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Characterization of two strains of tobacco mosaic virus

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CHARACTERIZATION OF TWO STRAINS OF TOBACCO MOSAIC VIRUS

By

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Chapter I

INTRODUCTION

Statement of Problem

My thesis research has been based on the characterization of two plant viruses using a variety of methods. During the winter of 1971, I became interested in studying a virus found commonly in the weedy plantain or ribgrass, Plantago media, on the lawns of the University of Montana. A plant virus isolated from a different species of ribgrass, P. lanceolata, had been reported in 1940 by Holmes, and since then, other strains of the virus have been reported. I felt it would be interesting to examine this virus to determine whether it was the classic Holmes' ribgrass virus or a new strain.

My study began during the summer and fall of 1971, with host range determinations. With the onset of winter the various plants needed for hosts grew very slowly, making research difficult. At that time, I decided to begin a second project which was to be completed while my host plants matured.

I chose a laboratory-oriented problem involving ultraviolet light inactivation of plant viruses, and photoreactivation after exposure to visible light. I chose a virus which had been isolated by a fellow graduate student, Mr. Lallan Giri, in cactus. This virus, designated Cactus Virus X 196 (CaVX 196), has properties which make it especially interesting for this type of study, as will be further elucidated.

During my investigations with ultraviolet light, I found this aspect of plant virology even more fascinating than the characterization I had planned for the ribgrass virus. Consequently, I decided to continue these studies and further attempt to invove the ribgrass virus by testing its ultraviolet sensitivity.

Review of Background Information

Though begun only about eighty years ago, the study of plant viruses has increased tremendously in the last quarter of a century. "New" viruses are reported frequently, leading to study and discovery of different strains of these viruses. The work has been quite recent, and the findings so rapid and numerous, that much virus research is still in the stage of classification and characterization. Though it is quite likely that a new discovery may actually be a known virus found in a different host or environment, carefully performed identification experiments, host range studies, electron microscopy, and serology can provide assurance as to the identity of the virus, as well as establishing the closeness of its relationship to other viruses.

Tobacco mosaic virus (TMV) is probably the most studied of the plant viruses. First described by a German scientist, Mayer, in 1886, and distinguished from bacteria by Iwanowski in 1892, it soon became the most popular experimental virus for many types of research because of its high concentration in host plants and its remarkable stability. Several strains, distinct from the original TMV, later became apparent. The following are cited in Smith, A Textbook of Plant Virus Diseases (1957): Tobacco distorting virus (Ainsworth and Smith), tobacco mosaic ringspot

strain (Smith), tobacco internal browning virus, tomato aucuba mosaic virus (Kunkel), tomato streak virus (Jarrett), and the ribgrass strain (Holmes).

This last type, the ribgrass strain of TMV, was first observed by Francis O. Holmes in 1940 in Princeton, New Jersey, in ribgrass (Plantago lanceolata) and the broad leaved plantain (P. major). Holmes performed extensive experiments to determine the identity of the virus, including heat resistance, desiccation, filterability, dilution end point, host range, serological tests, and electron microscopy. In his original paper, Holmes mentioned the possibility of substrains.

Substrains were indeed reported following Holmes' publication. Several European virologists reported viruses similar or identical to the original Holmes' ribgrass virus (HRV) (Juretić, Wrischer, and Polák (1969); Harrison, Scotland (1953); Schumann, Germany (1963)). In the same publication, the authors reported a substrain of HRV in Yugoslavia (HRV-Y), which was different from the classical strain. Characterization was made by host range study, serological relationships, and electron microscopy of both the virus and the inclusion bodies produced in cells.

Inclusion bodies of strains of HRV are distinctive, as described by Miličić, Štefanac, Juretić, and Wrischer (1968). Despite the closeness of the relation between HRV and TMV, the ribgrass does not produce the common hexagonal crystals. Instead, cells infected with HRV may produce "rounded plates." These are layers of virus particles stacked one on top of the other, with the particles themselves oriented perpendicular to the plane of the layer. The center layers are the largest,

and the surrounding discs become gradually smaller, so that a direct view reveals concentric lines, but a side view appears needle- or cigar-like.

In the late 1960's Dr. Meyer Chessin noticed that many of the broad leaved plantains (P. media) growing on the lawns of the University of Montana showed chlorotic mottling. He mechanically transmitted the virus to Nicotiana tabacum var. Turkish, in which a systemic infection, with mild mottling in upper, uninoculated leaves, resulted. Since the original HRV was found in New Jersey, some 2,500 miles from Missoula, there was a good possibility that a different substrain of the virus could be found in Montana.

Host Range Studies

Mayer (1886) was the first to publish data showing mechanical transmission of TMV from infected to healthy tobacco plants. In 1908 Clinton found that other members of the Solanaceae were susceptible to TMV infection. Almost all plant viruses known were first discovered in angiosperm species, but only relatively few of the possible host-virus combinations have been tested. It seems that most species of angiosperms are immune to most viruses (Matthews, 1970).

Several types of valuable information are obtained in conducting a host range determination, some of which are too often overlooked. The following are of considerable importance (Corbett and Sisler, 1964 and Matthews, 1970).

a. Characterization can be made of reactions or lack thereof on host plants which react with known viruses.

b. An attempt should be made to discover a suitable test plant or indicator species, which develops distinct symptoms soon after inoculation. If assays are to be performed, a local lesion host may be desirable.

c. A large variety of plants should be examined, many outside the family of the plant in which the virus was discovered. "New" viruses have often been found to be known virus strains normally associated with a different type of plant.

d. Absence of visible symptoms should not be immediately assumed to indicate absence of infection. If this occurs, it should be followed by back-inoculations to an indicator species to test for masked infection.

e. Failure of a virus to infect a certain plant species may be as important in identification as the production of severe symptoms. Species tested but not infected should always be reported.

f. Even though host range studies are usually unreliable in providing specific identification, in a few cases this method is actually the best criterion for such determination.

Effects of Ultraviolet Light on Plant Viruses

Recently it has become possible to use rates of ultraviolet inactivation and degree of photoreactivation in the identification of plant viruses. Inactivation rates are characteristic for each virus, and the only viruses yet known to be unphotoreactivable are of rigid morphology. Chessin, Zaitlin, and Solberg (1967) showed that the sensitivity of a strain of TMV isolated from Lychnis alba was essentially equal to that of the common (U-1) strain, thus eliminating several other strains of TMV.

Other experiments were done with clover yellow mesaic virus (Chessin, 1965). The first deliberate test of virus sensitivity to ultraviolet (UV) light occurred in 1926, when Mulvania reported that viruses (TMV in particular) were less sensitive to inactivation than bacteria.

The UV dose is usually administered to a virus solution which has been purified or merely clarified to various degrees from a mixture of plant host components. Preliminary studies are often done with clarified sap, but experiments must be performed using relatively purified material if results are to be comparable and repeatable, since unpurified solutions may contain components which may alter susceptibility to UV damage. Inactivation has been found to occur at doses which do not apparently alter the virus particle in any other way, physically, chemically, or serologically (Matthews, 1970). Neither do these doses produce mutant strains.

Measurements of inactivation by exposure to UV are usually presented in the form of "dose-response" curves, plots of the log of survival of the virus versus the dose of UV given. Such a graph normally gives a straight line and thus is characteristic of "first-order" reactions (Jagger, 1967). The rate of a reaction is proportional to the active masses of the reactants, but in most cases the concentration may be used instead of the active mass (Williams and Williams, 1967). Thus the rate of inactivation of a virus solution can be written

$$\text{Rate} \propto (\text{Virus}), \text{ or,}$$

$$\text{Rate} = k\ (\text{Virus}).$$

Since the exponent of the concentration term is one, the reaction is first-order. The importance of such a designation is that first-order

reactions are usually attributed to one-hit processes. In this case, a one-hit theory suggests that virus particles are inactivated by a single quantum of energy that chances to hit a region necessary for infectivity.

This process, however, is not necessarily operating in the inactivation of virus, as pointed out in 1960 by Kleczkowski. He found that quantum yields, or

$$\frac{\text{number of particles altered}}{\text{number of quanta absorbed}}$$

are very low. One TMV particle, for example, absorbs between 15,000 and 30,000 quanta to be inactivated. Kleczkowski preferred, therefore, a "disequilibrium" theory, which stated that irradiation results in a state of disequilibrium due to excitations in various parts of the virus particle. In this state the particle may be inactivated at any time, without any definite number of quanta being absorbed.

Different viruses show wide variability in the degree of inactivation by UV radiation. The degree of sensitivity may be expressed by taking the negative slope of the dose-response curve.

The actual mechanism of UV inactivation is not yet fully understood. Since it has been established that the nucleic acid of a virus is the infective portion, inactivation probably results from some change in its makeup. The protein coat seems to have no direct effect on infectivity but does offer some protection for the RNA from UV light inactivation. It has been found that the nitrogen bases are the important UV absorbers of the RNA and that the pyrimidines are about ten times as sensitive as the purines (Jagger, 1967). Thus, in plant viruses, cytosine and uracil are the most important targets for UV damage (Jagger, 1967).

At present, two theories are supported which explain the actual process of UV inactivation:

a. Dimerization of pyrimidine residues in the RNA chain, the older theory (Matthews, 1970).

b. Hydration of the double bond in cytosine and uracil between carbons in positions 5 and 6.

Both of these processes are reversible and thus could account for photoreactivable damage. Recent experiments comparing inactivation in light and heavy water (D_2O) (Tao et al., 1966) support the latter theory.

Photoreactivation (PR) is a phenomenon in which visible light has been found to reverse or counteract the effects of irradiation in many biological systems. Most plant viruses are no exception. However, it is interesting to note that an irradiated virus solution, when placed in the light, shows no change at all, but if the irradiated solution is first inoculated to a host plant and the plant is then exposed to visible light, PR may be demonstrated. Apparently the reaction is dependent on a light sensitive system within the host plant which is not found in the virus particle. Enzymes involved in the reaction have been reported in DNA systems. Minato and Werbin (1972) reported a DNA-photoreactivating enzyme isolated from blue-green algae.

The effect of PR becomes apparent when the proportion of virus particles remaining infective after irradiation is relatively greater in inoculated plants placed in the light than in those placed in darkness. The apparent rate of inactivation is slowed and is equivalent to decreasing the dose by a factor differing with each virus (Bawden, 1964).

The actual degree of PR may be represented by taking the ratio of the negative slopes of the straight line plots of survival in darkness to

those in light. It is interesting to compare some of these figures for viruses which have already been examined. Except for rigid rod shaped viruses, all plant viruses can be photoreactivated. However, even in the case of rigid rods, if the RNA is removed from its protein coat and tested separately, PR does occur (Bawden and Kleczkowski, 1955).

The occurrence of PR is believed to be related to the tightness of bonding between the protein coat and the nucleic acid core of the virus. Although flexible and rigid rods have basically similar helical designs, the RNA coil of the flexible rods is not as tightly held by the interactions between successive protein coils (Casper and Klug, 1962). Perhaps this is due to less specificity in the bonds. The isometric particles have very loose bonds between NA and protein, as reported by Casper and Klug. Other evidence supporting this theory of protection of the RNA of rigid rods by their protein coat is provided by the tests of free RNA of TMV, which is photoreactivable. Govier and Kleczkowski (1970) have found that the degree of protection depends on the wavelength of the radiation.

A final factor to consider is that PR increases infectivity after UV exposure but does not restore it to its original level. It is thought, therefore, that two types of change may be produced in the RNA, one reversible in visible light, the other not. In the case of the rigid rods, the protein seems to give protection from only the photoreactivable type of damage (Bawden, 1964).

In 1971 Lallan Giri at the University of Montana isolated a virus from wild cactus, designated Cactus Virus X 196. The virus, which had been found to be a rod of 546 nm, was intermediate in morphology between

the rigid (unphotoreactivable) and the flexible (photoreactivable) viruses, being slightly flexible. It seemed worthwhile to examine this virus to determine its photoreactivability, if any. After the experiments were begun, however, Mr. Giri began to suspect that this virus had disappeared from his host plants. Electron microscopy confirmed these suspicions and revealed that a second type of virus particle was in its place. Experiments to determine the identity of this virus are still in progress. Present information indicates that this virus, which is similar to TMV in length and morphology, was present with the other type of virus in the original cactus isolate, but that it was undetected until the other virus had been removed. The second virus is probably not present due to contamination by the classical TMV, since host reactions on N. sylvestris are different from any strain of TMV yet reported (Figure 1). PR experiments provide some evidence as to the morphology of the virus.

Figure 1. Nicotiana sylvestris infected with Cactus Virus X 196 showing chlorotic and necrotic lesions.

Chapter II

MATERIALS AND METHODS

Host Range Study of Virus Found in Ribgrass

Source of Virus and Method of Inoculation

The virus studied was obtained either directly from infected lawn ribgrass leaves or from inoculated N. tabacum plants in the greenhouse. The latter was consistently used as a virus source plant due to high rate of virus production and concentration, ease of cultivation, and facility with which crude sap was extracted from the leaves by grinding. Leaves harvested were usually used immediately but occasionally were frozen for use at a later date. Leaves were ground in 2 ml per leaf of 0.01 M PO_4 buffer of pH 7, with a mortar and pestle. Test plants were sprayed with carborundum powder as an abrasive and the crude juice was rubbed onto the leaves using a finger. Leaves inoculated were marked with a black pen.

Selection of Host Plants and Greenhouse Conditions

Host range plants chosen had been used by previous researchers for the identification of HRV and its substrains (Juretić, Wrischer, and Polák (1969) and F. O. Holmes (1940)). Plants used and symptoms produced are listed in the Results section. Plants were chosen for inoculation when leaves reached mature size but before the plant flowered.

Inoculated plants were kept on benches in the greenhouse. During the summer months the glass windows were whitewashed to minimize insolation

and to reduce the temperature. In winter the benches were steam heated to maintain temperatures above 15-18°C, and supplementary light was provided by incandescent lamps. Despite these precautions seasonal variations were unavoidable, and daylength ranged from 15 hours in summer to 9 hours in the winter. Temperatures were kept between 15.5-24 C during the winter; summer temperatures ranged from 15.5°C at night to about 37°C on some afternoons.

UV and Photoreactivation Experiments

Choice of the Virus and the Assay Plants

Preliminary work in these experiments involved the selection of a virus and a suitable assay plant. CaVX 196 was first chosen for the study because of its intermediate morpology between the rigid rods and the flexible rods. Nicotiana glutinosa L., a local lesion host for the virus (Giri, unpublished) was used almost exclusively as an assay plant. For one particular experiment using the crude sap of CaVX 196, there were no N. glutinosa available and N. sylvestris was substituted. This plant was unsatisfactory since infection produced chlorotic local lesions which coalesced to the point where counts were extremely difficult. The ribgrass virus' sensitivity to UV light was studied as well.

Preparation of Virus Solutions

a. The first seven experiments utilized a crude sap preparation of CaVX 196. Virus was obtained by crushing infected leaves of N. tabacum in .005 M potassium phosphate buffer, pH 7, one ml per gram of leaf material. The liquid obtained was clarified through four layers of cheesecloth and

centrifuged at 7710 xg for 10 minutes in a Sorvall Superspeed Centrifuge, model RC2-B. The clarified juice was then frozen in aliquots of 0.5 ml. Aliquots were thawed as needed and diluted 1/100 by adding 49.5 ml of .005 M potassium phosphate buffer.

b. Later experiments were performed using the CaVX 196 solution purified by a combination of the chloroform and butanol methods (Steere, 1956 and Tomlinson, 1964) followed by alternate cycles of high (60,000 rpm) and low (10,000 rpm) speed centrifugations. High speeds were attained in a Beckman Model E Ultracentrifuge, using rotor model 60Ti. Purity was determined by use of a Shimadzu multipurpose recording spectrophotometer.

c. Experiments with ribgrass virus involved the purification technique described for the CaVX 196. However, an additional heat treatment was needed to purify the solution. Before application of the heat the UV absorption spectrum revealed a peak at 290 mu which probably represented a protein impurity (Figure 2). This peak disappeared after diluting the virus 1/10 and heating at 54°C for 30 min. This treatment should have little or no effect on the infectivity of the virus (Price, 1940, cited in Bawden, 1964).

d. Irradiation of the virus solutions was done using a "Mineralight" AW lamp (UV Products, San Gabriel, California) with approximately 85% of its energy output at 2537 Å. Solutions of 10 ml (and later 5 ml) were placed in flat-bottomed Petri dishes. Distance between the surface of the lamp and the base of the dish was 6.5 cm. The dish was agitated slightly during irradiation to insure uniform exposure of the virus. After exposure to UV, 15 mg/ml celite was added to each dish as an

abrasive. Four dishes of solution were used for each test, and each dish was irradiated for a different length of time.

Inoculation

Plants were used when mature but before the onset of flowering. Four fully expanded leaves were chosen and all others removed. Often, plants were decapitated and placed in darkness for 24 hours prior to inoculation to increase infectivity (Kassanis, 1952). Inoculations were made between 10 AM and 2 PM, a procedure which increases uniformity of the tests as well as actual lesion numbers (Matthews, 1970).

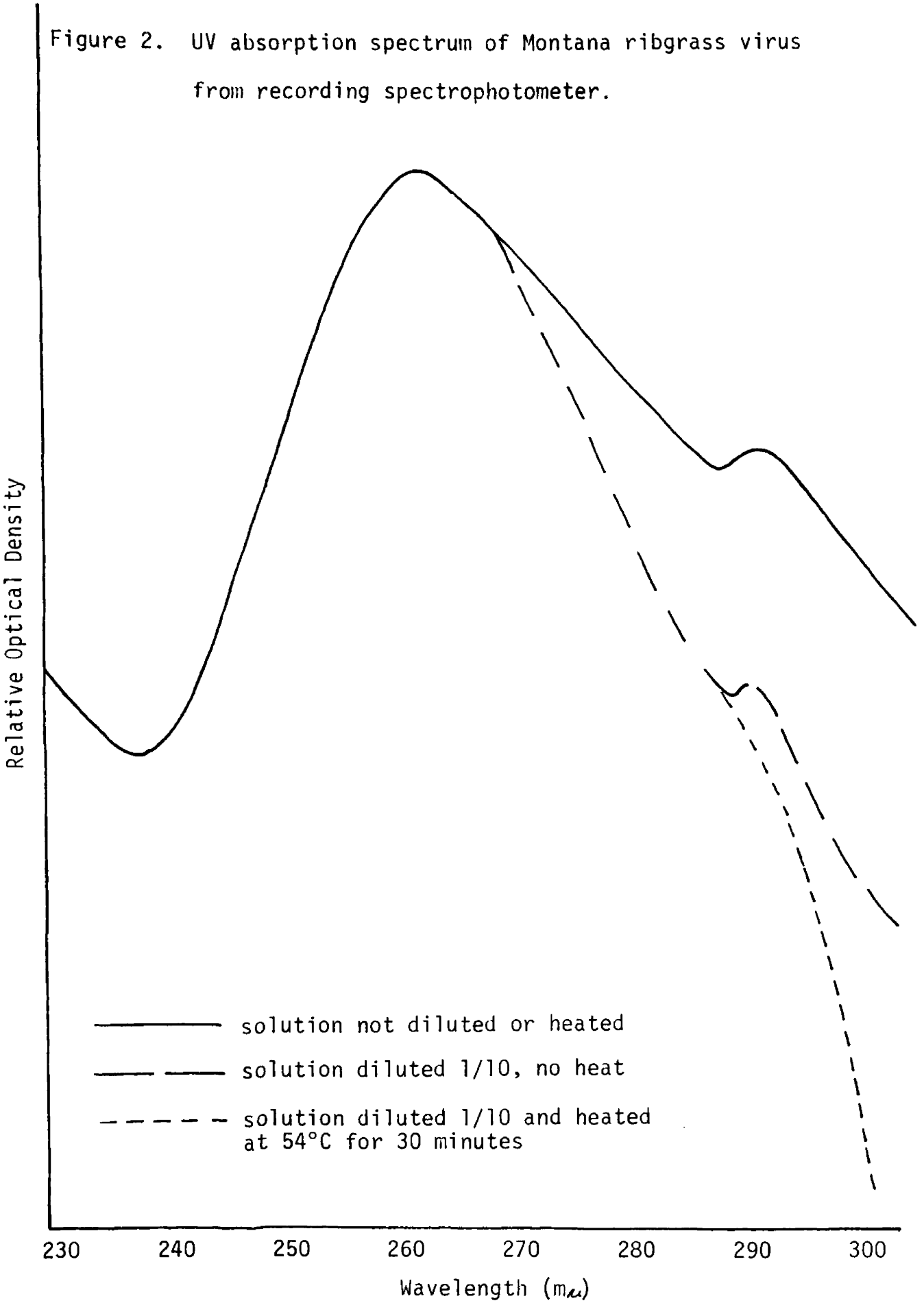
Inoculations immediately followed irradiation. The four solutions irradiated for different periods of time were applied in a randomized manner determined by the Latin Square method, to minimize differences between plants and leaf positions. Inoculum was applied with a finger, and leaves were rinsed with distilled water. Red light was used during inoculations since no PR occurs at this wavelength (Chessin, 1959). Plants were then kept in an environmentally controlled room for 24 hours, with temperatures maintained at 22-24°C. Half of the plants were exposed directly to constant illumination from "Cool White" fluorescent tubes. The rest were placed immediately in the dark. After the first four experiments, the light intensity was increased from 400-500 ft. candles to 1400-1500 ft. candles. Light intensity has been found to limit PR in some instances. Chessin (1958) showed that 2 hours at 380 ft. candles gave much PR for potato virus X. This intensity is intermediate between the 600 ft. candles found to be fully effective and the 80 ft. candles which was ineffective (Bawden and Kleczkowski, 1955). After 24 hours

all plants were brought into the light. Lesions were counted 3 days after inoculation. On two occasions lesions were recounted on the 4th day, and although lesion numbers were slightly higher, the slopes of the lines on the dose response curves did not change.

Photoreactivation

The degree of PR was estimated by plotting the log of the surviving virus (average number of lesions per leaf) separately for both light and dark treatments, against the UV dose in time (Matthews, 1970). Numerical slopes of the lines indicate degree of sensitivity to UV, and the ratio of the negative slopes of Dark divided by Light give the measure of PR. A ratio of 1 indicates no PR.

Figure 2. UV absorption spectrum of Montana ribgrass virus from recording spectrophotometer.



Chapter III

RESULTS

Host Range of Ribgrass Virus

The following species of plants were used in the host range study: Nicotiana tabacum L., N. glutinosa L., N. sylvestris Speg., Gomphrena globosa L., Chenopodium quinoa Willd., C. capitatum, C. foliosum Aschers., Phaseolus vulgaris L., Datura stramonium L., Brassica rapa (L.) Hartm., B. perviridis Bailey, Sinapis arvensis L., Petunia violaceae, Plantago lanceolata L., P. media L., and Ciphomandra betaceae Sendt. The results of the host range study and comparisons with Holmes' data for HRV, the Yugoslavian strain (HRV-Y) and TMV as reported by Grant, 1934, are presented in Table 1.

Table 1. Host Range of the Montana Ribgrass Virus and comparisons with symptoms produced by Holmes' Ribgrass Virus, HRV-Y, and Classical TMV.

Host Plant	HRV	HRV-Y	TMV	Montana HRV
Amarantaceae				
Gomphrena globosa L.	...	...	NLL	14 days-local necrotic lesions on inoculated leaves
Chenopodiaceae				
Chenopodium quinoa Willd.	NLL	NLL	NLL	4 days-Mottling on inoc. leaves 15 days-mottling, vein clearing on upper leaves
C. capitatum	...	...	...	4 days-chlorotic lesions inoc. leaves. Syst. mottle, veinal chlorosis
C. foliosum	+ VC B	+ VC B	...	4 days-chlorotic LL inoc. leaves 11 days- syst. mottle, blisters
Cruciferae				
Brassica perviriridis Bailey	...	M VC YL	...	No visible symptoms*
B. rapa L.	...	CLL VC	...	No visible symptoms*
Sinapis arvensis L.	...	+ M B	...	No visible symptoms*
Leguminosae				
Phaseolus vulgaris L.	---	...	NLL	No infection
Plantaginaceae				
Plantago lanceolata L.	+	---*	---	5 days-necrotic LL on inoc. leaves (4 of 16 plants) upper leaves no symptoms*

Host Plant	HRV	HRV-Y	TMV	Montana HRV
P. media L.	+M CLL	+M CLL	---	Systemic mottling-4 days
Solanaceae				
Ciphomandra betaceae Sendt.	...	NLL +M B	...	4 days-necrotic LL inoc. leaves
Datura stramonium L.	NLL	NLL	...	3 days-necrotic LL, crinkled leaves
Nicotiana flutinosa L.	NLL	NLL	NLL	3 days-necrotic LL
N. sylvestris	NLL	NLL	+BM	4 days-necrotic LL
N. tabacum L. Turkish	NLL +M	NLL +M	NLL +M	3 days-necrotic LL
Petunia violaceae	...	...	...	4 days-veinal chlorosis, necrotic leaf tissue

Table 1. Explanation of abbreviations

M	mottling
YL	systemic yellow lesions
CLL	chlorotic local lesions on primary leaves
NLL	necrotic local lesions on inoculated leaves
VC	vein clearing
B	blistering
+	systemic
...	species not used
---	no symptoms
*	virus recovered by back inoculations (masked)

Symptoms produced by the Montana ribgrass virus in species inoculated were similar to those produced by HRV and HRV-Y except in a few cases. Perhaps the most interesting difference occurred in Chenopodium quinoa, a species often used for both assay and buildup of plant viruses. HRV, HRV-Y, and the classical TMV produced chlorotic lesions on inoculated leaves only. The Montana virus (which is abbreviated HRV-M) also produced chlorotic local lesions consistently on inoculated leaves, but also produced chlorotic mottling and vein clearing in upper, uninoculated leaves. To verify that the virus causing the systemic infection was indeed the ribgrass virus, a back inoculation was made from the C. quinoa upper leaves to Nicotiana sylvestris, which is known to produce a systemic infection with TMV but only local necrotic lesions with ribgrass virus strains. Local lesions were produced, thereby verifying the systemic transport of HRV-M (Figures 3 and 4).

An interesting problem arose when N. sylvestris was first inoculated from the maintenance host, N. tabacum (Turkish). Unmistakable systemic infection similar to that reported for TMV (Smith, 1964), and completely contradictory to that reported for HRV (Holmes, 1940) resulted. Systemic mottling, dark green blisters, distortion of young leaves, and delayed black necrosis all occurred.

After these tests a pure ribgrass virus was apparently obtained by collecting more Plantago leaves from the university lawns and making inoculations from these to fresh N. tabacum plants. Since Holmes (1940) reports that TMV does not infect Plantago, this source could be assumed to be TMV free. Inoculation from these new plants to N. sylvestris produced localized necrotic lesions only.

Figure 3. Montana ribgrass-infected Chenopodium quinoa.
Right: inoculated leaf with necrotic lesions.
Left: uninoculated leaf showing chlorosis.

Figure 4. Chenopodium quinoa. Left: healthy leaf.
Right: uninoculated leaf with chlorosis.

Another interesting difference between HRV-M and HRV-Y occurred on all members of the Crucifereae tested. The Yugoslavian team reported systemic infections in Brassica perviridis, B. rapa, Sinapis alba, and S. arvensis, stating that symptoms produced agreed with those produced by the classical HRV. In tests using the HRV-M (excluding S. alba) the whole family produced no visible symptoms. However, back inoculations to N. glutinosa produced necrotic local lesions.

In testing Plantago lanceolata Juretić et al., reported different results for the HRV-Y than did Holmes for the original ribgrass virus. Holmes had found a definite systemic infection, whereas Juretić could see no symptoms in this species. However, they discovered a masked infection by back inoculations. HRV-M differed from both of these. Sixteen P. lanceolata plants grown from several sources of seeds were observed: (a) Two of the plants developed many small local lesions (Figure 5), (b) Two other plants produced few but very large necrotic lesions (Figure 6), and (c) The remainder showed no visible symptoms. Back inoculations from uninoculated leaves of each of the three groups were made. The first (a) and last (b) groups produced many local lesions, indicating that a systemic infection had occurred. The second (c) group produced no lesions. A summary of the back inoculations is made in Table 2.

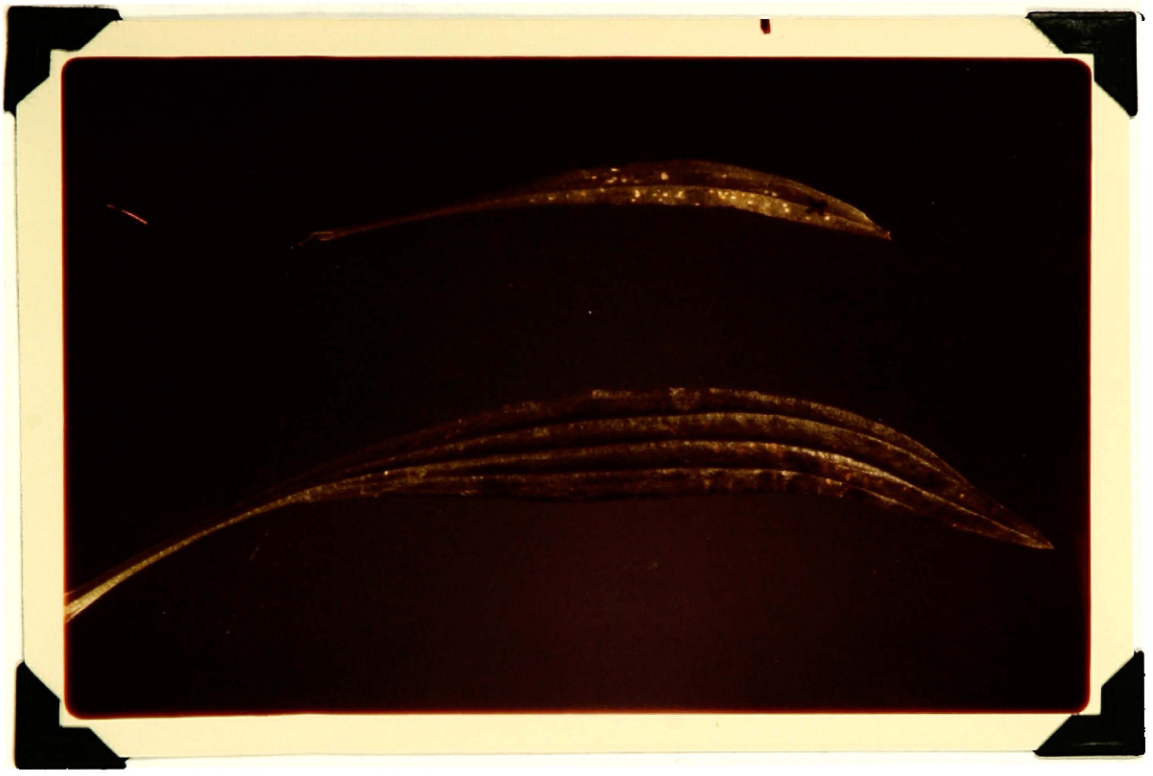


Figure 5. Plantago lanceolata. HRV-M inoculated leaves with many small local lesions.

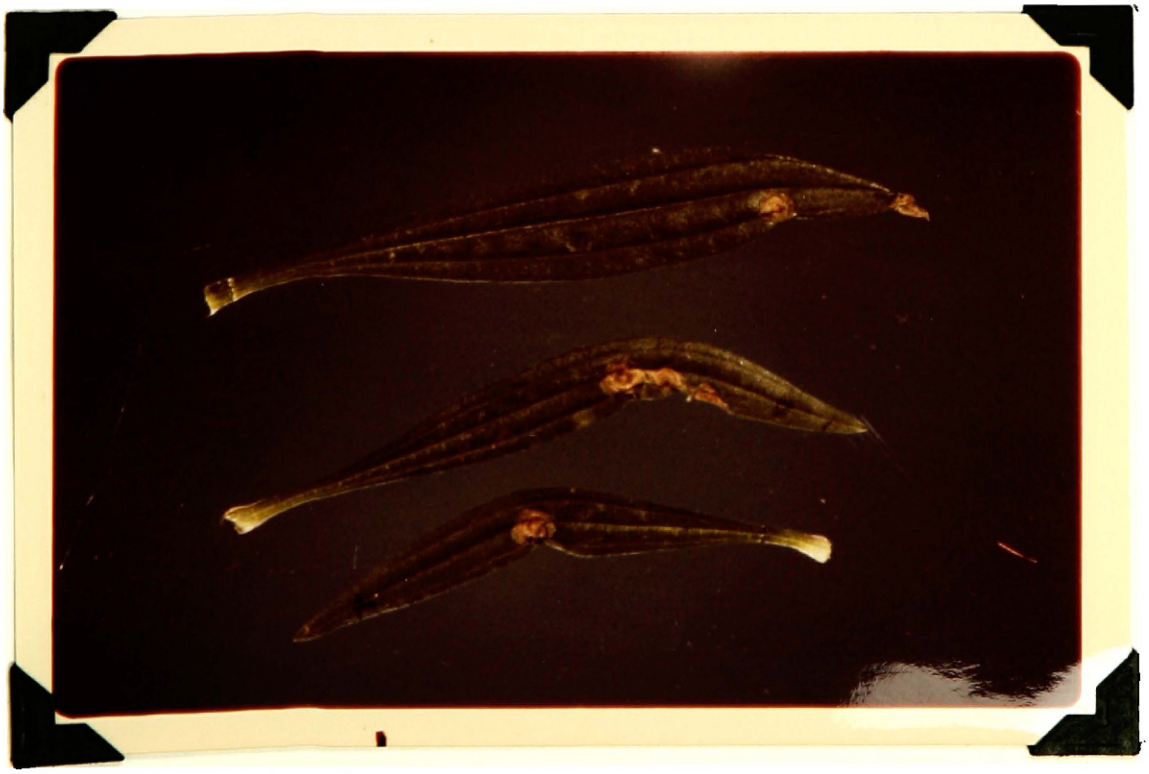


Figure 6. Plantago lanceolata. HRV-M inoculated leaves with a few large necrotic lesions.

Table 2. Results of some back inoculations from species inoculated with the Montana ribgrass virus.

From inoculated	To	Symptoms
Chenopodium quinoa (upper leaves)	*N. sylvestris*	Necrotic LL
N. tabacum (original maintenance hosts)	*N. sylvestris*	Systemic mottle, blistering, distortion
N. tabacum (new maintenance hosts)	*N. sylvestris*	Necrotic LL
Brassica perviridis	*N. glutinosa*	Necrotic LL
B. rapa	*N. glutinosa*	Necrotic LL
Sinapis alba	*N. glutinosa*	Necrotic LL
Plantago lanceolata		
a. Plants with many small lesions	*N. glutinosa*	Necrotic LL
b. Plants with a few large lesions	*N. glutinosa*	No symptoms
c. Plants with no symptoms	*N. glutinosa*	Necrotic LL

UV and PR Experiments

Experimental Conditions

CaVX 196 tests

Crude juice was used for the first seven experiments. The first test was made to determine the best range of exposure times. It was desirable to have countable, reasonably high numbers of lesions produced by the solution receiving the highest dose of UV radiation. It was found that exposure times greater than five minutes produce too few lesions to be reliable (Table 3). Exposure times were reduced in the second

experiment (Table 3, Figure 7). In the third experiment, N. sylvestris was used as an alternative assay plant to N. glutinosa. There was a possibility that a different host might produce a different reaction pattern in the lesion counts of light- and dark-exposed plants. It was found that N. sylvestris was an unsuitable host since the chlorotic spots produced in response to infection were not distinct and thus were very difficult to count (Figure 8). Therefore, N. glutinosa was used in all subsequent experiments (Table 3). In the fourth experiment exposure times were further reduced to concentrate on those for which larger numbers of lesions were produced (Table 3). Before the fifth test a new light was installed in the environmentally controlled room, increasing the light intensity from 400-500 ft. candles to 1400-1500 ft. candles (See Materials and Methods). It was thought that the intensity of light might have a limiting effect on the degree of PR, if any, in the light-exposed plants (Table 3). The sixth test again focused on times of exposure that produced large lesion numbers in the previous test. A repetition of this test was done to check an unusual dip in the "Dark" curve and to determine the similarity of the curves in all respects (Table 3).

Prior to further experimentation, the CaVX 196 was purified as previously described. Sample volumes were reduced to 5 ml for the sake of economy. Reduction of sample size made no difference in UV sensitivity (See Part C). Experiment 8 used this purified solution (Table 3). Exposure times resulting in large numbers of lesions occurred between 0-3 minutes of UV exposure. Lesion numbers for this test were counted on two successive days, and while actual numbers increased slightly, the

slopes of the dose response curves did not. For the ninth test exposure times were reduced to the range of 0-3 minutes (Table 3).

Ribgrass virus

The ribgrass virus was partially purified as described before any of the tests were performed. An exploratory experiment was done using only partially purified solution (no heat denaturation). This test showed that reliable numbers of lesions are produced in the range of 0-3 minutes of UV exposure only (Table 3). The second test used this range of exposure times, but the solution had been purified by application of heat (Table 3). Since lesion numbers were extremely low in this experiment, it was repeated using a virus solution of doubled concentration (Table 3). Lesion numbers were still very low, suggesting that there may have been some inactivation of the virus other than that caused by UV. Since the first experiment producing relatively large numbers of lesions was done using unheated solution, heat treatment may have caused some damage to the virus. The final experiment was done using unheated solution (Table 3, Figure 9).

It should be noted that the dose rate of UV for each experiment varies, indicating that the output meter had approximately a $\pm$ 10% error.

Table 3. Effect of UV treatment and post-UV illumination on virus survival.

Virus Solution	Sample Size (ml)	Exposure Time(min)	Dose Rate ($\mu W/cm^2$)	Temperature (°F)	Light Intensity (ft.candles)	Average Lesions/Leaf		
						Time	Light	Dark
CaVX 196								
1) Crude juice	10	0	395.2	75	400-500	0	24.00	10.80
		5				5	1.30	0.28
		10				10	1.00	0.37
		15				15	0.00	0.00
2) Crude juice	10	0	453.4	73	400-500	0	52.08	52.65
		2				2	13.91	12.38
		4				4	4.50	6.75
		6				6	3.67	2.00
3) Crude juice	10	0	424.3	76	400-500	0	45.80	46.20
		2				2	17.80	12.10
		4				4	3.00	4.40
		6				6	3.50	5.20
4) Crude juice	10	0	432.6	74	400-500	0	60.25	43.13
		1				1	30.90	34.87
		2				2	19.00	12.25
		3				3	5.75	5.62

Table 3. (Continued)

Virus Solution	Sample Size (ml)	Exposure Time(min)	Dose Rate ($\mu W/cm^2$)	Temperature (°F)	Light Intensity (ft.candles)	Average Lesions/Leaf		
						Time	Light	Dark
CaVX 196								
5) Crude juice	10	0	420.2	72	1400-1500	0	95.50	72.00
		1				1	73.42	37.00
		3				3	7.43	8.59
		5				5	1.91	1.48
6) Crude juice	10	0	396.5	75	1400-1500	0	79.88	91.63
		1/2				1/2	61.75	42.50
		1 1/2				1 1/2	43.37	39.63
		2 1/2				2 1/2	15.00	12.38
7) Crude juice	10	0	407.7	71	1400-1500	0	88.63	58.50
		1/2				1/2	39.25	43.13
		1 1/2				1 1/2	10.88	22.25
		2 1/2				2 1/2	4.88	17.25
8) Purified solution	5	0	399.4	76	1400-1500	0	16.75	9.38
		1				1	12.25	5.88
		3				3	1.88	1.50
		5				5	0.63	0.13

Table 3. (Continued)

Virus Solution	Sample Size (ml)	Exposure Time(min)	Dose Rate ($\mu W/cm^2$)	Temperature (°F)	Light Intensity (ft.candles)	Average Lesions/Leaf		
						Time	Light	Dark
CaVX 196								
						After 3 days		
9) Purified solution	5	0	424.3	76	1400-1500	0	48.10	79.00
		1 1/2				1 1/2	12.12	27.09
		2 1/2				2 1/2	6.75	13.62
		3 1/2				3 1/2	2.50	1.62
						After 4 days		
						0	55.50	103.21
						1 1/2	15.43	43.40
						2 1/2	8.62	24.00
						3 1/2	3.88	4.62
10) Purified solution	5	0	420.2	74	1400-1500	0	39.91	29.12
		1/2				1/2	29.00	13.24
		1				1	12.88	12.60
		2				2	7.08	6.00

Table 3. (Continued)

Virus Solution	Sample Size (ml)	Exposure Time(min)	Dose Rate ($\mu W/cm^2$)	Temperature (°F)	Light Intensity (ft.candles)	Average Lesions/Leaf		
						Time	Light	Dark
Ribgrass								
1) Purified solution (no heat)	5	0	416.0	73	1400-1500	0	64.00	47.38
		1				1	11.25	23.88
		3				3	1.38	0.13
		5				5	2.88	0.38
2) Purified solution (heated)	5	0	428.4	75	1400-1500	0	18.37	6.50
		1				1	5.00	0.62
		2				2	1.16	0.62
		3				3	1.88	0.38
3) Purified solution (heated)	5	0	453.4	74	1400-1500	0	2.75	5.13
		1				1	1.13	1.50
		2				2	1.13	0.05
		3				3	0.09	0.05
4) Purified solution (no heat)	5	0	416.0	76	1400-1500	0	121.75	104.63
		1				1	23.38	16.63
		2				2	3.50	7.88
		3				3	3.25	1.38

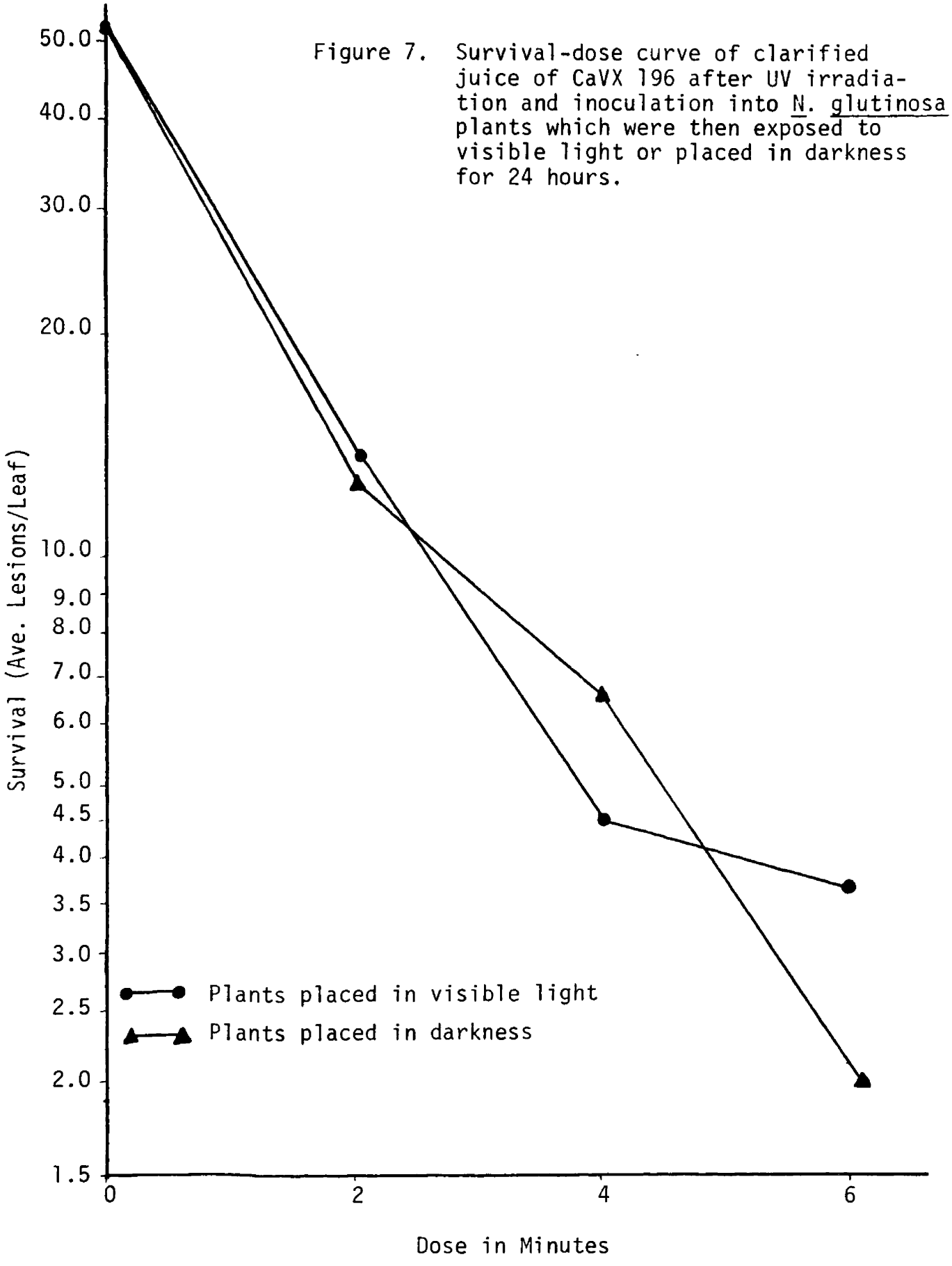


Figure 7. Survival-dose curve of clarified juice of CaVX 196 after UV irradiation and inoculation into N. glutinosa plants which were then exposed to visible light or placed in darkness for 24 hours.

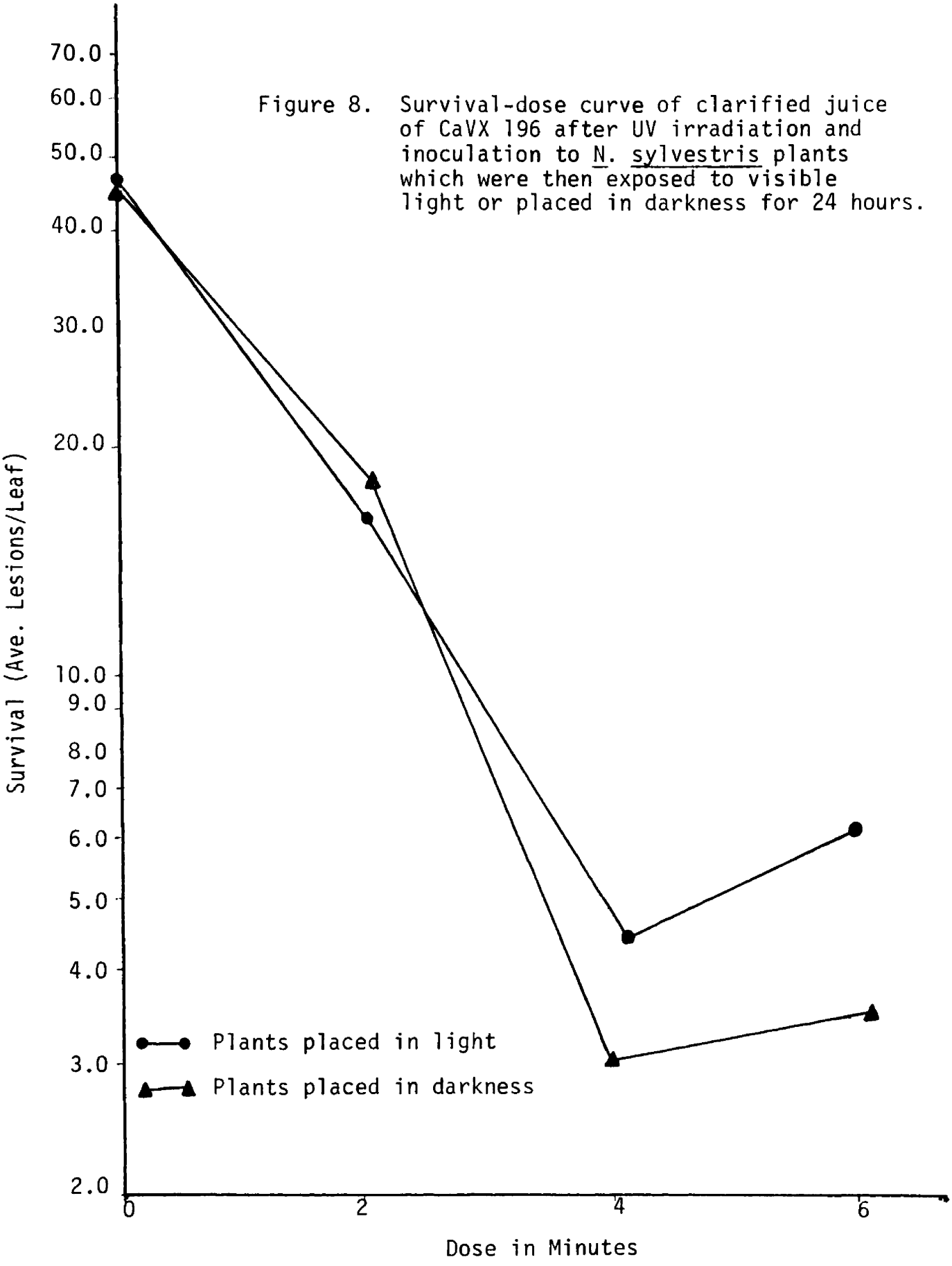


Figure 8. Survival-dose curve of clarified juice of CaVX 196 after UV irradiation and inoculation to N. sylvestris plants which were then exposed to visible light or placed in darkness for 24 hours.

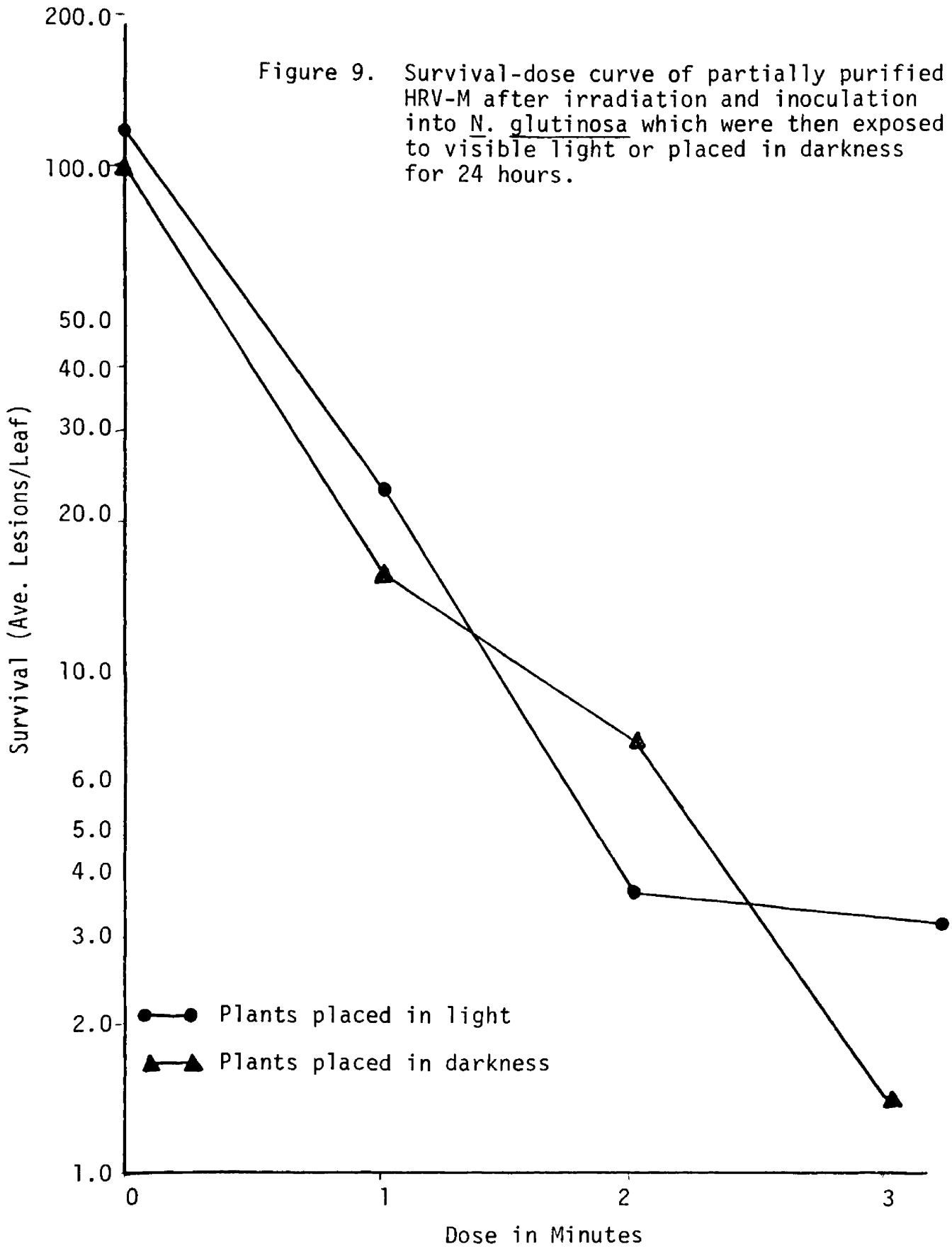


Figure 9. Survival-dose curve of partially purified HRV-M after irradiation and inoculation into N. glutinosa which were then exposed to visible light or placed in darkness for 24 hours.

Combined Results of UV and PR Experiments

CaVX 196

Data for experiments using unpurified solution were combined, as were data for the purified solution tests, and separate graphs were made for each. The most accurate straight line for each was determined by computer, using the least squares regression method (Figure 10). The slope of the line made for the unpurified solution was -.3296, and that of the purified solution was -.2999.

Slopes of the unpurified and purified solutions given above were assumed to be equal, allowing for experimental error. Data for all "Light" curves (both purified and unpurified) and data for all "Dark" curves were combined (Figure 11).

Ribgrass Virus

Data for all "Light" curves and data for all "Dark" curves were combined (Figure 12) and the most accurate straight line was determined (Figure 13).

Data for only the first and last experiments (those in which no heat treatment was given) were combined (Figure 12).

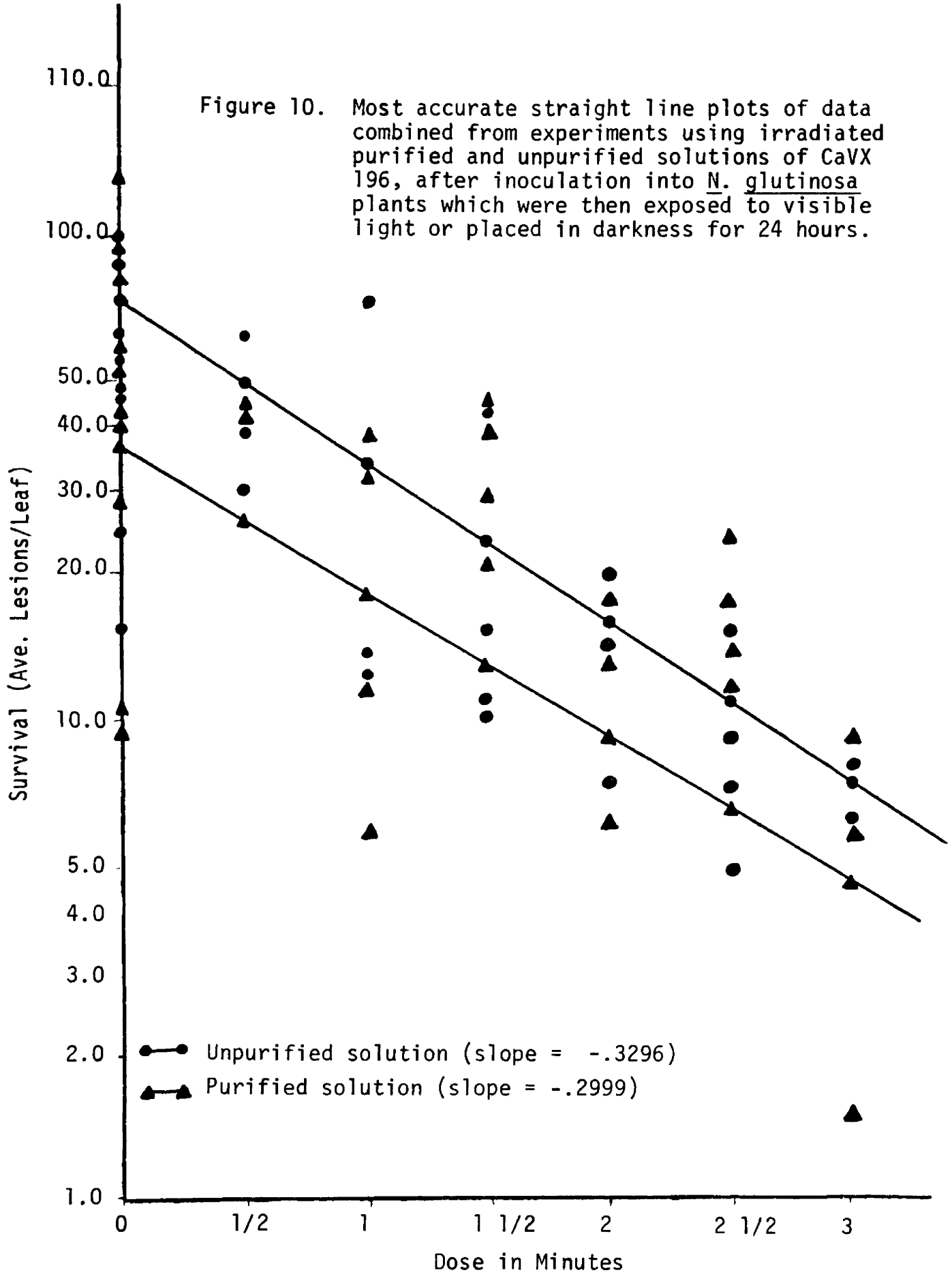


Figure 10. Most accurate straight line plots of data combined from experiments using irradiated purified and unpurified solutions of CaVX 196, after inoculation into N. glutinosa plants which were then exposed to visible light or placed in darkness for 24 hours.

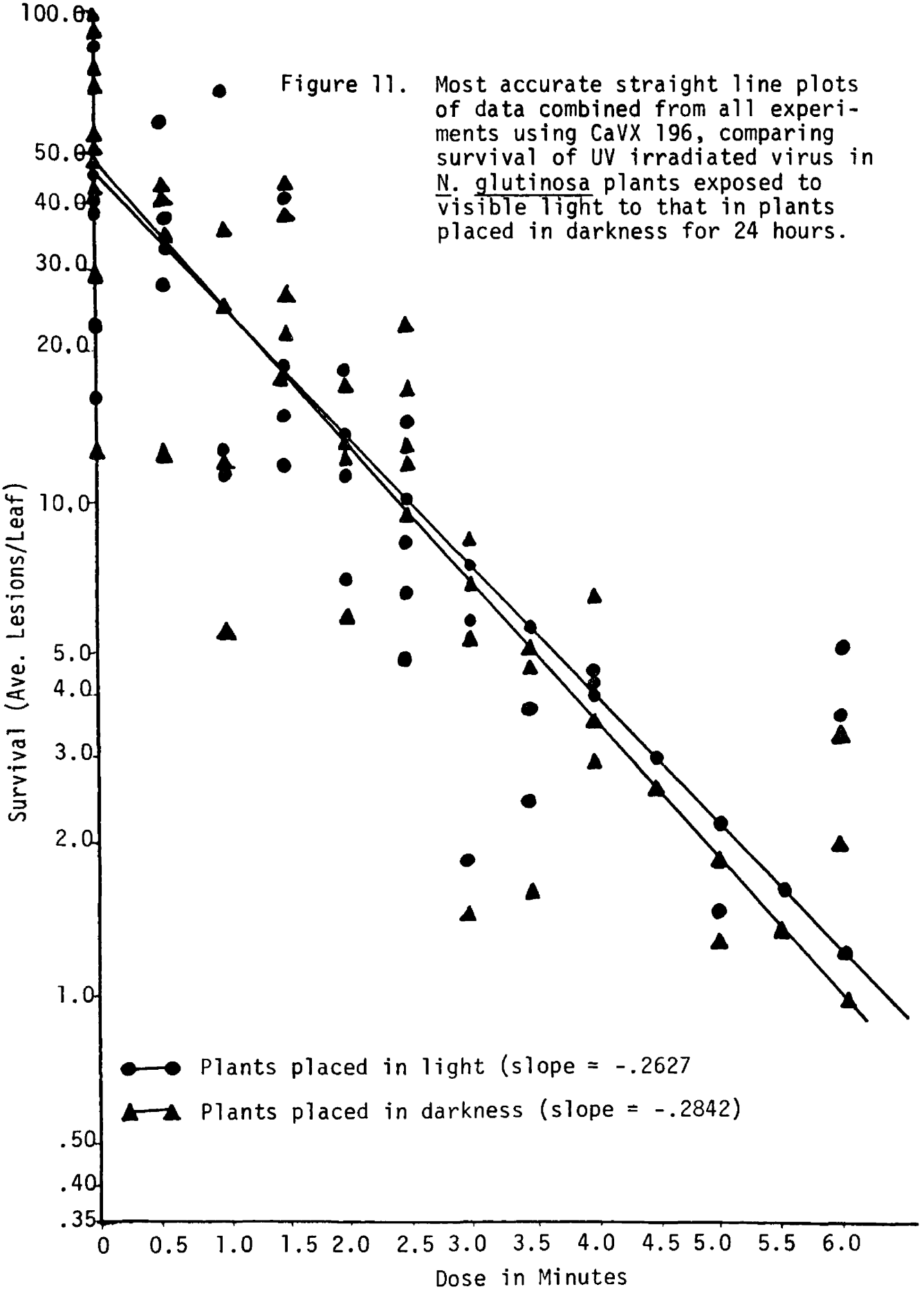


Figure 11. Most accurate straight line plots of data combined from all experiments using CaVX 196, comparing survival of UV irradiated virus in N. glutinosa plants exposed to visible light to that in plants placed in darkness for 24 hours.

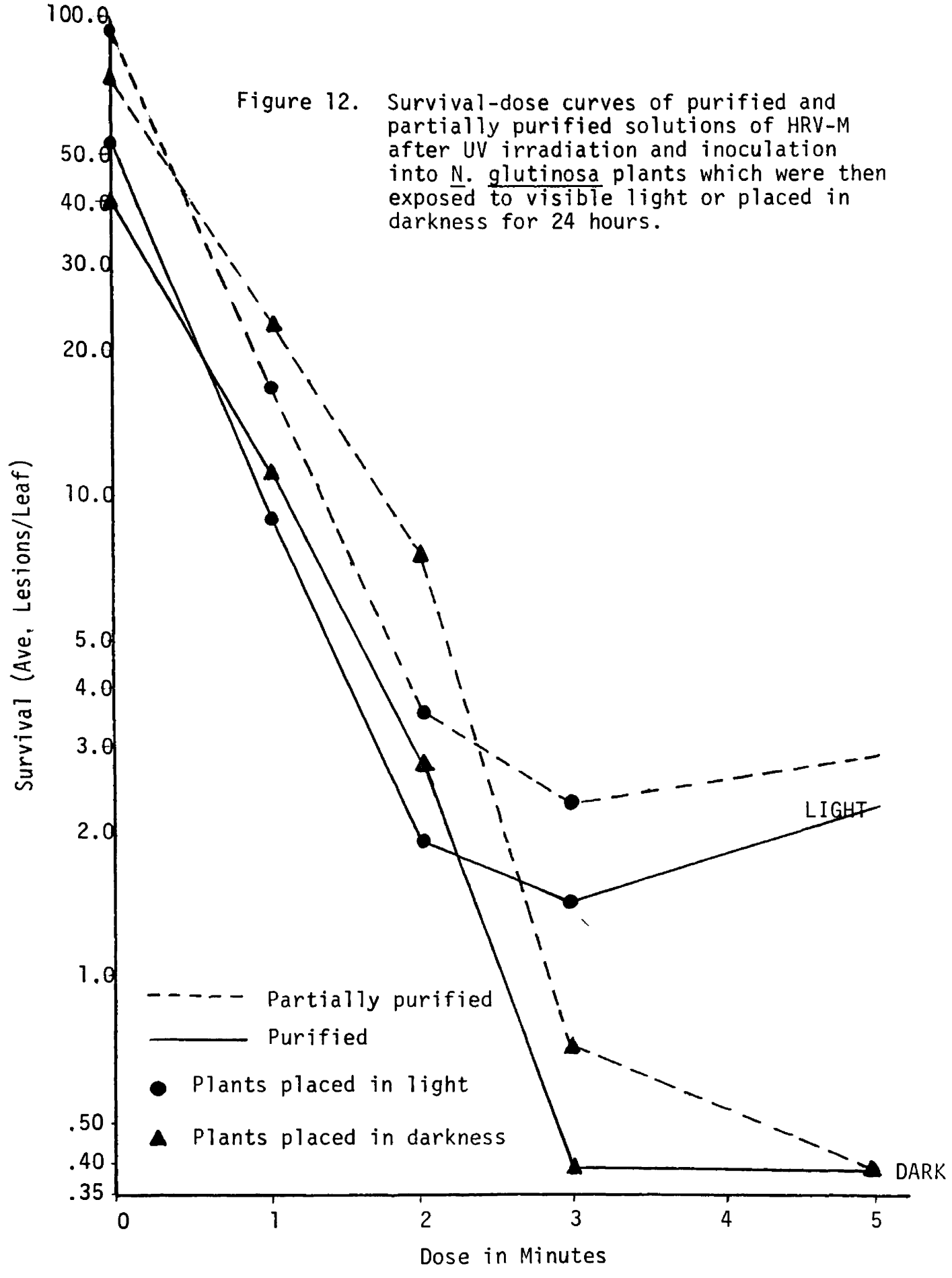


Figure 12. Survival-dose curves of purified and partially purified solutions of HRV-M after UV irradiation and inoculation into N. glutinosa plants which were then exposed to visible light or placed in darkness for 24 hours.

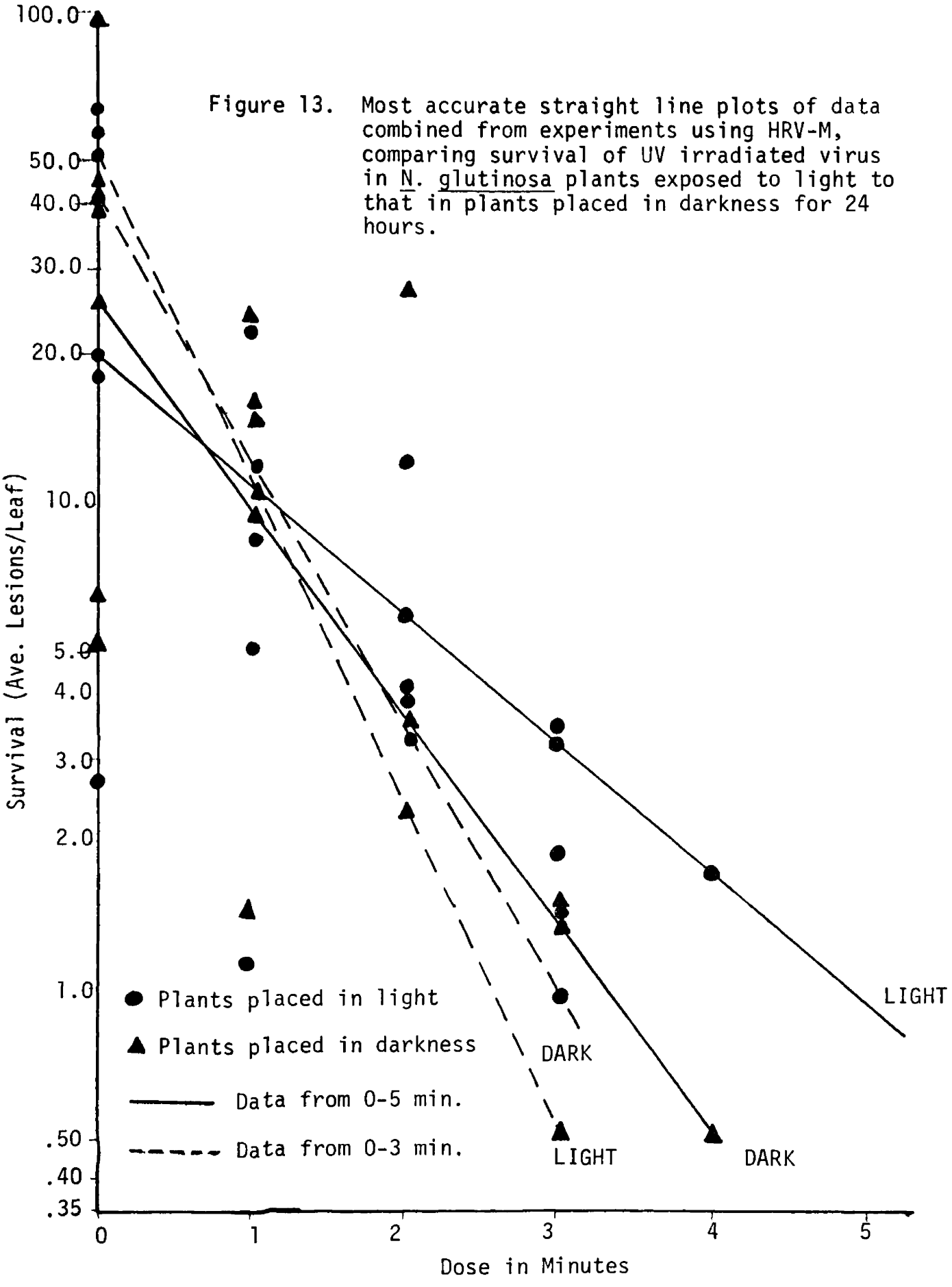


Figure 13. Most accurate straight line plots of data combined from experiments using HRV-M, comparing survival of UV irradiated virus in N. glutinosa plants exposed to light to that in plants placed in darkness for 24 hours.

Chapter IV

DISCUSSION

Host Range of HRV-M

Data and symptoms from host range studies indicated that HRV-M is a different strain from the classical HRV and the Yugoslavian strain. The production of systemic symptoms in Chenopodium quinoa has never been reported after infection with any ribgrass strain. HRV-M caused such an infection on all four occasions tested at different times of the year.

The production of masked infection in members of the Cruciferae family is also unique to the Montana strain of HRV. HRV and HRV-Y were both reported to cause systemic infection in members of this family.

The unusual symptoms produced by HRV-M in individuals of Plantago lanceolata differ significantly from the systemic mottling found by Holmes with HRV and from a masked infection produced by HRV-Y. The production of three different types of infection in plants of the same species may be due to genetic variability of the host in wild populations. Reference is made to the production of local necrotic lesions as a hypersensitive reaction, which often confines the disease to a few spots on inoculated leaves and gives effective "field resistance" to the disease (Matthews, 1970). A masked infection may be described as tolerance in which the virus spreads through the plant but the obvious disease symptoms are lacking. Resistance to systemic spread of a virus associated with local lesion production is often controlled by a single,

dominant gene (Klisiewicz, 1967). Some species of tobacco infected with TMV produce necrotic local lesions while others produce chlorotic local lesions followed by mesaic disease. Weber (1951) found that the difference in such varieties of plants may be due to control by a single dominant gene pair. F_1 hybrids from parents of the above characteristics had necrotic local lesions followed by systemic necrosis. It is possible that the P. lanceolata plants represented at least two genetic varieties and that perhaps those of group (a) are hybrids between those of groups (b) and (c). This would be an interesting problem to pursue.

Host reactions to plant viruses are good indicators of possible strain differences between viruses. However, they cannot be presumed to give definite proof, since environmental variables and the general condition of the host plants used may cause differences in symptoms produced. The host range study of HRV-M does give ample reason to suspect that it differs from HRV, but it would be important to perform other tests (serological, amino acid analysis, inspection of inclusions, etc.) to verify its relationship to Holmes' isolate.

UV and PR Experiments With CaVX 196

There seems to be no indication in any of the experiments done with CaVX 196, involving both purified and unpurified material, that PR occurs in this virus. Dose response curves (Figures 7 and 8) were visually examined after each experiment. None of these curves suggested a significant difference in slope between the "Light" and the "Dark" curves.

Other types of observations were made from these graphs of individual experiments. It is important to consider in any experiment involving

local lesion assays that the greater the numbers of lesions produced per leaf, the more reliable is the assay, within limits. In some experiments, high doses of UV caused so much inactivation that lesion numbers were one or less per leaf. It was established that for CaVX 196, exposure times beyond 5 minutes were not suitable.

It was also determined that light intensity to which inoculated plants were exposed had no apparent effect on either UV sensitivity or photoreactivability. There was no detectable difference between slopes of curves in experiments using light of 500 foot candles and those in which the light intensity was three times as great. This observation agrees with the report by Bawden and Kleczkowski (1955) that light of 600 foot candles is fully effective in PR.

An interesting situation occurred in several of the experiments. Lesion numbers per leaf tended to level off or even to increase at higher doses in some experiments (Figure 8). This phenomenon has not been reported in the literature, but Chessin (unpublished) has observed similar results in UV studies with other viruses. The rise, when present, seems to be associated with very small lesion numbers which were frequently less than one per leaf. Perhaps if Kleczkowski's (1953) method of adding a constant to the log of the lesion numbers were employed this rise would disappear, however this was not attempted.

After completion of the experiments, data were combined in various ways for several calculations. To determine whether purification had any effect on the sensitivity of the virus, all data for experiments run with clarified sap were combined, as were data for experiments done with the purified material. Using these figures the most accurate straight lines

were determined. (See Results and Figure 10.) The slope of the "Impure" line was -.3296, that for the "Purified" line was -.2999. These slopes were assumed to be the same, indicating that purification has no effect on UV sensitivity. This determination also showed that reducing the sample size from 10 ml (unpurified) to 5 ml (purified) had no significant effect on the UV inactivation.

Actual degree of PR was then calculated using data for both purified and unpurified solutions. Data for all experiments were combined and the most accurate straight lines determined (Figure 11). Slope of the "Light" curve was -.2627, that for the "Dark" curve was -.2842.

$$\text{Degree of PR} = \frac{\text{Slope Dark Curve}}{\text{Slope Light Curve}} = 1.082.$$

Considering expected experimental error, this figure indicates no PR with Cactus Virus X 196.

UV and PR Experiments With HRV-M

Visual examination of dose response curves drawn after each experiment with HRV-M gave no indication of PR (Figure 9). Several of the graphs, however, did show the interesting levelling-off or rise at the highest dose of UV. Some, as in Figure 7, showed this phenomenon only in the "Light" curve, others in both. For this reason, combining data for all experiments resulted in a graph (Figure 12) in which the "Dark" curve levelled off slightly but the "Light" curve turned upward. Nonetheless, from 0-2 minutes of exposure time the curves appeared quite similar, and there was no significant difference in slope.

When the most accurate straight lines for the combined data were calculated (Figure 13) the "Dark" curve had a much greater slope than the

"Light" curve, which would seem to indicate PR to the degree:

$$PR = \frac{.4343}{.2623} = 1.65.$$

Looking again at Figure 12, the difference in slope can be seen to be due to the differences in the upward bending of the "Dark" and the "Light" curves at high doses. When the most accurate straight lines were determined from the 0-3 minute exposure times on the graph they were quite similar (Figure 13) and the ratio of the "Dark" to the "Light" slopes was $\frac{.6470}{.5356} = 1.17$. This figure is below any of the figures for viruses reported to show PR.

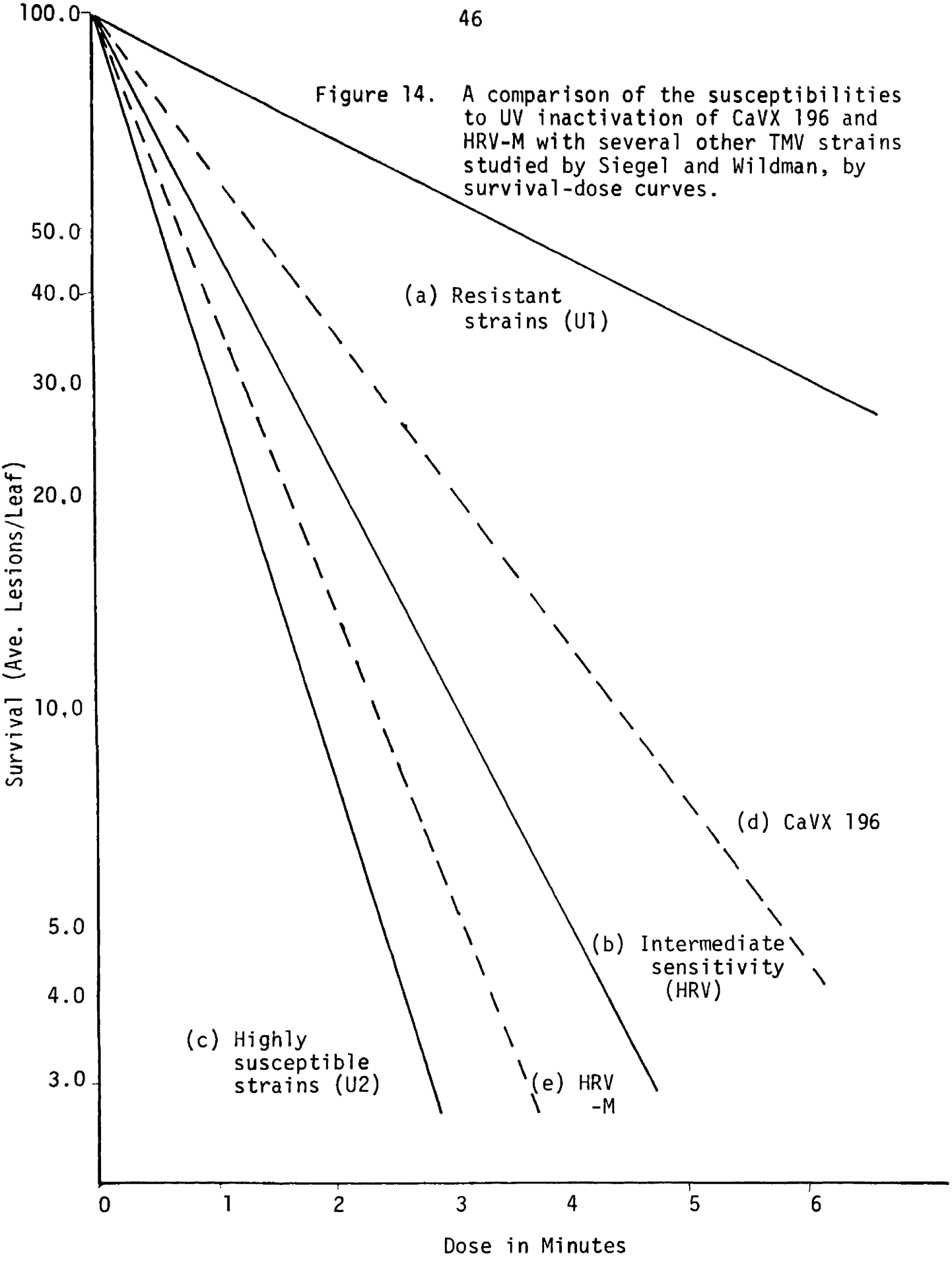
Two of the experiments with HRV-M were done using only partially purified material (See Materials and Methods and Figure 2) and two were done with purified solution. An extreme difference was noted in the numbers of lesions produced (Table 3). The partially purified solution produced many more lesions than did the solution which had received heat treatment. No heat inactivation assays have been reported for the ribgrass virus, except that Holmes (1940) stated that the virus was completely inactivated in 10 minutes at 93°C but not at 92°C. HRV is similar to the U2 strain of TMV (Siegel and Wildman, 1954), and it is known that the U2 strain is more sensitive to heat denaturation than the U1 strain (Siegel, et al., 1957). It is possible that HRV is also sensitive to heat and this would be worth investigating.

Siegel and Wildman (1954) found that strains of TMV differ greatly with respect to inactivation by UV light. The CaVX 196 and HRV-M may be compared to the eight strains used by Siegel and Wildman, as well as to each other. In Figure 14 the three lines from Siegel represent three

groups of viruses: (a) these resistant to inactivation, including the common U1 strain, (b) those intermediate in sensitivity, including Holmes' ribgrass virus, the U8 strain and (c) those highly susceptible to inactivation, including the U2 strain of TMV. These tests were made with a Westinghouse Sterilamp, Model 782L-30, with output mainly at 2537Å. However, Siegel and Wildman did not state the actual doses. The dotted lines represent CaVX 196 (d) and HRV-M (e), using straight lines determined by least squares. The line for HRV-M was determined from data for 0-3 minutes exposure time. This comparison is merely an estimation, since dosages have not been taken into account, but it can be noted that HRV-M is much less sensitive to UV light than CaVX 196, a relationship comparable to that between HRV and TMV (U1) determined by Siegel and Wildman, in which HRV is much less sensitive than the U1 strain. These observations are consistent with our hypothesis that HRV-M is closely related to HRV and that CaVX 196 is probably more closely related to the U1 strain than to the HRV and U2 strain (based on host range symptoms which are similar to those of the U1 strain on every plant tested except N. sylvestris).

It has become evident from the results of this study that there are several areas which bear further investigation. Additional PR experiments should be done with HRV-M to verify the degree of PR and to determine the cause of the upward bending in dose response curves. Dosages of experiments previously reported should be determined to make more accurate comparisons between CaVX 196, HRV-M and other TMV strains. Another possibility would be to obtain samples of the other strains and to do the experiments over to insure identical conditions. It is clear, however, that UV sensitivity can be a valuable tool in the identification of a virus or a virus strain.

Figure 14. A comparison of the susceptibilities to UV inactivation of CaVX 196 and HRV-M with several other TMV strains studied by Siegel and Wildman, by survival-dose curves.



Chapter V

SUMMARY

A strain of Holmes' ribgrass virus (HRV) was isolated from Plantago media L. in Montana for the first time. The virus was transmitted to sixteen species of host plants of six families. Symptoms produced by Chenopodium quinoa, Plantago lanceolata, and members of the Cruciferae family differed from those reported for the original HRV and for the Yugoslavian substrain, indicating that the virus found in Montana may be a different substrain.

UV light sensitivity and PR of two viruses were studied. Cactus Virus X 196, recently isolated from wild cactus by Giri, and HRV-M were purified, exposed to various doses of UV radiation (2537Å) and inoculated into local lesion assay hosts, half of which were illuminated with visible light, while the other half were placed in darkness. Data were summarized in the form of survival-dose curves, for which the most accurate straight lines were determined by computer. It was found that CaVX 196 is much less sensitive to UV inactivation than ribgrass virus, the slope of its survival dose curve being much less than that of HRV-M. This relationship is comparable to the observation by Siegel and Wildman (1954) that the U-1 strain of TMV is much less sensitive than classical HRV to UV inactivation. This information is not inconsistent with our hypothesis that HRV-M is closely related to HRV and that CaVX 196 is more closely related to the U-1 strain of TMV.

Neither CaVX 196 nor HRV-M show definite PR. Combined data from all experiments with CaVX 196 gave a PR ratio of 1.08, far below any figure reported for a photoreactivable virus. Survival-dose curves for HRV-M seemed to show PR at first examination, however, a possibly more representative presentation of the data give a PR ratio of 1.17, a figure also below any for viruses reported to show PR.

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