SOME VARIABLES AFFECTING THE USE OF COWPEA AS AN ASSAY HOST FOR CUCUMBER MOSAIC VIRUS

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Summary

A study was made of the factors affecting the production of local lesions by cucumber mosaic virus on cowpea (*Vigna sinensis* (L.) Endl. ex Hassk.).

The following facts have been established:

Cowpea is a satisfactory host for quantitative work with cucumber mosaic virus at dilutions of inoculum from 1/20 to 1/200. The half-leaf technique can be used to advantage.

Cucumber mosaic virus inoculum is most infectious at pH 8, has greatest longevity at pH 7, and will tolerate a pH range from 4 to 10.

Alauxite and carborundum (both 500-mesh) were superior to all other abrasives tested. Washing of inoculated leaves did not increase the number of lesions developed.

More lesions developed on cowpea plants placed under fluorescent lights at 200 f.c. than on plants placed either in the glass-house or under higher light intensities.

The rapid drying of inoculated leaves increased the number of lesions developed.

The use of leaf-culture techniques has not made possible a reduction in the variation between plants.

I. Introduction

Sill and Walker (1952) recently published a description of the technique they developed using cowpea (*Vigna sinensis* (L.) Endl. ex Hassk.) as an assay host for cucumber mosaic virus and reviewed the relevant literature. In virus investigations at Waite Institute a similar technique had been developed independently. In the main our findings have confirmed those of Sill and Walker but additional factors which are of importance in quantitative work have been investigated and are published as an extension of Sill and Walker's work.

II. Materials and Methods

The strain of cucumber mosaic virus used was obtained from Mr. L. L. Stubbs, of the Department of Agriculture, Victoria, and had been isolated by him from naturally infected spinach. Inoculum for all experimental work was extracted from 3-weeks-old cucumber seedlings within 10 days of their being inoculated.

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All cowpea seedlings used were of the variety Blackeye from seed of Californian origin which was obtained through Mr. W. Hartley, of the Division of Plant Industry, C.S.I.R.O., Canberra.

All cowpea leaves were inoculated by means of two strokes towards the tip with a ground-glass spatula, as this was found to result in the production of more lesions than other methods. This is not in agreement with the findings of Sill and Walker (1952), who used a circular stroking method with a glass spatula on all leaves, but the most successful inoculation technique may be expected to vary with different workers. Plants used were raised individually in small pots of John Innes compost, placed in semi-darkness in the laboratory when 13-15 days old, inoculated the following day, and placed in an artificially illuminated growth chamber immediately after inoculation.

Experiments involving only four treatments were carried out by the half-leaf method, using 16 or 20 replicates of each treatment in a randomized block design so that each treatment occurred on every plant, and occurred an equal number of times in each leaf position. Where larger numbers of treatments were necessary, experiments were carried out either in an incomplete block design, or by whole-leaf comparisons where it was imperative that no run-over of inoculum should occur.

III. Experimental

The work of Sill and Walker (1952) has been independently confirmed in the following particulars:

(1) The variety Blackeye is one of the most susceptible varieties of cowpea to cucumber mosaic virus;

(2) Cowpea seedlings are most susceptible when 14-16 days old;

(3) Their susceptibility is greatly increased by keeping the plants in darkness for 24 hr prior to inoculation;

(4) Tobacco (*Nicotiana tabacum*) is a more infective source of cucumber mosaic virus than either *N. glutinosa* or cucumber (*Cucumis sativa*). Additional data have been obtained from the present work and are discussed in detail below.

(*a*) *Effect of Dilution*

In quantitative work with plant viruses using the local lesion technique, it was shown by Best (1935), working with tobacco mosaic and tomato spotted wilt viruses, that the numbers of local lesions produced are directly proportional to virus concentration only over a limited portion of the dilution range; i.e. only over the "straight line portion where a change in relative concentration is accompanied by an equivalent change in resultant lesions." No dilution curve for cucumber mosaic virus has been published although a knowledge of the effect of dilution is essential for all quantitative work. Hence an experiment was carried out using 24 replicates of each dilution in an incomplete block design covering the range from undiluted infective sap to a dilution of 1 in 5120. The results are graphed in Figure 1.

These results were verified by repeated experiments. The sharp rise in infectivity on first diluting infective sap is probably due to several factors; firstly, buffering is known to increase the infectivity of this virus; secondly, dilution would result in the dispersion of virus aggregates and thus result in a higher concentration of infective units; and thirdly, dilution would also result in the dilution of toxic constituents of plant sap which inhibit virus infection.

The steeply sloping portion of the curve occurs over the dilution range from 1/10 to approximately 1/150. At lower dilutions, 1/5-1/10, and at all dilutions greater than 1/200 only small changes in lesion numbers result from large changes in dilution.

In quantitative work with cucumber mosaic virus at Waite Institute, a dilution of 1/50 is generally used, being near the centre of the straight-line portion of the curve, and producing lesions in numbers sufficiently large for accurate counting. It is, however, preferable where possible to use several dilutions.

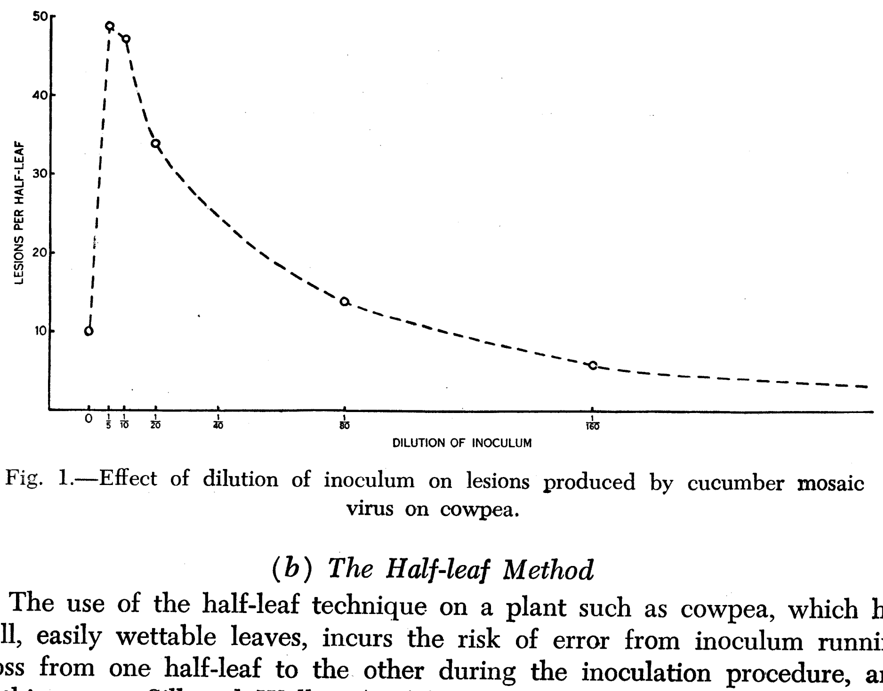

Fig. 1.—Effect of dilution of inoculum on lesions produced by cucumber mosaic virus on cowpea.

(*b*) *The Half-leaf Method*

The use of the half-leaf technique on a plant such as cowpea, which has small, easily wettable leaves, incurs the risk of error from inoculum running across from one half-leaf to the other during the inoculation procedure, and for this reason Sill and Walker (1952) used whole-leaf comparisons in their investigations. As the half-leaf method allows the comparison of four treatments on every plant, thus increasing the experimental accuracy by spreading the effects of the between-plant differences on double the number of treatments, and frequently making possible the elimination of these differences in the analysis of the results, it was thought that the advantages of using this technique might outweigh the disadvantages. Experiments were devised to determine the magnitude of the error due to run-over of inoculum. Table 1 shows the results of experiments in which lots of 20 plants were inoculated on one half-leaf with a

1/40 dilution of infective cucumber sap and on the other half-leaf with distilled water.

TABLE 1

AMOUNT OF RUN-OVER OF INOCULUM USING THE HALF-LEAF METHOD

Inoculum Used	Average Lesions per Half-leaf				
	Experiment				
	1	2	3	4	Mean
Infective inoculum	61	80	146	103	98
Distilled water	0·4	0·1	0·3	0·2	0·2

Table 2 shows the results of an experiment to determine whether there was any significant difference in the number of local lesions produced by a weak inoculum, firstly when the same inoculum was used on the other half of each leaf, and secondly when a strong inoculum was used on the other half of each leaf.

TABLE 2

EFFECT OF RUN-OVER OF A STRONG INOCULUM ON NUMBER OF LESIONS PRODUCED BY A WEAK INOCULUM ON THE SAME LEAF

Treatment		Average Lesions per Half-leaf		
		Leaf Half		
		Left	Right	Mean
Weak with weak.	Weak	2·0	2·3	2·1
Weak with strong.	Weak	2·5	2·6	2·6
	Strong	187	171	179

Differences between treatments or treatment means not significant at $P = 0{\cdot}05$.

The results of both experiments indicate that a slight "run-over" from one half-leaf to another does occur but it is not of sufficient magnitude to produce a significant difference in the results of any treatment. It is concluded that the half-leaf method is the most satisfactory for use in quantitative work with cucumber mosaic virus on cowpea, except in experiments such as dilution curves or pH-infectivity determinations where it is imperative that no "run-over" should occur.

(*c*) *Effect of Abrasives*

Various abrasives have been used to facilitate the mechanical inoculation of plant viruses since the first demonstration of their value by Rawlins and Thompkins (1934), who showed that several viruses which were otherwise difficult to transmit mechanically could be readily transmitted with the aid of carborundum powder. Costa (1944) first reported the use of carborundum powder in the inoculation of cowpea var. Black with cucumber mosaic virus. He also showed marked differences to exist in the effectiveness of different grades of carborundum and alauxite in promoting infection of a tobacco hybrid by tobacco mosaic virus.

As not all of the abrasives used by Costa were available in Australia, and because of the recent tendency by some virus workers to use celite in preference to carborundum (Kalmus and Kassanis 1945; Hutton and Peak 1951) several of the available abrasives were tested to determine which was the most efficient for quantitative work with cucumber mosaic virus on cowpea. The results are set out in Table 3.

TABLE 3

COMPARISON OF EFFICIENCY OF ABRASIVES IN INOCULATING COWPEAS

Abrasive	Average Number of Lesions per Half-leaf				
	Experiment				
	1	2	3	4	Mean
None	7	109	25	7	37
Increase over controls with					
Carborundum 3F	39***	168***	60***	97***	91
Alauxite 400	55***	175***	57***	61***	87
Hyflo Super Cel	24***	41*	21*	35***	30

* Significant at $P = 0 \cdot 05$.
*** Significant at $P = 0 \cdot 001$.

Earlier experiments had shown 3F carborundum and 400 alauxite to be superior to any coarser grades of either abrasives though the finer (500-mesh) grades of both are slightly superior. Carborundum and alauxite are clearly most efficient inoculation aids and both resulted in up to a tenfold increase in the number of lesions developed; both were far superior to celite (Hyflo Super Cel).

(*d*) *Washing off the Inoculum*

Since the first report of Holmes (1929) that the washing of inoculated leaves with a jet of water immediately after inoculation increases, rather than decreases the number of lesions produced, workers have adopted the technique of washing inoculated leaves shortly after inoculation. Samuel and Bald (1933) showed that washing off the inoculum immediately after inoculation resulted in a large increase in local lesions developed when undiluted infective sap was used as inoculum, but only a slight increase when dilute buffered inoculum was used. They support the suggestion of Holmes that washing off removes some plant constituents in the inoculum which have toxic effects on the slightly damaged cells of the inoculated leaf. In dilute inoculum the constituents would be correspondingly diluted before inoculation, and thus less effective.

TABLE 4

EFFECT OF WASHING INOCULATED LEAVES AFTER VARIOUS TIME INTERVALS

Time Interval	Average Lesions per Half-leaf				
	Experiment				
	1	2	3	4	Mean
Immediately	51	170	57	64	85
30 Sec	67**	192	64	85*	102
5 Min	76**	223**	76**	79	114
Unwashed	56	211*	72**	79	105

* Significant at $P = 0 \cdot 05$.
** Significant at $P = 0 \cdot 01$.

A series of experiments was carried out to determine the importance of this factor in local lesion production by cucumber mosaic virus on cowpea. The results tabulated in Table 4 indicate that this factor is not of critical importance. Variations of up to 30 min in the interval between inoculations and washing off produced small differences in the number of lesions developed. It is concluded that in quantitative work washing off should not be carried out as it adds another variable to all experiments and enhances the risk of "run-over" from one half-leaf to another. The lack of agreement between these results and those of previous workers is attributed to the use of dilute buffered inoculum (1/50) in which any toxic constituent would be correspondingly diluted before inoculation.

(e) *Effect of pH on Infectivity of Inoculum*

Published data on the effect of pH on the infectivity of cucumber mosaic virus appear conflicting. Stanley (1935) reported that "cucumber mosaic is inactivated as the pH is increased or decreased from 5.7." Nienow (1948) reported the optimum pH for the inoculation of cowpea leaves to be 6.5. Bearing indirectly on this Uppal (1934) reported buffers of pH 6.6-7.2 to be the most effective in eluting the virus.

As these reports are thought to be rather variable investigations were carried out to determine at what pH inoculum would induce most lesions on cowpea. The effect measured is undoubtedly one affecting both the plant and the virus, but for the purpose of these investigations it is information on this interaction which is sought.

Inoculum buffered in composite buffer (Best and Samuel 1936) at pH 8 consistently produced significantly more lesions than inoculum buffered at either higher or lower pH.

Several experiments were carried out to compare the infectivity of cucumber mosaic virus at pH values from 2.0 to 12.0.

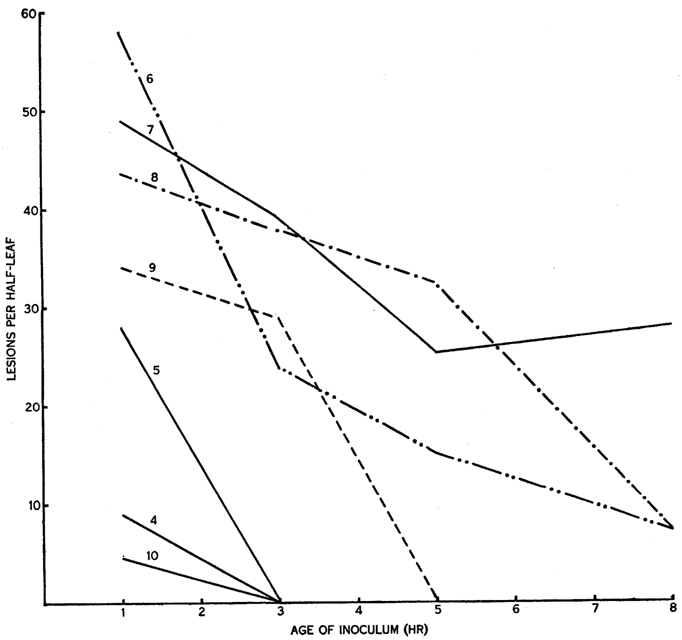

Fig. 2.—Longevity of cucumber mosaic virus *in vitro* at pH values from 4 to 10.

It was found that the values obtained varied in different experiments, depending on the period of time for which the inoculum was kept at the different pH values. It was concluded that the only satisfactory method of investigating the pH range of the virus is to determine the longevity of inoculum at different pH values. It is clear from the experimental results shown in Figure 2 that the pH range of cucumber mosaic virus varies markedly with the period the virus is exposed to that pH. Inoculum less than an hour old is infective at all pH values from 3.0 to 10.0, while inoculum 8 hr old is infective only over the pH range from 6.0 to 8.0. It follows that it is best to carry out all quantitative work with cucumber mosaic virus inoculum buffered to pH 7.0-8.0, for within this pH range the decline in infectivity over a period is least.

(*f*) *Effect of Illumination Conditions on Lesion Development*

Numerous workers have demonstrated the marked effect of light intensity on virus multiplication and on symptom development by virus-infected plants. The influence of this factor on lesion production by cowpea was investigated.

It was found at an early stage that more lesions developed on plants placed under fluorescent lights than on those replaced in the glass-house after inoculation. Illumination conditions under the artificial lights were found to affect greatly the number of lesions developed and experiments were conducted to determine the most favourable illumination conditions for lesion production. The results are tabulated in Table 5. A low light intensity of 200 f.c. has consistently resulted in the production of more lesions than an 800 f.c. intensity, while a continuous illumination at either intensity resulted in consistently more lesions than either 12 hr illumination per 24 hr or continuous darkness.

TABLE 5

EFFECT OF POST-INOCULATION LIGHT INTENSITY OF LESION PRODUCTION

Treatment	Average Lesions per Leaf				
	Experiment				
	1	2	3	4	Mean
800 f.c.	47	238	53	51	45
200 f.c.	114***	416***	80***	97***	77

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

The results indicate that a light intensity as high as 800 f.c. inhibits the multiplication of cucumber mosaic virus, possibly through accumulation of products of photosynthesis, which at a lower light intensity may be produced only at a sufficient rate to supply the needs of the plant respiration at that temperature.

(*g*) *Rapid Drying of Inoculated Leaves*

Recently Yarwood (1952) reported that the number of lesions produced by tobacco mosaic virus on *N. glutinosa* could be increased more than sevenfold by rapidly drying the inoculated leaves within 10-15 sec of inoculating.

Several experiments were carried out with cucumber mosaic virus on cowpea in which one leaf of each plant inoculated was washed and dried by means of a stream of warm air within 20-30 sec of the completion of the inoculation; the other leaf of every plant was washed and allowed to dry in the ordinary way; this taking about 30 min. The results of four experiments are set out in Table 6.

Rapid drying has consistently resulted in a significantly higher lesion production by cowpeas, but the effect is not sufficient to greatly increase the sensitivity of the reaction, and in quantitative work the addition of another factor which could increase the variation between plants is most undesirable.

(*h*) *Leaf-culture Techniques*

An attempt was made to reduce the variability between cowpea plants by detaching all leaves soon after inoculation and keeping them alive in leaf culture on tap water, or on a 10 per cent. sucrose solution in petri dishes until the lesions developed were countable. The technique used was one of several described by Yarwood (1946). Using this method more lesions were produced on detached leaves in petri dishes than on leaves still attached to the plant, but the lesions produced tended to be of a more spreading nature, making accurate counting difficult. In addition, the variation in the results of lesion counts on leaves in the petri dishes was no less than the variation in lesion counts of leaves attached to the plant, indicating that the more constant post-inoculation environment was not important.

TABLE 6

EFFECT OF RAPID DRYING OF INOCULATED LEAVES ON LESION PRODUCTION

Treatment	Average Lesions per Leaf				
	Experiment				
	1	2	3	4	Mean
Dried	77***	34***	74*	77	66
Undried	58	18	53	71	50

* Significant at $P = 0 \cdot 05$.
*** Significant at $P = 0 \cdot 001$.

(*i*) *Statistical Analysis*

In studies of the frequency distribution of local lesions produced by tobacco mosaic and tomato bushy stunt viruses, Kleczkowski (1949) found both to be skew and 'leptokurtic,' the standard error increasing with the mean, and he has put forward a transformation, $y = \log_{10}(x + c)$, where x is the number of lesions and c a constant.

An experiment was carried out to determine the value of the constant c, using Kleczkowski's method. Fifty cowpeas were inoculated on all four half-leaves with a 1/50 dilution of infected cucumber sap.

Analysis of results of the lesion counts showed the regression of the standard error on the mean to be highly significant. A value of 2.6 has been calculated for the constant c, and this transformation has been used for the statistical analysis of all results.

IV. Acknowledgments

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