxic to trees. In several instances, bark necrosis had spread over half of the branch length by July 1. By mid-July, much of the foliage of formycin B treated branches was smaller than normal and chlorotic. Often there were various degrees of curling and twisting along the midrib of the leaves and necrotic patches developed in the leaf margins. On branches injected with terramycin during the autumn, it was observed that by the middle of May, leaf expansion was about one week behind that of the rest of the tree and of the other trees. In addition, the expanding leaves were noticeably chlorotic.

Spring injections of amantadine HCl and methisazone resulted in typical circular necrotic areas of bark around the injection site, however, no other signs of phytotoxicity were apparent on the treated branches.

Effects of Summer Injections of Antiviral Compounds into MacIntosh Trees on Apple Mosaic Virus Infectivity and Foliar Symptom Expression

In July 1979, two year old apparently healthy MacIntosh trees were injected with each of the antiviral compounds and one week later they bud-inoculated with apple mosaic virus. The results of this experiment are summarized in Table 8. In August 1980, one year after treatment, the six trees injected with ribavirin showed no virus symptoms whereas all other treated and control trees showed typical apple mosaic symptoms. The percentage of leaves with symptoms on trees treated with 2-thiouracil was not significantly different from that of

TABLE 8

Effect of Injections of Antiviral Compounds in Young Apple Trees on Apple Mosaic Virus Infectivity and Foliar Symptom Development

Treatment		Number of Trees			Percent of leaves with symptoms per tree[a.b]		Disease severity index[a,c]	
Chemical	Amount (mg/tree)	Treated 1979	With symptoms 1980	With symptoms 1981	1980	1981	1980	1981
Ribavirin	4	6	0[d]	2[d]	0*	13*±24.4*	0*	0.21*±0.39
2-Thiouracil	4	6	6	6	47 ±14.0	54 ± 9.2	0.66*±0.21	0.78 ±0.17
Terramycin	5	6	6	6	27*± 8.5*	37*± 9.6*	0.39 ±0.06	0.51 ±0.16
Control*		6	6	6	40 ±11.1*	67 ±10.2	0.42 ±0.12	0.69 ±0.11

[a] Each number is the average of 6 replications ± standard deviation.

[b] Percent of leaves with symptoms = number of leaves with symptoms per total number of tree leaves.

[c] Disease severity = (% leaves with light symptoms x 1) + (% leaves with moderate symptoms x 2) + (% leaves with severe symptoms x 3) ÷ 100.

[d] Indexing results showed apple mosaic virus present in symptomless trees two years after chemical treatment.

[*] Treatment is significantly different from respective control (P = 0.05).

the control trees but the severity of the symptoms on the 2-thiouracil treated trees was significantly greater than that of the control group. Trees treated with terramycin had a significantly lower percentage of leaves with symptoms than did untreated control trees. However, the average disease severity (0.39) for the terramycin injected trees was not significantly lower than the mean disease score (0.42) for the control trees. This indicates that even though fewer leaves in terramycin treated trees showed mosaic symptoms, the symptoms tended to be more severe than those on the foliage of control branches.

By August 1981, two years after the initial injections, two of the six ribavirin treated trees were showing typical mosaic symptoms while the remaining four trees were still symptomless. When these six trees were indexed by bud inoculation on Golden Delicious seedlings, the results showed that apple mosaic virus was present in all six trees. The percentage of foliar symptoms and symptom severity in the 2-thiouracil and formycin B treated trees were lower than those of the control trees, but the differences were not significant.

Effects of Injections of Antiviral Compounds into Scar Skin Infected Red Delicious Tree Branches on Fruit Symptom Expression

In October 1980 and May 1981, Red Delicious apple tree branches, on which the fruit showed typical scar skin symptoms in August 1980, were injected with antiviral compounds. The results of these experiments are summarized in Tables 9 and 10. Fruit on all injected and control branches showed typical symptoms. The severity of symptoms on the fruit of branches injected with antiviral compounds either in October 1980 or in May 1981 did not differ significantly from the severity of fruit symptoms in uninjected branches. Many injected branches showed signs of phytotoxicity similar to those described previously for MacIntosh branches injected at the same time, including bark necrosis around the injection wounds and foliar chlorosis and necrosis. There were no apparent signs of phytotoxicity on the fruit of treated branches.

Effects of Injected Antiviral Compounds on Symptom Expression of Apple Mosaic Infected Golden Delicious Grafts in the Greenhouse

When young Golden Delicious grafts grown in the greenhouse which had already been inoculated with and

TABLE 9

Effect of Autumn Injections (1980) of Antiviral Compounds on Fruit Symptom Expression in Scar Skin Infected Red Delicious Branches

Treatment		Percent of fruit with symptoms per branch[a,b,d]	Disease severity index[b,c,d]
Chemical	Amount (mg/branch)		
Ribavirin	16 mg	100	1.67±0.37
	24 mg	100	1.00±0.00
Formycin B	16 mg	100	1.17±0.22
	24 mg	100	1.63±0.50
Terramycin	24 mg	100	1.30±0.63
	40 mg	100	1.58±0.38
Control		100	1.42±0.42

[a] Each number is the average of 6 replications ± standard deviation.

[b] Percent of fruit with symptoms = number of fruit with symptoms per total number of branch fruit.

[c] Disease severity index = (% fruit with light symptoms x 1) + (% fruit with moderate symptoms x 2) + (% fruit with severe symptoms x 3) ÷ 100.

[d] Statistical analysis showed no significant difference between treatments and control at $P = 0.05$.

TABLE 10

Effect of Spring Injections (1981) of Antiviral Compounds on Fruit Symptom Expression in Scar Skin Infected Red Delicious Branches

Treatment		Percent of fruit with symptoms per branch[a,b,d]	Disease severity index[b,c,d]
Chemical	Amount (mg/branch)		
Ribavirin	16 mg	100	1.60±0.41
	24 mg	100	1.10±0.46
Formycin B	1 mg	100	1.50±0.58
	2 mg	100	1.58±0.74
Terramycin	24 mg	100	1.48±0.50
	40 mg	100	1.49±0.53
Control		100	1.50±0.58

[a] Each number is the average of 6 replications ± standard deviation.

[b] Percent of fruit with symptoms = number of fruit with symptoms per total number of branch fruit.

[c] Disease severity = % fruit with light symptoms x 1) + (% fruit with moderate symptoms x 2) + (% fruit with severe symptoms x 3) ÷ 100.

[d] Statistical analysis showed no significant difference between treatments and control at P = 0.05.

were showing symptoms of apple mosaic virus, were injected with antiviral compounds, the following results were obtained (Table 11). Ribavirin at 0.3 and 0.5 mg per graft resulted in complete suppression of virus symptoms in foliage produced after injection. Many newly emerging leaves of the plants injected with ribavirin were slightly chlorotic and narrower than normal leaves but these symptoms tended to disappear in about 2-3 weeks.

Terramycin did not suppress foliar symptom development in the virus-infected plants. One of 30 plants injected with 0.3 mg of terramycin did not show virus symptoms while 100% of the plants injected with 0.5 and 1.0 mg of the antibiotic showed typical mosaic symptoms. Terramycin-injected plants developed much more severe symptoms than did control plants or plants treated with the other chemicals. Leaves of terramycin-injected plants that showed mosaic patterns had larger and brighter yellow or white areas and smaller green areas compared to leaves of the other treated or untreated inoculated grafts.

Of 30 plants injected with 0.005 mg of formycin B, 18 showed apple mosaic symptoms after treatment. Similarly, 16 out of 30 plants injected with 0.01 mg of formycin B showed symptoms after injection. However, one month after injection of 30 grafts with 0.05 mg of formycin B, 13 of

TABLE 11

Effect of Antiviral Compounds on Virus Symptoms in Apple Mosaic Infected "Golden Delicious" Grafts

Treatment		Number of plants		Percentage of plants with symptoms
Chemical	Amount (mg/graft)	Treated	With symptoms	
Ribavirin	0.1 mg	43	10	0.23
	0.3 mg	39	0[a]	0.00
	0.5 mg	43	0[a]	0.00
Terramycin	0.3 mg	30	29	0.97
	0.5 mg	29	29	1.00
	1.0 mg	30	30	1.00
Formycin B	0.005 mg	30	18	0.60
	0.01 mg	30	16	0.53
	0.05 mg	30	17[b]	1.00
Control		12	12	1.00

[a] Indexing results showed apple mosaic virus present in symptomless trees 5 months after treatment.

[b] The remaining 13 trees were dead.

the plants had died and the remaining plants (17) showed typical apple mosaic symptoms. After treatment with formycin B, newly emerging leaves were slightly chlorotic and marginal necrosis developed on much of the foliage.

A set of greenhouse grown Golden Delicious grafts were injected in October 1980 with either ribavirin, formycin B, or terramycin. The trees were then placed outdoors for overwintering. In the spring, the trees were transferred to the greenhouse again and were observed for symptom development during spring and summer. The results are summarized in Table 12. All but five of the treated trees showed typical mosaic symptoms. There were no noticeable signs of phytotoxicity on any of the newly emerging leaves or any of the foliage later on.

Results of a Bioassay Procedure to Detect the Presence of Ribavirin in Injected Apple Trees

Pseudomonas aeruginosa was used as the test organism in a bioassay to detect the presence of ribavirin in the leaves and stems of injected Golden Delicious grafts growing in the greenhouse. In laboratory experiments, *P. aeroginosa* was sensitive to filter discs impregnated with as little as 0.05 mg of ribavirin. When stem cross-sections from grafts injected with 0.05, 0.5, 0.3 and 0.1 mg

TABLE 12

Effect of Antiviral Compounds on Virus Symptoms of Apple Mosaic Infected "Golden Delicious" Grafts Expressed in the Greenhouse Following Autumn Injections in the Greenhouse and Outdoors Overwintering

Treatment		Number of plants	
Chemical	Amount (mg/graft)	Treated	With symptoms
Ribavirin	0.1 mg	13	12
	0.3 mg	12	12
	0.5 mg	11	11
Terramycin	0.3 mg	13	13
	0.5 mg	13	13
	1.0 mg	13	13
Formycin B	0.005 mg	12	10
	0.01 mg	12	10
	0.10 mg	10	10
Control		12	12

of ribavirin were plated onto petri dishes inoculated with *P. aeruginosa* no inhibitory effect upon bacteria became apparent. Filtered leaf sap from the injected grafts also failed to produce any inhibition zones.

CHAPTER V

DISCUSSION

Of the antiviral compounds tested, ribavirin was the most effective, in many cases completely suppressing apple mosaic symptom expression. Injections of ribavirin during autumn into apple mosaic infected orchard trees resulted in foliage free of virus symptoms the following year (Tables 1 and 6). Injections of ribavirin during July into healthy MacIntosh trees which were later inoculated with apple mosaic virus also produced trees free of apparent virus symptoms the following year (Table 8). In addition, greenhouse-grown, apple mosaic-inoculated Golden Delicious grafts injected with 0.3 and 0.5 mg of ribavirin produced symptomless foliage subsequent to the injections (Table 10). Indexing results, however, showed that the virus was still present in the symptomless trees and branches. These results support earlier research (Hansen, 1979; Schuster, 1976, Secor and Nyland, 1978; Cassel, 1981) which showed that in plant viruses ribavirin suppressed symptom development but it did not completely and permanently inactivate the virus.

How ribavirin brings about suppression of apple mosaic symptoms is not clear. As was reported before

(Sidwell et al, 1979) ribavirin appears to inhibit guanosine biosynthesis and also RNA-dependent RNA polymerase activity. Inhibition of virus multiplication by such mechanisms could explain ribavirin's effectiveness when injected into apple trees both prior and subsequent to virus infection.

Both young apple trees and individual apple tree branches injected with ribavirin often exhibited symptoms of phytotoxicity which included necrotic areas near the point of injection of necrotic streaks on the stem above the point of injection and narrowing of the leaf blades and slight chlorosis of the newly emerging foliage. The distribution of the toxic effect, however, was limited to the branch injected with the compound, which indicates that movement of the chemicals, at least in relatively high concentrations, was limited to the branch injected with the compound, which indicates that movement of the chemicals, at least in relatively high concentrations, was limited to the treated branch and did not spread to adjacent branches. Whether foliar symptoms are due to the inhibition of guanosine biosynthesis by ribavirin, which would result in a reduced availability of nucleotides to the plant cells as well as to the virus, or to other affects of ribavirin, is not known.

Spring injections of 2-thiouracil into apple mosaic infected MacIntosh branches resulted in a greater increase in the percentage of leaves with foliar symptoms and disease severity two years after chemical injection than did ribavirin and control treatments. At 24 mg per branch, ribavirin resulted in a noticeable decrease in foliar symptoms and symptom severity (Table 2). 2-thiouracil has been reported to enhance symptom expression in certain host plant/virus combinations (Dawson and Kuhn, 1972). It is possible that 2-thiouracil may have enhanced apple mosaic symptom development, thereby making the difference in foliar symptoms and symptom severity over a two year period more apparent between ribavirin and 2-thiouracil treated branches.

Formycin B injections into MacIntosh tree branches during autumn 1980 and spring 1981 had no significant inhibitory effect upon apple mosaic virus symptoms expression (Tables 2,6, and 7). However, mosaic symptoms were suppressed in about 50% of the young Golden Delicious grafts grown in the greenhouse and injected with the compound (Table 10).

Formycin B was very phytotoxic at the dosages used in the field and greenhouse treatments. Injections of 0.05 mg into greenhouse-grown apple tree grafts resulted in the death of 13 out of 30 treated trees. Perhaps higher dosages of formycin B are required to inhibit apple mosaic virus multiplication, however, such dosages injected into a young apple tree or individual tree branch are likely to kill the treated tree.

Autumn injections of terramycin resulted in significantly higher percentages of foliar symptoms and foliar disease severity relative to the control group (Table 6). However, none of the spring injections of terramycin produced significantly different levels of foliar symptoms and symptom severity (Table 7). Similarly, Golden Delicious grafts injected with the compound in the greenhouse also showed typical mosaic symptoms which in many cases were more striking than the symptoms on grafts of other treatments, including the control one.

Tetracyclines are known inhibitors of protein synthesis. It is possible that the increased severity of mosaic symptoms on the terramycin treated plants is the result of enhancec chlorosis due to combined inhibitory effects upon chloroplast development resulting from virus infection and terramycin inhibited plant protein synthesis.

Often after injection with an antiviral compound, young apple trees and individual apple tree branches exhibited necrotic areas around the point of injection. The necrotic areas near the point of injection show that the compound was present in them, at least for some time at a concentration that killed the cells involved. It is not known what percentage of the injected chemical was trapped temporarily or permanently in these areas and therefore how much of the chemical actually was distributed throughout the tree or branch. The necrotic streaks along stems or branches showed the path which the majority of the compound followed in its spread through the tree. From the size and arrangement of the streaks, it would appear that the initial distribution of the concentrated compound at least was quite erratic. The folar symptoms of phytotoxicity with the injected chemical, however, indicate that eventually a fairly uniform distribution of these compounds occurs throughout the tree or branch.

The season in which infected plants are injected with antiviral compounds appears to be an important factor in the suppression of symptoms in the orchard. Timing of the injection appears to affect the final distribution of the compound throughout the branch or the tree. The initial distribution of the injected compound is directly affected by the pressure exerted on the solution at the injection point. In the second phase of distribution the compound is carried via the conductive system of the plant at the normal pressure of the transpiration stream and the amount and condition of foliage influence the rate of its movement.

Four of the twelve symptom-showing branches injected with ribavirin in autumn 1980 (Table 6) were completely free of virus symptoms the following August, whereas none of the branches injected with ribavirin in spring 1981 (Table 7) were completely devoid of symptoms in August. These differences might be due to the possibility that autumn injections result in a more complete and timely distribution of the antiviral compound in the treated plant than do spring injections. Shabi et al (1974) have shown that when injections are performed in autumn before leaf drop, the injected solution is carried throughout the tree due to the rapid transpiration caused by the foliage remaining on the tree. At bud break, the following spring, the compound is probably present in the areas of the buds, is then taken up by the newly expanding foliage and, consequently, the foliage is protected from the effects of further multiplication of the virus.

Spring injections of ribavirin reduced the percentage and severity of foliar symptoms but failed to completely suppress virus symptoms in the individual branches. One possible explanation for the failure of the spring ribavirin injections to produce symptomless branches may be that the level of the antiviral compound present in the foliage was too low to effectively inhibit the multiplication of the virus. Research has shown that higher concentrations of compounds tend to accumulate in leaves during the spring on trees injected in the autumn compared to trees injected in the spring (Shabi et al, 1974; Keil and Civerolo, 1979). Such a pattern of distribution for spring injected compounds could also explain the absence of increased foliar symptoms and extreme symptoms severity in terramycin-injected branches (Table 7).

It is also possible that overall distribution is slower for spring injected chemicals than for autumn injected chemicals, due to the maturity and therefore greater transpiration of leaves in the autumn. Delayed distribution and accumulation of an effective concentration of the antiviral compound in the foliage during the spring may provide a length of time sufficient for the virus to replicate and spread systemically into the newly emerging foliage.

Injections of young trees carried out prior to their inoculation with apple mosaic virus appeared to be quite effective (Table 8). Trees injected with ribavirin were completely free of virus symptoms the year after treatment. Two years after treatment, only two of the six trees showed any mosaic symptoms. Thus it appears that ribavirin has a residual effectiveness of at least two years since indexing of the trees two years after treatment showed virus to be present in the symptomless trees also.

In the bioassay to detect the presence of ribavirin in the foliage and wood of treated plants, *Pseudomonas aeruginosa* did not appear sufficiently sensitive to the antibacterial effects of the ribavirin present. The levels of ribavirin present in the foliage of the young greenhouse-grown grafts were probably too low to be detectable by the bacteria. From the phytotoxic effects observed at higher concentrations, it would appear that levels at which ribavirin in the foliage would be inhibitory to *Pseudomonas aeruginosa* would most probably be lethal to the young tree.

Antiviral injections into greenhouse-grown Golden Delicious grafts during late autumn were not effective in suppressing mosaic symptom expression the following summer (Table 12). In these experiments, it was extremely difficult to inject the graft with the chemical and the small amount (0.5 ml) of chemical solution was taken up very slowly by the plant. It is possible that the plants were approaching dormancy at the time of injection and, therefore, the distribution of the compound was delayed until spring. How this explains the failure of ribavirin to effectively suppress symptom expression in the young plants the following summer, however, is not clear.

None of the antiviral compounds injected into scar skin infected Red Delicious trees in either autumn (Tables 3 and 9) or spring (Tables 4 and 10) appeared to have any obvious effect upon symptom expression on the fruit. It is quite possible that the scar skin pathogen is not affected by these compounds or that very little of the injected compounds were present in the fruit. Since the compounds move through the plant mainly via the transpiration stream, it is possible that the relatively larger leaf surface and higher transpiration rate of the leaf relative to the fruit results in little, if any, compound moving into the fruit.

CHAPTER VI

SUMMARY

Several synthetic chemicals with known antiviral activity were injected under pressure into apple trees infected with apple mosaic, caused by a virus, and scar skin disease, believed to be caused by a virus but as yet unproven. Apple mosaic virus causes a chlorotic yellow to creamy white mosaic-like pattern on the foliage of infected trees. Trees infected with scar skin show symptoms only on the fruit which becomes covered with mottled, cracked, corky-like scar tissue. The compounds used against these disorders included ribavirin, 2-thiouracil, formycin B, and terramycin, a tetracycline antibiotic.

Ribavirin completely suppressed virus symptom expression when injected during autumn into orchard trees infected with apple mosaic virus and also into greenhouse-grown Golden Delicious grafts infected with the virus. When young apple trees were injected with ribavirin and then inoculated with apple mosaic virus, all trees remained symptomless for one year and some of them for two years after receiving treatments. Indexing showed that virus was present in all trees that remained symptomless as a result of treatment with the compound. When ribavirin was injected during the spring into apple mosaic virus infected orchard trees, the percentage and severity of foliar symptoms were significantly reduced, however, total control of symptom expression was not achieved.

Terramycin injections of outdoor trees in the autumn or of grafts in the greenhouse significantly increased the percentage and enhanced the severity of foliar symptoms on the treated trees. Spring injections of terramycin did not appear to significantly alter foliar symptom expression. Formycin B did not have any apparent effect upon symptoms, however, it was the most phytotoxic compound at the dosage levels administered.

Injections of orchard trees in the spring appeared to have a lesser effect upon virus symptoms than did autumn or late summer injections. This may be due to a poorer final distribution of spring-injected compounds compared to those injected in the autumn.

Injections of the antiviral compounds into orchard apple trees infected with scar skin disease failed to produce any noticeable effects upon the fruit symptoms of affected trees.

Attempts to bioassay for the presence of ribavirin in the foliage of treated trees using Pseudomonas aeruginosa as the test organism were not successful.

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