An Ultrasonic Inoculation Method for Tobacco Mosaic Virus Bioassay

Calvin R. Lamborn, George W. Cochran, and John L. Chidester

Associate Director of Research, Gallatin Valley Seed Co., Twin Falls, Idaho 83301; and Professor and Research Associate, Department of Botany, Utah State University, Logan, Utah 84321, respectively.

Utah Agricultural Experiment Station Journal Paper No. 1045. Portion of a Ph.D. thesis by the senior author.
Accepted for publication 23 March 1971.

ABSTRACT

An ultrasonic inoculation method for a sensitive bioassay of tobacco mosaic virus on halves of detached primary leaves of bean is described. The pressure of the leaf against the apparatus, the speed of an inoculation passage, and the power level of the ultrasonic energy all influenced the sensitivity of the bioassay. One thousand-mesh Carborundum proved to be the best abrasive for the method. When the volume of inoculum used per half-leaf was as small as 1 µliter, the inoculation efficiency was greatest. This inoculation method consistently gave straight line curves through 1,000-fold dilutions, and increased the sensitivity of the bioassay above finger-rubbing 88-fold. The ultrasonic method was quick, easy, and reproducible from operator to operator and from day to day. Phytopathology 61:1015-1019.

Additional key words: local lesions.

Successful mechanical inoculation with plant viruses requires a technique that will introduce virus particles through wounds into the epidermal cells of leaves. The degree of wounding is important. Insufficient wounding of a cell will not allow virus to enter, whereas excessive wounding kills the cells and thus prevents successful infection.

The use of abrasive powders such as Carborundum or Celite was the first major step in improving the wounding procedure (5). Such abrasives increased the sensitivity of the plant virus assay about 100-fold (1). A physical force must be applied through these abrasives to induce a suitable wounding of the epidermal cells. The many methods of applying this force have been reviewed by Yarwood & Fulton (7). Nearly all of these methods fall far short of optimal results because the amount of force applied cannot be controlled reproducibly from person to person, from plant to plant, from leaf to leaf, or even from area to area on the same leaf by the same inoculator.

In 1963, attempts were begun to improve the standardization and regulation of the amount of force applied to the abrasive powders in the tobacco mosaic virus (TMV) inoculation process. One of the authors (Cochran) suggested that the cylindrical surface of a vibrating ultrasonic probe might serve as a controllable energy source for virus inoculation. This approach appeared feasible, and methodology was developed. This paper describes studies of a number of parameters that influence the inoculation results. Preliminary results have been reported previously (2, 3, 4).

MATERIALS AND METHODS.—The virus used in the assay was the common strain of TMV obtained from the Rockefeller Institute for Medical Research in 1946. It was purified using the chromatographic method of Venekamp & Mosch (6), and was resuspended in 0.1 M ammonium acetate at pH 7. The concentrated virus was stored in a refrigerator at 4 C and was used throughout the 5-year study reported here. For each experiment, the concentrated virus was first diluted to a standard A_{260} of 0.01, then further diluted in 10-fold steps. In most experiments, a 100-fold dilution of the standard inoculum was used containing 30 nanograms of virus/ml.

Virus was assayed on bean, *Phaseolus vulgaris* L. 'Scotia'. Plants were grown in vermiculite and subirrigated with Hoagland's nutrient solution at 20 C in a growth chamber with a 16-hr day and an 8-hr night. Light was supplied from fluorescent cool-white tubes at an intensity of 300 ft-c at leaf level. Primary leaves were removed from the plants for inoculating 9-11 days after seeding. In later experiments, when the chamber temperature was dropped to 15 C, the seeds were first germinated and the plants were grown to the crook stage at 25 C. This procedure gave primary leaves suitable for experimental use 14 to 16 days after planting.

The ultrasonic apparatus used for most of the studies was a Branson Sonifier (Model LS-75). A Branson Model (W-300) was used in later experiments when higher levels of power were tested.

Half a detached primary bean leaf was chosen as the basic measuring unit of the bioassay. All procedures developed were designed to handle and inoculate these half-leaves with a min of effort and with max reproducibility.

Detached half-leaves were held on a thin layer of 1.25% water-agar gel in clear polystyrene plastic boxes (17.8 × 8.3 × 4.4 cm) prior to inoculation and after postinoculation wilting. Four randomly selected half-leaves were placed in each box after being cut from bean plants that had been immersed for 1 min in hot water at 45 C. Free water on the half-leaf surface was blotted away with a dry paper towel, and Carborundum dust was applied uniformly to the uncovered leaves in a settling chamber (Fig. 1-A). Approximately 0.3 mg of Carborundum/cm^2 of leaf surface was applied.

Prior to inoculation, the four half-leaves were transferred to a small dry paper towel (cut to fit in the plastic box), and a measured amount of inoculum was delivered to each with a micropipet (Fig. 1-B). In most experiments, the amount of inoculum used for a half-leaf was 0.005 ml, and a total of 12 half-leaves was used/treatment. Each half-leaf was then inoculated by passing it under the vibrating cylindrical surface of the

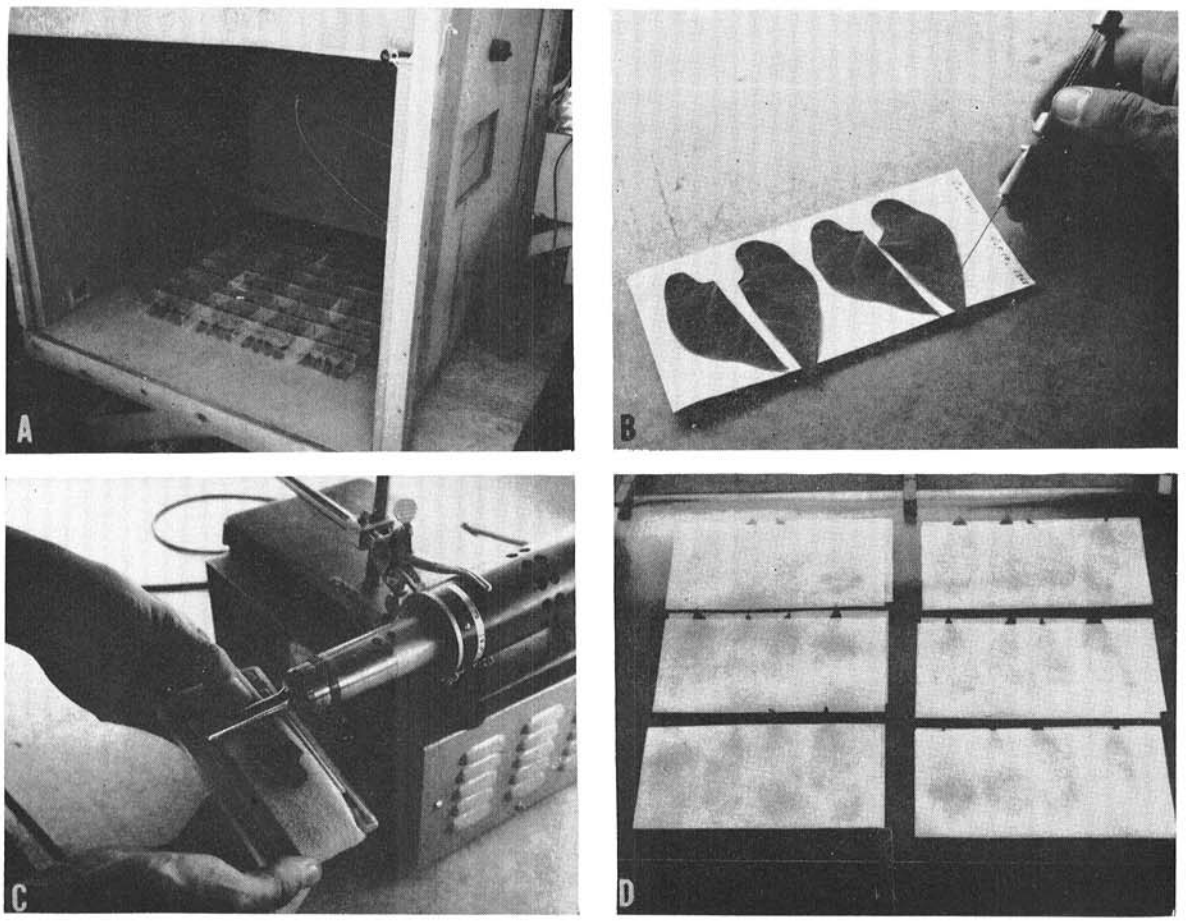


Fig. 1. Ultrasonic inoculation procedures used with Scotia bean half-leaves and dilute solutions of tobacco mosaic virus. **A)** Chamber for Carborundum dust application; **B)** application of inoculum to half-leaves; **C)** inoculation by the passage of a half-leaf against the vibrating ultrasonic probe with controlled speed and pressure; **D)** controlled wilting of inoculated leaves while held between sealed glass plates.

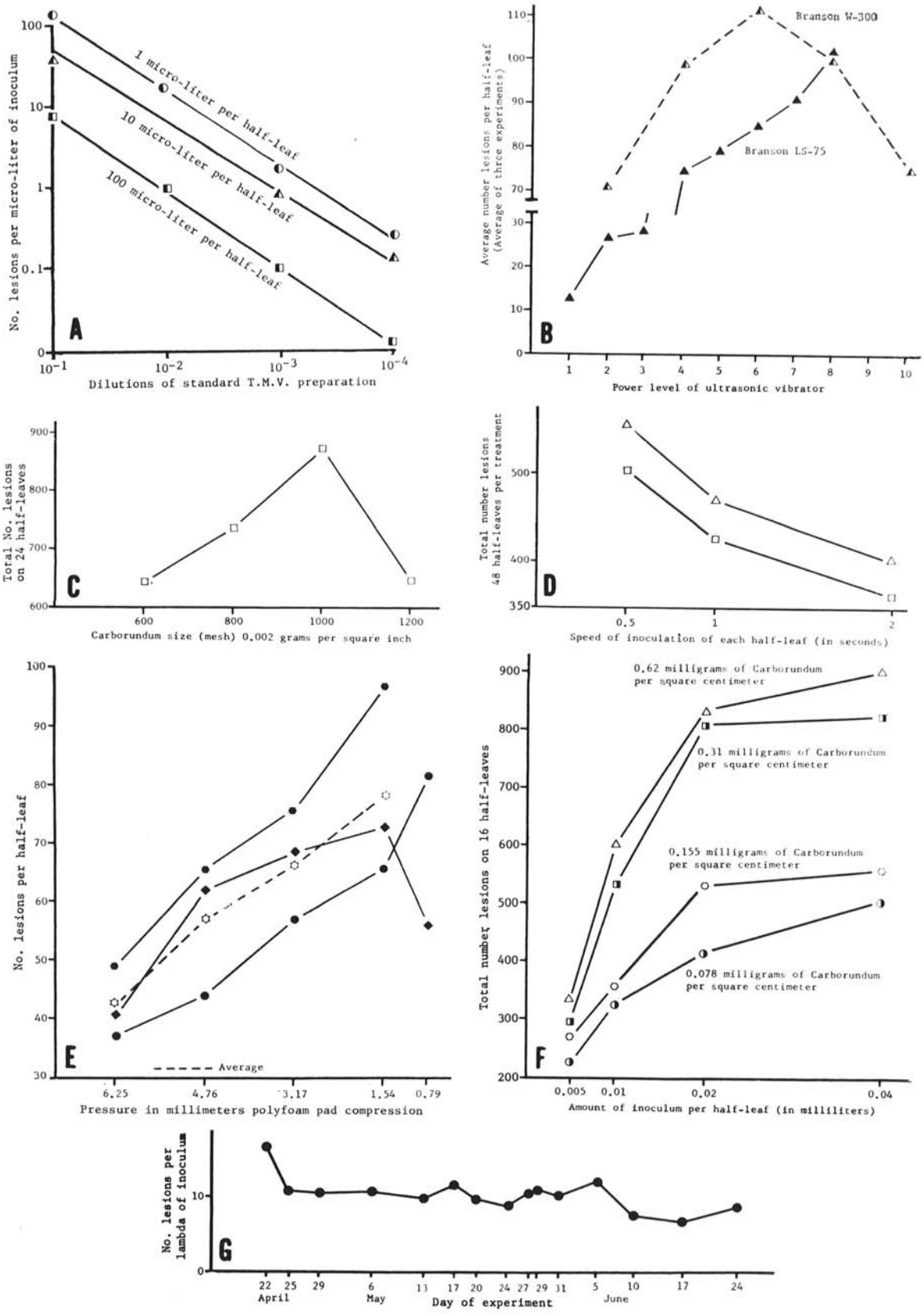
ultrasonic probe, with pressure against the probe applied by a uniformly compressed Polyfoam pad (Fig. 1-C). After inoculation, the half-leaves were returned to the absorbent paper, covered with a second piece of absorbent paper, and placed in a confined-atmosphere wilting chamber with a volume of 470 cc. This chamber (Fig. 1-D) consisted of two glass plates spaced 4 mm apart by a tight rubber gasket at the perimeter. A full chamber carried 24 half-leaves sandwiched between 1,550 cm^2 of absorbent paper. The degree of wilting induced in the leaves was fixed by the moisture equilibrium established between the confined air, the half-leaves, and the absorbent paper. The inoculated leaves were incubated in the wilting chamber in darkness at 30 C for 18 hr.

At the end of the wilting period, each piece of absorbent paper carrying four half-leaves was placed on the agar surface in a polystyrene box, covered with a tight-fitting lid, and incubated at 20 to 25 C under 300 ft-c of continuous daylight fluorescent lights. Lesions could be counted 2 to 3 days after inoculation. The vibrating ultrasonic probe was cleaned and sterilized between treatments by dipping in a water-detergent mixture, rinsing with distilled water, passing slowly through a flame, and cooling with a final distilled water rinse.

RESULTS AND DISCUSSION.—A number of variables in the methodology appeared to influence the ultimate sensitivity of the viral bioassay. These were choice of assay plant, type of abrasive used, size and amount of

→

Fig. 2. Relationship of various inoculation parameters to infectivity of a tobacco mosaic virus preparation (30 nanograms/ml) on detached Scotia bean half-leaves grown under standardized conditions, inoculated ultrasonically, and post-inoculation-wilted for 18 hr at 30 C. **A)** Dilution curves with measured inoculum volume; **B)** effect of ultrasonic power level; **C)** effect of Carborundum size; **D)** relationship of speed of inoculation; **E)** effect of inoculation pressure; **F)** relationship of amount of Carborundum with various amounts of inoculum; **G)** variability with a standardized inoculation procedure during a 2-month period.


A
No. lesions per micro-liter of inoculum
1 micro-liter per half-leaf
10 micro-liter per half-leaf
100 micro-liter per half-leaf
Dilutions of standard T.M.V. preparation
B
Average number lesions per half-leaf (Average of three experiments)
Branson W-300
Branson LS-75
Power level of ultrasonic vibrator
C
Total No. lesions on 24 half-leaves
Carborundum size (mesh) 0.002 grams per square inch
D
Total number lesions 48 half-leaves per treatment
Speed of inoculation of each half-leaf (in seconds)
E
No. lesions per half-leaf
Average
Pressure in millimeters polyfoam pad compression
F
Total number lesions on 16 half-leaves
0.62 milligrams of Carborundum per square centimeter
0.31 milligrams of Carborundum per square centimeter
0.155 milligrams of Carborundum per square centimeter
0.078 milligrams of Carborundum per square centimeter
Amount of inoculum per half-leaf (in milliliters)
G
No. lesions per lambda of inoculum
22 25 29 6 13 17 20 24 27 29 31 5 10 17 24
April
May
June
Day of experiment

Carborundum, amount of inoculum, pressure exerted on the Carborundum particles, power level of the ultrasonic apparatus, and speed of inoculation. Attempts were made to determine the optimal values for each parameter as well as their interactions. As the investigation progressed, frequent checks were made to determine the effect of the changing of one parameter on the optimum values of the others.

Choice of the assay plant.—In preliminary experiments, *Phaseolus vulgaris* L. 'Scotia' and 'Pinto' were compared with *Nicotiana glutinosa* L., *Nicotiana tabacum* L. 'Havana 423', and *Nicotiana tabacum* L. *'Xanthi nc'*, using ultrasonic and the usual mechanical inoculation methods with and without various pre- and postinoculation leaf treatments. The various treatments that were tested appeared to have little influence in altering lesion counts in the tobacco assay hosts. Because Scotia bean showed markedly increased size and numbers of lesions in response to preinoculation heat and postinoculation dessication treatments combined with the ultrasonic inoculation method, it was chosen as the assay host for further investigations.

Ultrasonic power level.—The relationships of ultrasonic power level to the number of lesions induced are indicated in Fig. 2-B. Because the Branson LS-75 lesion response curve appeared to be still rising at the top power level setting, a more powerful Branson W-300 was tested using the same interchangeable metal probe. A peak response was obtained at about midrange with the W-300. Because this peak response was only slightly better than that of the highest power on the LS-75, it was concluded that the LS-75 top power level was close to optimum. These response curves illustrate the direct relationship of ultrasonic energy levels to the initiation of infectable wounds. At the highest power levels with the W-300, increasing power resulted in fewer lesions. The ultrasonic frequency of 20,000 cycles/sec does not change with power level changes. The amplitude of vibration varies with power level and with the position of the vibrational nodes in the metal probe. Because different leaf areas pass under different portions of the vibrating probe, the determination of precise amplitudes of vibration for the inoculation procedure goes far beyond the scope of the present investigation.

Pressure between ultrasonic probe and leaf.—The amount of pressure between the vibrating ultrasonic probe and the leaf is important because it is related to the average distance between the epidermal cell surfaces and the surface of the vibrating probe. When this distance approaches the average diameter of the abrasive powders, one might expect to get the optimal wounding effect. As pressure is increased, this average distance decreases and the efficiency of the inoculation increases. Data from three experiments illustrating this effect are shown in Fig. 2-E. A controllable pressure was generated by compressing a 12.7-mm Polyfoam pad behind the leaf at the instant of inoculation. The pressure is recorded as the mm of space into which this pad was compressed. Because compression to 0.79 mm sometimes resulted in leaf injury and variable results, a compression to 1.54 mm was adopted as a realistic standard value.

It was difficult to pass a half-leaf under the probe with the max pressure without tearing it unless the probe was energized. The ultrasonic vibrating action reduced the friction to a negligible value.

Speed of inoculation.—Early in these experiments it was observed that one passage under the ultrasonic probe gave optimal infection. Two passes gave fewer lesions than one, and three passes gave fewer lesions than two. These observations suggested that the duration of the wounding action might be a factor of some importance. Tests were conducted in which the passage of the half-leaf under the ultrasonic probe was timed with a metronome. With practice it became possible to pass a half-leaf under the ultrasonic probe in 0.5 sec. Comparisons were then made of 0.5, 1, and 2 sec duration inoculation passes. The data from two experiments are shown in Fig. 2-D. The assay sensitivity varied directly with the speed of the inoculation passage. It was not possible to inoculate half-leaves at rates faster than 0.5 sec with repeatable results. The higher rates gave higher lesion counts.

Type of abrasive used.—With 600-mesh Carborundum rated at 100, the effectiveness of several other abrasives in inducing lesions with identical inoculation procedures was as follows: aluminum oxide (600-mesh), 53; Celite (diatomaceous earth), 31; Novicite (625-mesh crushed quartz), 24; and zinc oxide (derived by burning zinc), 12.

Size of Carborundum.—The effect of the size of the Carborundum particles was restudied after the optimal inoculating pressure was determined. Two μliters of inoculum were used/half-leaf. The use of 1,000-mesh Carborundum gave the greatest number of lesions (Fig. 2-C). The Carborundum size appears to be an important consideration. Two modes of vibration are induced by the cylindrical surface of the probe, a longitudinal mode which is parallel to the long axis and a shear mode which is perpendicular to the longitudinal mode. Both vibrational modes are probably transmitted to suitably sized Carborundum particles which puncture the epidermal cell walls and permit virus invasion.

Amount of Carborundum.—The amount of Carborundum in relation to the volume of inoculum applied to each half-leaf was studied. Data from a typical experiment are shown in Fig. 2-F. The greatest sensitivity (lesions per unit volume of inoculum) was obtained with the largest amount of Carborundum and the smallest volume of inoculum. The largest amount of Carborundum and the largest volume of inoculum gave the greatest number of lesions per half-leaf, but at a considerable loss in sensitivity. Because 0.62 mg of Carborundum/cm^2 of leaf surface interfered with lesion counting and sometimes induced excessive leaf injury, 0.31 mg/cm^2 were adopted as the standard amount in most experiments.

Amount of inoculum.—One of the advantages of using the ultrasonic inoculation method described here is that it works well with small amounts of inoculum. The vibrating action of the ultrasonic probe spreads a small

drop of inoculum over a max of leaf area. When tests were run with different volumes and concentrations of inoculum, the dilution curves were nearly parallel and proportional to virus dilution (Fig. 2-A). The differences noted were in the efficiency of the bioassay per unit volume of inoculum. The smallest inoculum volume gave the highest bioassay efficiency. The ultrasonic bioassay described here permits an investigator to describe the infectivity of a TMV preparation in terms of lesions per μliter of inoculum. When a volume of 1 μliter/half-leaf was used, there was not enough liquid to cover the entire leaf surface. Under these conditions, the volume of the inoculum and not the size of the half-leaf becomes the limiting factor.

Bioassay variation related to personnel.—One of the great sources of variability in mechanical inoculation of viruses has been the variability observed when different people inoculate leaves. As much as a 9-fold spread was observed in the final lesion counts in an experiment where five experienced persons inoculated equal numbers of bean leaves with the same TMV inoculum by the finger-rubbing method. There was also a highly significant difference in the results of how each person rubbed the two halves of the same leaf (3).

Four people using the ultrasonic technique described in this paper obtained the following results: average number of lesions/half-leaf, 10.6, 11.5, 9.5, 10.7. The first two people had extensive experience while the latter two had no previous experience. These results suggest that the use of this ultrasonic inoculation procedure for TMV will do much to eliminate bioassay variability because of personnel.

Day-to-day variability of the ultrasonic TMV bioassay.—During a 2-month period in this study, an inoculated control was run on all experiments. This control consisted of 12 bean half-leaves all inoculated ultrasonically with new virus dilutions made from the same virus stock solution. Figure 2-G shows a plot of the number of lesions per μliter of inoculum in 15 experiments. The low variability in these controls shows the potential repeatability of this ultrasonic TMV bioassay method. If a straight line is drawn for a best fit of the plotted points, it slopes downward with time duration. This downward slope reflects a decrease in inoculum infectivity which can occur in purified TMV solutions because of particle aggregation or other factors.

Ultrasonic inoculation vs finger rubbing with the same inoculum.—The effectiveness of ultrasonic over the finger inoculation method was compared periodically as a gauge of progress. In each case the same person did the finger inoculation to minimize the variation commonly experienced with this method (3).

In the earliest tests, the ultrasonic method gave 7 times as many lesions as did finger rubbing with the same inoculum (2, 3). After studying the variables influencing the results and developing a standardized technique in which each variable was controlled at or near its optimal level, new tests were made in comparison with finger rubbing. In six experiments, the ultrasonic method produced 93, 127, 108, 47, 47, and 109 (average of 88) times as many lesions as finger rubbing with the same inoculum.

LITERATURE CITED

1. BERAHA, L., M. VARZANDEH, & H. H. THORNBERRY. 1955. Mechanism of the action of abrasives on infection by tobacco mosaic virus. Virology 1:141-151.
2. LAMBORN, C. R. 1964. An improved method for assaying infectivity of tobacco mosaic virus. Phytopathology 54:898 (Abstr.).
3. LAMBORN, C. R. 1964. Inoculation method and treatments which increase the sensitivity and precision of the infectivity assay of tobacco mosaic virus. M. S. Thesis, Utah State Univ., Logan. 35 p.
4. LAMBORN, C. R., G. W. COCHRAN, & J. L. CHIDESTER. 1969. Improved ultrasonic inoculation techniques for tobacco mosaic virus. Phytopathology 59:13 (Abstr.).
5. RAWLINS, T. E., & C. M. TOMPKINS. 1936. Studies on the effect of Carborundum as an abrasive in plant virus inoculations. Phytopathology 26:578-587.
6. VENEKAMP, J. H., & W. H. M. MOSCH. 1964. Chromatographic studies on plant viruses. II. The use of polyethylene glycol in clarification and purification of tobacco mosaic virus. Virology 22:503-507.
7. YARWOOD, C. E., & R. W. FULTON. 1967. Mechanical transmission of plant viruses, p. 237-266. *In* K. Maramorosch & H. Koprowski [ed.]. Methods in virology. Academic Press, N. Y.