# Systematic-Host Assay of Sugarcane Mosaic Virus

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### ABSTRACT

Sugarcane mosaic virus was quantitatively bioassayed by systemic infection of seedlings of *Sorghum bicolor* with a degree of accuracy previously considered attainable with plant viruses only by local-lesion assay. Two inocula differing from each other by 10% in viral concentration consistently gave highly significant differences in percentage of test plants infected. Mechanical methods for planting and inoculating permitted handling large numbers of test plants with relatively small effort. The number of plants required was reduced by certain precautions for controlling random variation: (i) the use of sized, disease-free seed; (ii) uniform filling of pots with thoroughly mixed soil; (iii) fumigation of the soil in place in the pots; (iv) uniform depth of planting of the seeds; (v) uniform irrigation of the growing test plants; and (vi) elimination of position effects by growing the test plants in pots arranged in a circle on a rotating table. Data on the number of primary infection sites per systemically infected plant suggest that sometimes the methods for handling large numbers of test plants might profitably be used to extend the range of local-lesion assays. These data also suggest that mass handling techniques could make possible the efficient use of systemic infections for the separation and purification of virus strains, just as single local lesions are commonly used for this purpose. Phytopathology 61:526-531.

*Additional key words:* Infection sites, local-lesion assay, *Sorghum, Saccharum.*

The first local-lesion assay of a virus on a grass host was recently reported by Jean & Sehgal (8). Their test plant, a cultivar of *Sorghum bicolor,* developed both local necrotic lesions and systemic mottling. The virus, maize dwarf mosaic virus (MDMV), is related to sugarcane mosaic virus (SMV) (16, 18, 19). but it is not clear that the relationship warrants classifying MDMV as a strain of SMV. A local-lesion host was also reported for strain E of SMV (4, 5), but this system has been explored only tentatively for assay. The local-lesion host (also a sorghum) for this strain of SMV developed local necrotic lesions without systemic symptoms.

In reporting the first attempt at quantitative bioassay of a plant virus, McKinney (12) noted the need for large populations of test plants. Shortly thereafter, Holmes (6) described techniques enabling him to utilize 2,700 test plants for detecting a 2-fold difference between two viral inocula of tobacco mosaic virus (TMV). He suggested that greater accuracies could be attained if larger numbers of test plants could be used. But in the year following his report of this systemic-host assay, Holmes (7) reported the local-lesion assay of TMV. This method, capable of providing data on a large number of infection sites with use of few test plants, was refined and extended by many workers (10, 15, 17, 20). It came to be regarded as the only accurate method of plant-virus bioassay. Bawden (1) refers pointedly to the inaccuracy and infeasibility of systemic-host assays. Little or no attention has been devoted to the problem of improving the usefulness of systemic-host assays, even though some viruses lack a known local-lesion host. The common procedure has been to delay quantitative work while searching for a suitable local-lesion host. As late as 1964, Roberts (14) noted that only 15-20 of the 400-odd plant viruses had been subjected to quantitative assay. Matthews (11) suggests that there may be some distortion of our overall knowledge of plant viruses because most experimental work has been done with those viruses that have good hosts for local-lesion assay.

It has been demonstrated by several workers that the local-lesion assay, when executed with sufficient care, is capable of detecting 10% differences in viral concentration between two inocula (14). Such accuracy is unusual, however, according to Bawden (1), who notes that it is usually vain to try to detect differences smaller than a factor of two, and that even larger differences go undetected in most routine assays.

The purpose of this paper is to describe a systemic-host assay for SMV capable of detecting 10% differences in viral concentration between inocula compared two at a time, or 2-fold or smaller differences among inocula compared 8-10 at a time. The test plant was a cultivar of *Sorghum bicolor* (L.) Moench. In one experiment, it was possible to compare local-lesion assay with systemic-host assay with data for both methods coming from the same plants; this particular strain-host system gives, on occasion, both local lesions and systemic infection. Secondary purposes are to report this comparison of local-lesion and systemic-host assays, and to note some of the implications of the numbers of primary infection sites per plant in systemic infections. The systemic-host assay described here was developed over a considerable period with gradually increasing accuracy. Only a few of the later experiments are presented to illustrate the capability of the current method.

MATERIALS AND METHODS.—Original seed of the test plant, Mn 1056 sorghum, were supplied by the U.S. Sugar Crops Field Station, Meridian, Miss. Test plants were grown, 25/pot, in square plastic pots, 9.5 × 9.5 cm at the top. Uniform filling of pots was essential in preventing pot-to-pot variability in the rate of drying of the soil after irrigation. A method was developed for rapid filling of pots to within 1% of a predetermined wt. The soil was thoroughly mixed in a mechanical mixer before the pots were filled. The filled pots were fumigated with methyl bromide. Completely healthy

test seedlings were never obtained until the soil was fumigated in place in the pots.

To handle test plants in the numbers required by the assay, a rapid method of planting was essential. A suction planter for planting seeds in pots or other containers was described by Brown et al. (2). A modification of this device, designed for the particular pots to be used (Fig. 1-A), was made of heavy-gauge sheet metal. Suction was provided by a vacuum sweeper. The planter was used essentially as directed by its inventors, except that instead of the vacuum box being inverted to load the perforated plate with seed, the seeds were picked up directly from a shallow box simply by touching the perforated plate to the surface of the seed. Besides the essential speed, the planter also provided precision spacing and depth of planting.

Growing test plants on the greenhouse bench resulted in excessive variation within treatments. Three uniformity trials established that the patterns of variation (position effects) were unstable and could not be controlled through experimental design. Failure to achieve uniform irrigation was apparent, and may have been the chief source of variation in the uniformity trials. Position effects were eliminated by growing the plants in pots arranged in one to three concentric circles around the periphery of a slowly rotating (3 rpm), circular table, 150 cm in diam (Fig. 2). Rotation of the table under timer-controlled misting nozzles resulted in highly uniform dispersal of water over the pots, but water that puddled slightly on the table top was unevenly absorbed through the holes in the bottoms of the pots. This was corrected by nesting each pot containing plants in an empty pot. A uniformity trial on the turntable revealed no position effects.

Irregular emergence and growth of seedlings were also sources of undesirable variability. Examination of the underground portions of seedlings revealed that all were parasitized to some degree by fungi. All field-grown sorghum seeds examined bore pericarp lesions, and could not be freed of fungi by surface treatment with sodium hypochlorite. Seeds produced in the greenhouse during the winter months were free of lesions, and gave rise to disease-free seedlings when planted in properly fumigated soil. To obtain additional uniformity, the seeds were sized on sieves. Only medium and large seeds were used, and these were kept separate for each particular assay. Germination was virtually 100%. The seedlings emerged almost simultaneously, and grew uniformly on the turntable.

Inoculum for all experiments was obtained from 13-day-old Mn 1056 sorghum plants, 7 days after they had been inoculated. The two youngest visible leaves and almost all of the unexpanded tissues above the growing point were used as sources of inoculum. Weighed tissue was triturated in a blender for 1 min with a set quantity of cold buffer, and the pulp was removed by straining the liquid through cheesecloth. The buffer consisted of equal volumes of 0.01 M $K_2HPO_4$ and $KH_2PO_4$, and was adjusted to 0.005 M with the addition of anhydrous $Na_2SO_3$. Further addition of 0.01 M $KH_2PO_4$ brought the pH to 7.0.

Test seedlings were inoculated in the three-leaf stage,

and were suitable 6 days after seeding when greenhouse temperatures were above 32 C. Pots of test seedlings to be inoculated were moved on a conveyor belt past a high-pressure spray of inoculum from an automotive-type paint sprayer (Fig. 1-B). This method of inoculation requires standardization of several variables: (i) air pressure operating the spray gun; (ii) distance from the spray nozzle to the plants; (iii) height above the soil line at which the center of the spray cone strikes the plants; (iv) rate of inoculum output by the spray gun; and (v) rate of movement of the plants through the spray (conveyor-belt speed).

Previous work (3) had shown that air pressure and distance were related so that a change in one could be compensated by an appropriate change in the other. The important factor was the intensity of the blast striking the plants, higher intensities giving higher infection rates up to the limit the plants were able to withstand. However, in the present research, max economy of inoculum (assessed by number of infected plants per ml of inoculum) was obtained at the shortest practicable distance. The distance was set at 7.5 cm (from nozzle to center of pot), and air pressure was regulated to that value (4.6 kg/cm$^2$) which plants could just withstand without breaking off and blowing away.

Maximum infection rate was obtained with the spray cone directed as low (close to the soil line) as possible without blowing soil out of the pots. This was about 2 cm above the soil line. The spray gun was bolted in place so that its position relative to the plants was constant from test to test.

Preliminary tests showed that the inoculum output of the gun and the speed of the conveyor belt were related in such a way that a constant rate of infection was maintained while these factors were varied, provided that they were varied so as to give a constant volume of inoculum per plant. Inoculum output was set at 1.6 ml/sec; belt speed was set to move one pot/sec past the spray gun. Doubling or halving the inoculum output did not affect the infection rate when the belt speed was also doubled or halved in order to maintain an inoculum volume of 1.6 ml/pot of plants inoculated.

Previous results (3) had indicated no benefit from inclusion of 240-mesh silica as an abrasive in the inoculum. A preliminary experiment reconfirmed this result for 420- and 600-grit silicon carbide; consequently, no abrasive was used in the experiments reported here.

Since position effects were not detected on the turntable, a completely randomized design was used. The percentage of infected plants in each pot was calculated; these percentages were subjected to the arc sin transformation for statistical treatment. Local-lesion data, expressed as number of lesions per pot, were transformed by first adding 0.5 to each figure, then extracting the square root. In tests involving assay of more than two inocula, significant differences among means were detected by Duncan's new multiple range test. In tests comparing only two inocula, significant differences between means were identified by the standard t-test for unpaired observations.

RESULTS.—*The relationship of concentration of inoculum to percentage of plants infected.*—Results of this
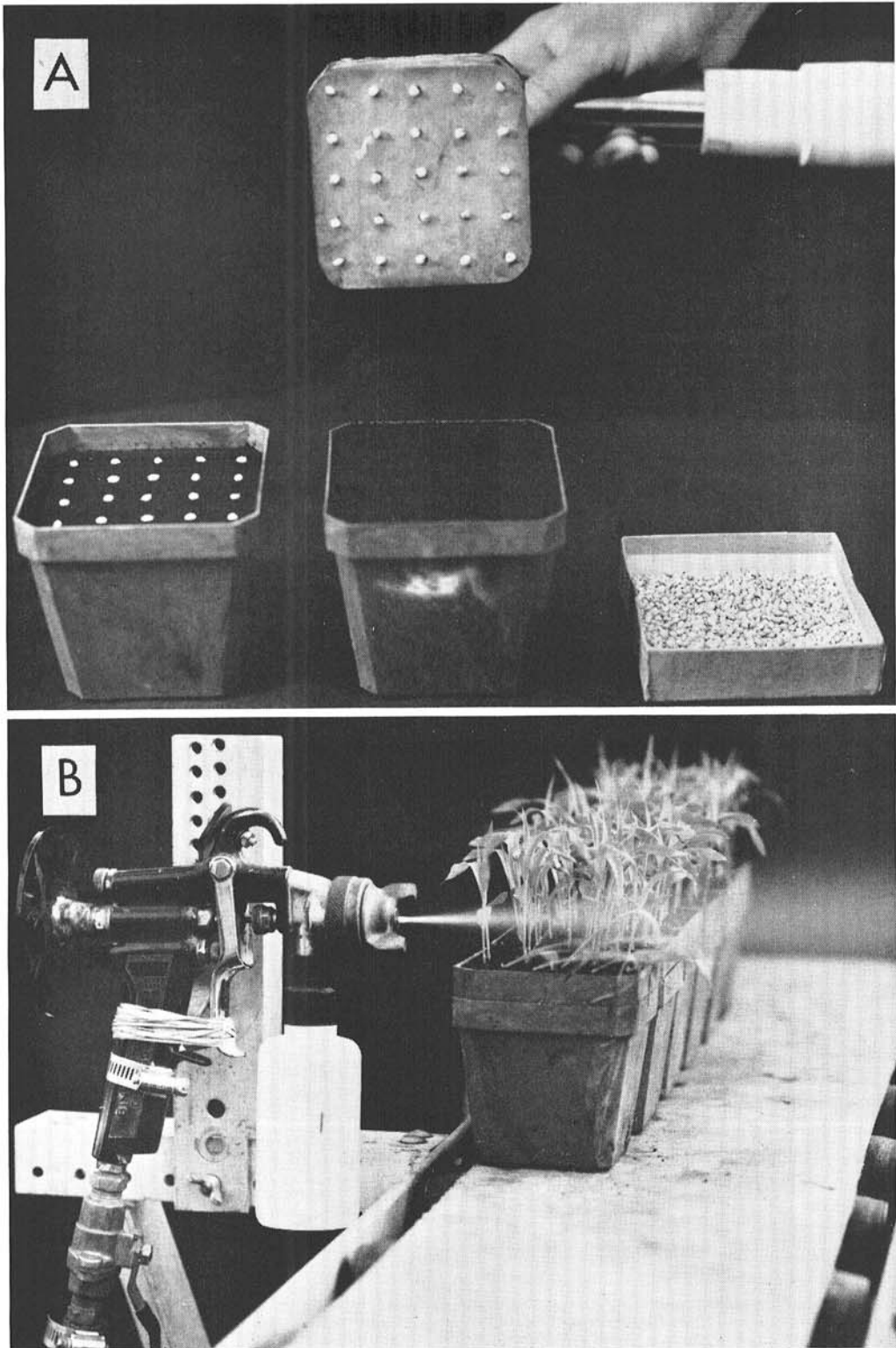
**Fig. 1.** A) Planting seed of the sorghum test plant. Seed are picked up from the box (right), carried on the perforated plate of the vacuum box (top), pressed into the surface of the soil in the pot (center), and left in a plane (left) ready to be covered. B) Apparatus for inoculating test plants. The large black disc at the rear of the paint sprayer is marked in degrees, and was added to facilitate resetting liquid output. Rubber bands are used to hold sprayer air valve open; a lever-operated valve was added to air hose below sprayer.
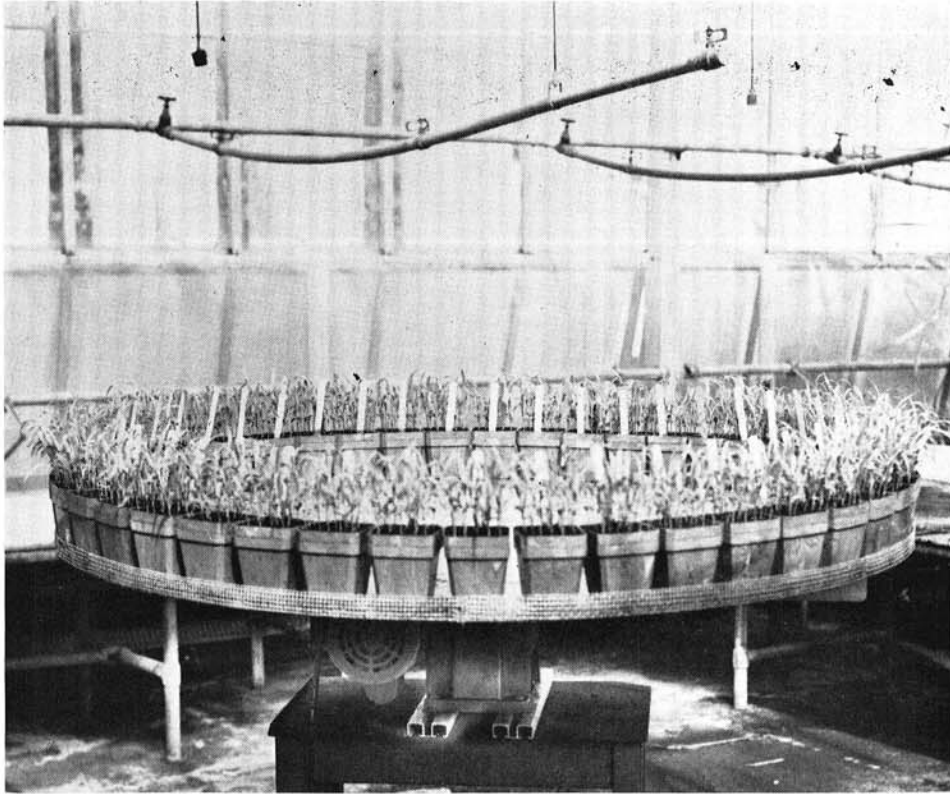
**Fig. 2.** Turntable for eliminating positional environmental effects on sorghum test plants. Table rotates at 3 rpm. Timer-controlled irrigation nozzles are shown above table. Each pot is nested in an empty pot to eliminate absorption of water puddled on the table under the pots.

experiment, summarized in Figure 3, indicate the general shape of the concentration-infection curve, and illustrate the degree of accuracy obtained in tests comparing several inocula.

A stock inoculum at a concentration equal to 20 g of leaf tissue/liter (1/50, w/v) was diluted to give additional concentrations of 16, 12, 8, 4, 2, 1, and 0.1 g/liter. Each inoculum was sprayed on 10 pots of seedlings.

Per cent infection reached 98 at an inoculum concentration of 12 g/liter (Fig. 3). Below this point, all differences in per cent infection between successive concentrations of inocula were significant at the 1% level. One of these significant differences was due to a 1.5-fold difference in virus concentration (between 8 and 12 g/liter). Other significant differences in per cent infection were due to 2-fold differences in virus concentration except for the 10-fold difference between 0.1 and 1.0 g/liter.

*Effect of reducing inoculum concentration by 20%.*— From a stock inoculum at a dilution of 1/500 (w/v), a second inoculum was made to 80% of this concentration; each of these inocula was sprayed on 26 pots of test seedlings.

The higher concentration infected 58.3% of the test plants; the lower concentration infected 44.0%. This difference was significant at the 0.1% level.

*Effect of diluting inoculum concentration by 10%.*—

A stock inoculum was prepared at a dilution of 1/500 (w/v). A second inoculum was obtained by diluting the stock to 90% of its former strength; thus the two inocula differed from each other by 10% (1.1-fold). Each inoculum was sprayed on 35 pots of seedlings in each of two tests.

The percentages of systemically infected test plants
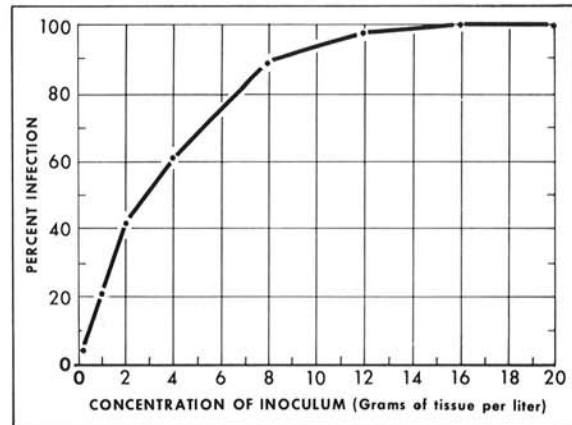


**Fig. 3.** Relationship between per cent of sorghum test plants infected and concentration of sugarcane mosaic virus inoculum. Each point on the curve represents 250 inoculated plants.

were 44.2 and 51.0 for the higher, and 37.5 and 43.0 for the lower concentration, respectively. In each test, the difference in per cent infection of test plants resulting from a 10% difference in virus concentration was highly significant.

*Comparison of local-lesion and systemic-host assays.* —The first test in the previous experiment provided an unusual opportunity to compare local-lesion and systemic-host assay on the same test plants, and with the same number of primary infection sites involved in both assays. The primary infection sites normally remain invisible with this particular host-strain combination, but apparently because of lower greenhouse temperatures during this experiment, they appeared as well-developed necrotic lesions. Virtually all plants showing systemic symptoms also showed at least one local lesion, and vice versa; thus, local lesions and primary infection sites were essentially identical in this experiment. Of the total plants in the test, 39.7% showed at least one local lesion, and 40.8% showed systemic symptoms. This slight discrepancy reflects the error in counting lesions. Lesions at the extreme edge of leaves were both inconspicuous and difficult to distinguish from random blemishes. The number of local lesions per plant was far below the usual range for a normal local-lesion assay. As a test average, there were 0.58 local lesions/plant. This deficiency in local lesions per plant was compensated by the number of plants. Data analyzed were local lesions per pot (25 seedlings/pot).

The higher concentration of inoculum gave an average of 15.6 local lesions/pot; the lower concentration gave 13.5 lesions/pot. This difference was significant, but only at the 5% level of probability.

In this test, with about 40% of the plants infected (average of both treatments), 66.3% of the infected plants were infected at a single primary site, 23.6% at two sites, 6.2% at three sites, 2.8% at four sites, 0.4% at five sites, 0.3% at six sites, and 0.03% at seven sites, respectively. No plant bore more than seven local lesions.

DISCUSSION.—The accuracy of the systemic-host assay of SMV is equal to that reported for local-lesion assay of any other virus. The general method should be applicable to some other viruses, particularly MDMV. Grass seedlings may be particularly suitable as test plants in systemic-host assays; suitable test plants outside the Gramineae may prove to be as rare as local-lesion hosts now seem to be within the family. However, any plant might work if it (i) is susceptible in the young seedling stage; (ii) will withstand the spray blast required for adequate infection; and (iii) has seed suitable for mechanical planting.

The use of two turntables made possible the completion of one assay/week with results from one test available 1 day before inoculation of plants in the next test; thus, the results of one test could influence treatments in the next. In the usual schedule, plants were inoculated 6 days after the seed were planted, and data were taken 6 days after inoculation. It was possible during the warm months (greenhouse temperatures ranging from 32 to 42 C) to inoculate 4 days after planting and take data 5 days after inoculation with no loss of ac-

curacy. However, the 6- plus 6-day schedule was convenient, and worked year-round.

An assay involving 80-100 pots required 8-10 man-hours, including time for statistical treatment of data. Ten pots of seedlings/inoculum consistently detected 2-fold differences in virus concentration, 26 pots were clearly more than adequate for detecting a 20% difference (one test), and 35 pots consistently detected 10% differences. These accuracies apply in the region from about 20 to 90% infection of test plants. Roughly, a 10-fold increase in virus concentration was required to increase the infection from 20 to 90%.

The methods used for handling large numbers of test plants in the assay of SMV could be applied to some local-lesion assays, either to increase accuracy or to extend the range in the direction of lower viral activity. An apparent example would be the local-lesion assay of MDMV as reported by Jean & Sehgal (8). The local-lesion density of 0.58 lesions/plant observed in the comparative study was well below the useful range for normal local lesion assays, but the data were useable because of the large number of test plants inoculated. At higher local lesion densities, larger numbers of test plants could be expected to increase accuracy, provided that the gain due to numbers were not counteracted by the increased time required for carrying out the inoculation. The susceptibility of test plants is likely to vary during a prolonged period of inoculation (11). With the methods used in this work, even large experiments require only a few minutes for inoculation.

As pointed out by Holmes (7), the advantage of the local-lesion assay lies in the ability of few test plants to supply data on large numbers of successful transmissions of the virus. This advantage declines with declining viral activity, and disappears entirely at an activity level corresponding to an average of one primary infection site per test plant. The local-lesion density of 0.58 corresponded to a systemic infection rate of 40%, a rate near opt for systemic-host assay. In this low range of viral activity, systemically infected plants probably can be counted more rapidly and accurately than local lesions unless the lesions are unusually prominent and distinctive.

The use of single local lesions as sources of inoculum for separation of virus strains, isolation of mutants, etc., is well established (13). To a limited degree, a "dilution method" has also been used in conjunction with systemic hosts for this same purpose (12). For example, Jensen (9) infected 24 of 1,247 test plants with a virus inoculum at a dilution of $10^{-7}$. He supposed that at least some of the plants should have been infected by a single virus particle. It has not been conclusively established that a single particle is capable of infecting, but if a systemic infection results from a single primary site, the infected plant is functionally equivalent to a single local lesion for purposes of isolation, except that it furnishes a much larger quantity of virus for the next step in the procedure.

The usefulness of the dilution method clearly depends upon the ease of obtaining plants systemically infected through a single site. The frequency of single-site infection in a population of inoculated systemic hosts is

related to the percentage of plants infected. As the percentage of infection declines, the percentage of infected plants that are infected at a single site increases. For any given percentage of infection below 100, there is a corresponding expected percentage of the infected plants that are infected at a single site. This expected percentage can be calculated on the basis of a Poisson distribution, (E. J. Koch & J. U. McGuire, Biometricians, ARS, USDA, Beltsville, Md., *personal communication*). The assumptions necessary to validate application of the theory are (i) that the test plants are reasonably uniform in susceptibility to infection; and (ii) that the acquisition of a few infection sites by a plant does not appreciably alter its probability of acquiring more infection sites. In work reported here, even at the relatively high infection rate of 40%, the majority (66%) of the infected plants were infected at a single site. At the infection rate (2%) obtained by Jensen (9), 99% of the infected plants are expected to be infected at a single site. Thus, it appears that the use of the dilution method and a systemic host for isolation of virus strains can be highly efficient if the means are available for growing and inoculating large numbers of test plants with reasonable effort.

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