THE EFFECT OF SEED EXTRACTS ON THE INFECTIVITY OF PLANT VIRUSES AND ITS BEARING ON SEED TRANSMISSION

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[*Manuscript received September* 28, 1954]

Summary

Substances inhibiting infection by cucumber mosaic and tobacco mosaic viruses have been found to occur in the seeds of some of their hosts.

The following evidence is considered to show conclusively that the action of these substances is to inhibit infection rather than to inactivate the viruses:

(1) The substances are capable of instantaneous effect.
(2) Their effect is dependent upon the host used.
(3) Non-infective mixtures of inhibitor and inoculum can be made infectious by dilution.
(4) Their effect is greatest on concentrated inocula.
(5) They will affect lesion production when applied up to 2 days before inoculation.
(6) The inhibitor from cucumber seeds affects lesion production when applied to the under surface of cowpea leaves.

Both of the inhibitors are proteins.

The bearing of these results on the theories that have been advanced to explain the rarity of seed transmission of plant virus diseases is discussed.

I. INTRODUCTION

The rarity of seed transmission of plant virus diseases, and particularly of some which are highly infectious, has long lacked an adequate explanation. Bennett (1936) advanced a very satisfactory explanation for the lack of seed transmission of beet curly top virus. He showed this virus to be chiefly restricted to the vascular tissues of its hosts and thus unable to invade the embryos of the seeds. This explains satisfactorily the lack of seed transmission of all plant viruses that are restricted to the vascular tissues of their hosts, but it does not account for the rarity of seed transmission of a far greater number of plant virus diseases which are unrestricted in the tissues of their hosts and which can move quite independently of vascular tissue. Bennett, assuming that plant viruses spread through plant tissues only via plasmodesmata, suggested that these viruses are not seed-transmitted because of the lack of plasmodesmatal connections from the embryo to the parent plant. This suggestion also assumes that those viruses which are seed-transmitted are capable of between-cell movement other than through plasmodesmata.

Recently, Caldwell (1952), working with aspermy disease of tomato, in which infected plants produce few seeds and less than 1 per cent. of normal pollen, suggested: "The presence of virus in the microspore-mother-cell results

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in a complete interference with the normal stages of meiosis . . . " This hypothesis could well explain the lack of seed transmission of plant viruses which cause a reduction in the amount of seed and pollen produced, but there are many plant viruses which induce no such effect.

Attempting to explain the lack of seed transmission of tobacco mosaic virus Duggar (1930) suggested that the virus was inactivated by some "specific protein or other specific material" in the seeds. Experimental evidence in support of this theory was published by Kausche (1940) who showed that the addition of aqueous extracts of tobacco seed to purified tobacco mosaic inoculum could reduce its infectivity by as much as 50 per cent. Kausche suggested that the inactivator has a surface effect on the tobacco mosaic virus molecule which makes it non-infective, but his results can be equally well interpreted as demonstrating the existence in tobacco seed of a substance or substances capable of inhibiting infection of *Datura stramonium* by tobacco mosaic virus.

Work described here has been carried out to determine whether inhibitors or inactivators of plant viruses do exist in the seeds of their hosts, and to determine the nature of their action.

II. Materials and Methods

The strain of cucumber mosaic virus used was obtained from Mr. L. L. Stubbs, of the Department of Agriculture, Victoria. Inoculum for all experimental work with cucumber mosaic virus was obtained from 3-week old cucumber seedlings within 10 days of their being inoculated. The seedlings were ground in composite phosphate buffer at pH 7, filtered through muslin, and diluted with more buffer until the preparation consisted of 1 volume of infective sap in 24 volumes of buffer. All cowpea seedlings used for assay of cucumber mosaic virus were of the variety Blackeye from seed of Californian origin. The technique used for inoculating cowpeas has been described previously (Crowley 1954). All experiments carried out with cowpeas involved either two or four treatments. Those with two treatments were carried out by whole leaf comparisons, using 20 replicates. The experimental points in Figures 1 and 3 were obtained in this manner. Experiments involving four treatments were carried out by the half-leaf technique with 16 replicates of each treatment in a randomized block design arranged so that each treatment occurred on every plant, and occurred an equal number of times in each leaf position. Extracts of seed tissues used in work with cucumber mosaic virus, except where otherwise stated, were prepared by grinding 1 g of tissue in 20 ml of distilled water and filtering through muslin before use. Five ml of this extract were than added to 5 ml of virus inoculum.

The inoculum used in all work with tobacco mosaic virus was obtained by diluting a sample of purified virus preparation (kindly supplied by Dr. R. J. Best) in 1000 volumes of composite phosphate buffer at pH 7. All *Nicotiana glutinosa* plants used were raised in a warmed insect-proof glass-house, and when approx. 10 weeks old were dusted with carborundum powder and inoculated with a glass spatula. All experiments with tobacco mosaic virus were

carried out by the half-leaf technique, all treatments occurring an equal number of times on left and right halves. Twenty or more replicates were used in all experiments. The experimental points in Figures 2 and 4 are means of 24 replicates, six, each with four leaves, being used for each dilution. The eight treatments used to obtain the results presented in Table 7 were each replicated 40 times in five 8 × 8 latin squares in which each treatment occurred on every plant and occurred an equal number of times in each leaf position. Extracts of tobacco seed used in work with tobacco mosaic virus were prepared

TABLE 1

EFFECT OF SEVERAL DILUTIONS OF AQUEOUS CUCUMBER EMBRYO EXTRACTS ON LESION PRODUCTION BY CUCUMBER MOSAIC VIRUS ON COWPEA

Extract From	Mean Number of Lesions per Half Leaf			
	Material Added to Inoculum			
	Extract	Extract/10	Extract/100	Control Dist. Water
Cucumber embryos	0***	8***	71***	176
Cucumber testas	0***	6***	90**	138
Wild cucumber embryos	2***	136*	171	182
Wild cucumber testas	55***	152	147	152

* Difference from control significant at $P = 0{\cdot}05$.
** Difference from control significant at $P = 0{\cdot}01$.
*** Difference from control significant at $P = 0{\cdot}001$.

by grinding 2 g of Blue Prior tobacco seed in 10 ml of phosphate buffer at pH 7 over a period of 3-5 hr and then filtering through muslin. After it was found that the inhibitor was heat-stable the extract was partially purified by heating in boiling water for 10 min and again filtering. Portions of this extract were then added to equal volumes of virus inoculum.

III. EXPERIMENTAL

(*a*) *Effect of Seed Extracts on Plant Viruses*

Two viruses, tobacco mosaic and cucumber mosaic, were selected for this work; tobacco mosaic virus because it had been used for similar work by Kausche, and because of all the plant viruses known the rarity of seed transmission of the virus is possibly the most puzzling; cucumber mosaic virus because it provided an example of a highly infectious plant virus, seed-transmitted in one host (wild cucumber, *Echinocystus lobata*) and not seed-transmitted in another (cucumber, *Cucumis sativa*). It was hoped that a comparison of

the results of work using seed extracts of these two hosts on one virus where seed transmission occurred in only one of the hosts would give an indication of the validity of Duggar's theory of inactivation by seed extracts. However, recent trials at the Waite Institute have shown that the seed transmission of cucumber mosaic virus in wild cucumber is much less frequent than reported by Doolittle and Gilbert (1919). Only one infected seedling occurred amongst more than 500 seedlings raised from seeds of infected plants.

The results of experiments carried out with both of these viruses are shown in Tables 1 and 2.

TABLE 2

EFFECT OF BUFFERED AQUEOUS EXTRACT OF TOBACCO SEED ON LESION PRODUCTION BY TOBACCO MOSAIC VIRUS ON *N. GLUTINOSA*

Treatment	Number of Lesions per Half Leaf
Inoculum + buffer	141
Inoculum + extract	37***

*** Difference significant at $P = 0{\cdot}001$.

The results demonstrate the existence of some water-soluble constituent in seeds which can greatly reduce the number of lesions produced by both cucumber mosaic and tobacco mosaic viruses on their respective hosts.

With cucumber mosaic virus, embryo extracts were much more effective than testa extracts in reducing the number of lesions produced on cowpea. Cucumber extracts were regularly found to be more effective than wild cucumber extracts.

With tobacco mosaic virus, whole seeds were used as the small size of seeds did not permit the dissection of large numbers. The addition of tobacco seed extract to tobacco mosaic virus inoculum consistently resulted in a significant reduction in the number of lesions produced; but the extracts used here were four times as concentrated as those reported by Kausche to produce a similar effect. This is attributed to the fact that Kausche used a different variety of tobacco seed.

(*b*) *Nature of the Action*

It is impossible to tell in any simple manner whether a treatment which reduces the number of lesions produced by a given virus inoculum produces its effect by an effect on the host used for measuring infectivity, or by inactivating the virus. A number of ways in which the two processes can be distinguished have been suggested by several workers (Caldwell 1935; Slagle, Wolcyrz, and Price 1952; Bawden and Freeman 1952). Inhibitors, whose effect is primarily to affect the susceptibility of the host used, can be distinguished from virus inactivators by possessing the following characteristics: they are capable of instantaneous effect; their effect is dependent on the host used;

they have greater effect on concentrated inocula; the effect is diminished by dilution; they have an effect when applied prior to inoculation or when applied to the under surface of leaves.

TABLE 3

INSTANTANEOUS EFFECT OF CUCUMBER EMBRYO EXTRACT IN INHIBITING LESION FORMATION BY CUCUMBER MOSAIC VIRUS ON COWPEA

Inoculation Time	Mean Number of Lesions per Half Leaf	
	Extract	Control
Immediately after mixing	9·8	94·4
4 Hr after mixing	4·8	24·6

The experiments described below, using all these methods, were carried out to determine the nature of the action of aqueous extracts both from cucumber embryos and tobacco seed.

TABLE 4

INSTANTANEOUS EFFECT OF TOBACCO SEED EXTRACT IN INHIBITING LESION FORMATION BY TOBACCO MOSAIC VIRUS ON *N. GLUTINOSA*

Inoculation Time	Mean Number of Lesions per Half Leaf	
	Extract	Control
Immediately after mixing	26·9	85·1
5 Hr after mixing	22·2	84·7
Immediately after mixing	26·4	94·9
24 Hr after mixing	20·1	89·1

(i) *Reaction Time.*—The results in Tables 3 and 4 show that the effect of both of the extracts used is just as great immediately (within 30 sec) after addition to the inoculum as it is several hours later. The decline in the infectivity of the treatments was consistently found to be of the same order as the decline in the infectivity of the controls, and was not more than normally occurs through a decline in the infectivity of inoculum with age.

(ii) *Effect of Host Used.*—The results of a typical experiment in which four different hosts were used to measure the infectivity of the inocula are set out in Table 5 and show that the effect of cucumber embryo extract is dependent on the host used for infectivity measurements. It is not thought possible

that these differences in the results are due to differences in the susceptibility of the different hosts, as in trials carried out concurrently with this work the dilution end-point of inoculum was found to be 1 in 10,000, using both cucumbers and cowpeas.

Similar trials could not be carried out with tobacco mosaic virus because no local lesion host other than *N. glutinosa* was available and even on this host it was impossible to inhibit local lesion production completely. Hence the use of any host producing systemic symptoms on infection would be futile, because 100 per cent. infection would always result.

TABLE 5

INFECTIVITY OF MIXTURES OF CUCUMBER MOSAIC VIRUS AND CUCUMBER EMBRYO EXTRACTS TO DIFFERENT HOSTS

	Host			
	Cowpea	Cucumber	*N. glutinosa*	Tobacco
	Mean Number of Lesions per Leaf	Proportion of Plants Infected		
Treatment	0	20/20	2/2	1/2
Control	69	20/20	2/2	2/2

(iii) *Dilution of Extract-Inoculum Mixtures.*—Gupta and Price (1950), in studying the nature of the effect of fungal extracts on plant viruses, showed that non-infective mixtures could be made infective simply by dilution and they concluded that the fungal extracts did not inactivate the virus but that "either the inhibitory agent enters into a reversible combination with the virus or that it alters host susceptibility."

The results of several experiments carried out with two viruses, using a series of dilutions of an extract-inoculum mixture, are graphed in Figures 1 and 2.

The ratio of extract concentration to inoculum concentration does not alter throughout the series of dilutions, yet the reduction in lesion production induced by both inhibitors becomes progressively less as the mixture is diluted. In fact, the converging nature of the dilution curves indicates that it would theoretically be possible to overcome completely the effect of either inhibitor simply by sufficiently diluting a mixture of inoculum and extract. Thus, either the inhibiting constituents of the extracts are combined with the virus in some way that is readily dissociated by dilution, or their effect is on the host used for the infectivity tests and their tolerance of dilution is less than that of the virus.

(iv) *Effect of Inoculum Concentration.*—Caldwell (1935) showed that substances inhibiting infection by a virus could be distinguished from substances inactivating a virus because inhibitors have their greatest effect on concentrated

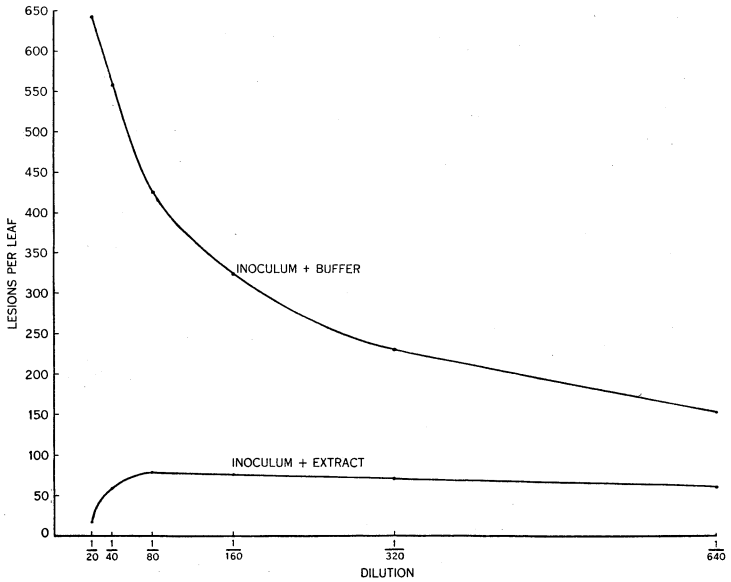

Fig. 1.—Effect of diluting a mixture of cucumber mosaic virus inoculum and cucumber embryo extract with buffer, on the infectivity of the mixture to cowpea.

TABLE 6

EFFECT OF CUCUMBER SEED EXTRACT ON LESION PRODUCTION BY COWPEA WHEN APPLIED BEFORE OR AFTER APPLICATION OF CUCUMBER MOSAIC VIRUS INOCULUM

Time of Application of Treatment Relative to Time of Application of Inoculum	Mean Number of Lesions per Half Leaf Following Treatment with:	
	Cucumber Seed Extract	Distilled Water
−48 Hr	1·1***	145
−24 Hr	0***	60
+24 Hr	191	194

*** Differences significant at $P = 0{\cdot}001$.

inocula, whereas inactivators have their greatest effect on diluted inocula. Figures 3 and 4 show the results of experiments where constant amounts of extracts were added to a series of dilutions of cucumber and tobacco mosaic virus inocula. With both viruses the reduction in lesion numbers was consistently found to be greatest with concentrated inocula and it is concluded that the action of both seed extracts used is purely that of an inhibitor of virus infectivity.

(v) *Separate Applications of Extract and Inoculum.*—Two attempts were made to determine whether the action of the extracts was primarily on the host, or on the virus, by inoculating plants with the extract and the virus inoculum

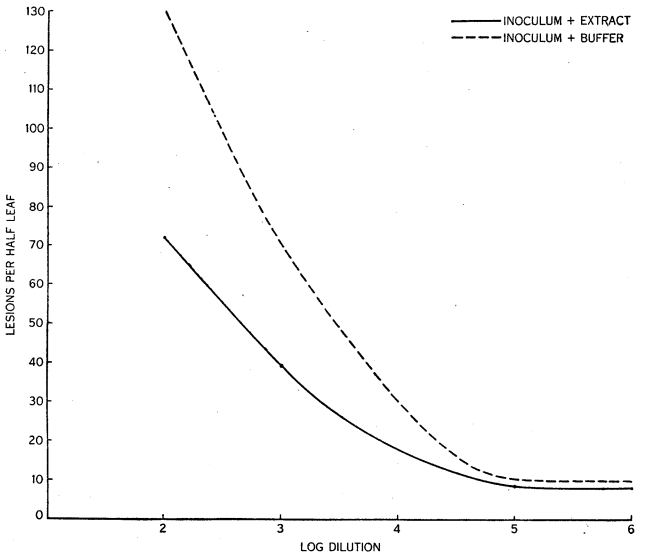

Fig. 2.—Effect of diluting a mixture of tobacco mosaic virus inoculum and tobacco seed extract with buffer, on the infectivity of the mixture to *N. glutinosa.*

separately. The first method was to treat the cowpea and *N. glutinosa* plants with the extract at various periods before and after inoculation with the virus inoculum.

TABLE 7

EFFECT OF TOBACCO SEED EXTRACT ON LESION PRODUCTION BY *N. GLUTINOSA* WHEN APPLIED BEFORE OR AFTER APPLICATION OF TOBACCO MOSAIC VIRUS INOCULUM

Time of Application of Treatment Relative to Time of Application of Inoculum	Mean Number of Lesions per Half Leaf Following Treatment with:	
	Tobacco Seed Extract	Distilled Water
−48 Hr	27·2**	63·5
−24 Hr	25·7**	73·3
Mixed with inoculum	3·8**	62·4
+24 Hr	56·0	58·6

** Differences significant at $P = 0{\cdot}01$.

The results in Tables 6 and 7 show that both the seed extracts used were able to induce a highly significant reduction in the number of local lesions produced by their respective hosts when applied 1 or 2 days prior to the application

of the virus inoculum. From this it is concluded that both inhibitors are capable only of interfering with infection by their respective viruses. They are incapable of interfering with virus multiplication because both are incapable of producing any effect after infection has taken place.

The second method of separate application of the extracts and inocula was to apply the extract to the under surface of the leaves 24 hr before applying the virus inoculum to the upper surface.

TABLE 8

EFFECT OF UNDER-SURFACE PRE-TREATMENT WITH CUCUMBER EMBRYO EXTRACT ON LESION FORMATION BY CUCUMBER MOSAIC VIRUS ON COWPEA

Leaf Under-Surface Pre-treated with:	Mean Number of Lesions per Leaf		
	Dilution of Extract		
	Undiluted	1/10	1/100
Extract	23	27*	32
Distilled water	35	33	29

* Difference significant at $P = 0{\cdot}05$.

The results in Table 8 show that extracts of cucumber embryos can induce a significant reduction in the number of local lesions produced on cowpeas when applied in this manner. This also indicates that the effect of the cucumber embryo extract is to reduce the susceptibility of cowpeas to infection. In similar experiments carried out with tobacco mosaic virus a significant reduction in lesion numbers could not be induced by applying tobacco seed extracts to the under surface of *N. glutinosa* leaves. This is attributed firstly to the fact that tobacco seed extracts were always far less effective than cucumber embryo extracts, and secondly to the hairy nature of the under surface of *N. glutinosa* leaves that makes them most difficult to wet.

(*c*) *Nature of the Inhibitors*

Investigations were carried out to determine the nature of the constituents present in cucumber and tobacco seeds which are responsible for the inhibition.

The inhibitor from cucumber embryos was found to be heat-labile, non-dialysible, and can be precipitated from solution by alcohol or half-saturated ammonium sulphate. The inhibitor from tobacco seed was heat-stable, non-dialysible, and was precipitated from solution by 60 per cent. alcohol, but not by three-quarters-saturated ammonium sulphate. In tests kindly carried out by Dr. R. J. Swaby, of the Division of Soils, C.S.I.R.O., both substances were identified as proteins by their electrophoretic mobility and their staining reaction with bromphenol blue (Swaby, unpublished data). This finding is at variance

with the conclusion reached by Kausche that the virus-inhibiting substance present in tobacco seeds belongs to the amino-alcohol group, but he presented no evidence that either supports his claim or is at variance with that presented here.

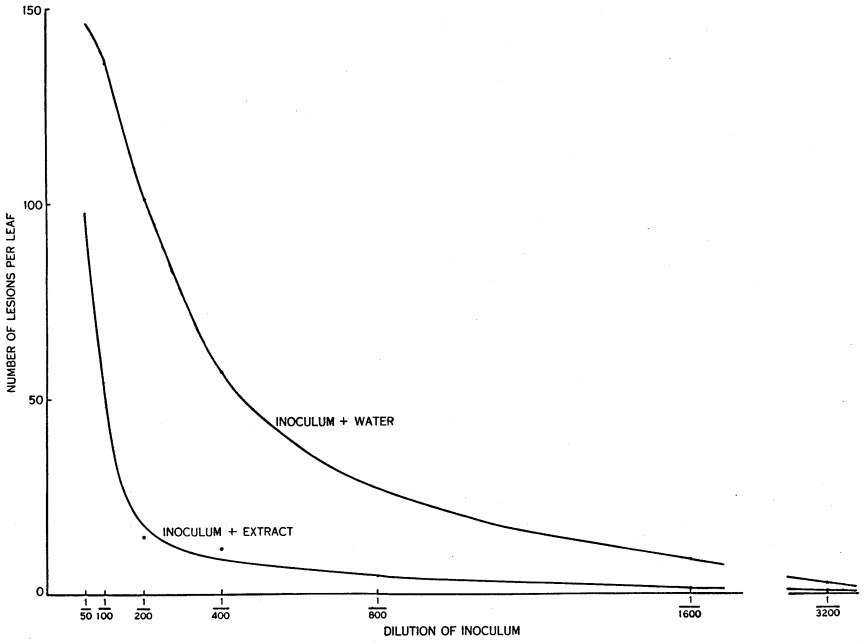

Fig. 3.—Effect of a constant amount of cucumber embryo extract on infectivity of several dilutions of cucumber mosaic virus to cowpea.

IV. Discussion

The presence of an inhibitor of cucumber mosaic virus in the seeds of cucumber, and of tobacco mosaic virus in the seeds of tobacco, has been demonstrated. Six separate lines of evidence all indicate that the effect of these substances is, by some action on the host, to inhibit infection by the virus.

It is not thought possible that the presence of these inhibitors in the embryos of seeds can prevent the infection of embryos by plant viruses, for the inhibitors are also present in other tissues which undoubtedly can be infected. In studies carried out recently at Waite Institute on the distribution of viruses in the tissues of seeds, cucumber mosaic virus was found to be present in 92 per cent. of the testas, and only 4 per cent. of the embryos of mature seeds of wild cucumber, despite the fact that the inhibitor is present in approximately the same concentration in both tissues (see Table 1). Sill and Walker (1952) reported the presence of an inhibitor (possibly the same one as described here) of cucumber mosaic virus in all of the tissues, except the corolla, of cucumber plants. Yet no one would dispute the fact that the virus can infect these tissues, and multiply in them. It is far more likely that the action of the inhibitors is upon the local lesion host used for measuring virus infectivity. Its action could

be either to attach itself to the receptor sites in the cells at which virus multiplication is presumed to commence; or, as suggested by Bawden and Freeman (1952), for an inhibitor from the fungus *Trichothecium roseum* to "so alter the physiology of the host cells that they no longer support virus multiplication."

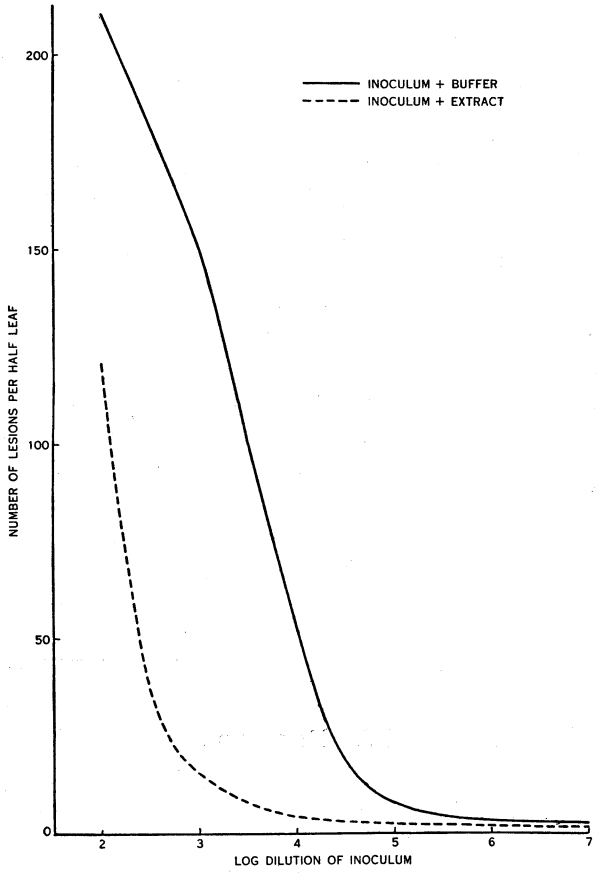

Fig. 4.—Effect of a constant amount of tobacco seed extract on infectivity of several dilutions of tobacco mosaic virus to *N. glutinosa.*

It might seem possible that inactivators could be present in the embryos of seeds in addition to the inhibitors described here, but if this were so it should be possible to demonstrate their presence by incubating seed extracts with viruses for some hours. Their presence would then be manifested by a greater decline in infectivity than would occur through normal aging of the inoculum. This was found not to be the case (see Tables 3 and 4).

It would seem that Duggar's theory that inactivators in seeds prevent the seed transmission of plant viruses must be abandoned as an explanation for the rarity of seed transmission of plant virus diseases, unless the inactivator is some transitory product of the metabolism of embryos. Such a transitory

product could be an enzyme or group of enzymes which breaks down virus particles, together with the other proteins of the endosperm, prior to their absorption by the developing embryo, and re-synthesis into embryo proteins. Inactivators of such a nature could inactivate viruses without ever accumulating to such an extent that their presence could be detectable by the usual infectivity techniques.

V. Acknowledgments

The author wishes to acknowledge the helpful suggestions made by Dr. N. T. Flentje and Dr. R. J. Best on many aspects of the work; the help of Dr. R. J. Swaby in identifying the two proteins studied; of Dr. C. G. Hansford and Dr. N. T. Flentje in the preparation of the manuscript; of Mrs. I. Mathison for much help in the statistical design and analysis of experiments, and of Miss H. Lewis for able technical assistance.

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