The Detection of Symptomless Virus-Infected Tissue in Inoculated Tobacco Leaves

J. A. Foster and A. F. Ross

Department of Plant Pathology, Cornell University, Ithaca, N.Y. 14850. Present address of senior author: Department of Plant Pathology, Ohio Agricultural Research and Development Center, Wooster 44691.

Portion of a Ph.D. thesis submitted by the senior author to the Graduate School, Cornell University, Ithaca, N.Y. 14850.

Supported in part by National Science Foundation Grant GB-13856.

The technical assistance of T. Hammond and G. Nemoto is gratefully acknowledged.

Accepted for publication 2 January 1975.

ABSTRACT

Necrotic lesions were induced by three different procedures in Turkish tobacco (*Nicotiana tabacum*) leaves inoculated with tobacco mosaic virus (TMV), a virus that normally does not cause local necrosis in that host. The exposure of TMV-inoculated leaves to hot water at 50 C for 40 seconds 2-5 days after inoculation consistently collapsed necrotic rings or spots at the initial infection sites, except when tobacco plants were grown in the greenhouse during hot sunny summer days. The collapse of infected areas in inoculated leaves of cuttings during uptake of 0.5 M NH_4Cl starting 2 days after inoculation was consistent in leaves grown during summer, but rare in leaves which developed in winter. Despite some injury of the treated leaves, lesion development was also observed in TMV-inoculated leaves that had been grown at 20 C, then maintained at 50-54 C for 9 minutes, and immediately immersed in 20% ethanol at −4 C for 30 seconds. Each treatment induced lesion formation within 1-3 days but did not prevent systemic virus movement. Of these treatments, the hot water treatment proved the simplest and most dependable method of detecting the initial sites of TMV infection. Heat treatment also induced necrotic lesions in other host-virus combinations, such as potato virus X (PVX) or cucumber mosaic virus (CMV) in tobacco, that normally do not result in necrotic local lesions. That heat-induced necrosis of the virus-infected areas results from a rapid change in leaf temperature, rather than from the actual leaf temperature reached, is suggested by the facts that hot water (but not hot air at the same temperature) induced lesions, that cold liquid induced lesions only after plants were subjected to high temperatures, and that greenhouse conditions during hot summer days prevented complete heat collapse in the host-virus combinations tested.

Phytopathology 65:600-610

After a healthy susceptible leaf is mechanically inoculated with a plant virus, small virus-infected areas develop in the rubbed leaf. Each area is presumably centered on an initially infected cell and enlarges as multiplication and subsequent cell-to-cell movement of the virus occur. If these areas become visible, they are called local lesions. The initial infection sites are usually not visible during tobacco mosaic virus (TMV) multiplication in the inoculated leaves of tobacco (*Nicotiana tabacum* L.) cultivars without the *N* gene.

The existence of these small infected areas in a symptomless inoculated leaf was first appreciated by Holmes (9, 10) when during the summer months he noticed small yellowish areas in tobacco leaves previously inoculated with the common strain of TMV. This observation led to the development of the starch-lesion technique (10), by which differences in starch retention between healthy and virus-infected cells can be detected in symptomless inoculated leaves. Since the development of this procedure, many other physiological differences between healthy tobacco tissue and tobacco tissue inoculated with TMV have been reported; but, until recently, none has been utilized for detecting the initial TMV infection sites in symptomless inoculated leaves.

The purpose of our research was to develop techniques for the detection of the initial TMV infection sites in symptomless inoculated leaves by accentuating differences between healthy and infected cells. This objective was pursued by exposing the inoculated leaf to environmental and/or chemical treatments severe enough to cause disruptions in the metabolism of the leaf. This investigation was undertaken on the assumption that development of additional methods for revealing the initial infection sites would improve and extend the usage of those research procedures presently restricted to local lesion hosts, and would also aid in identifying physiological differences between infected and healthy cells.

When this research was started, the starch-lesion procedure (10) was the only dependable method for detecting the initial TMV infection sites in inoculated nonhypersensitive tobacco leaves. Since the preliminary 1968 report of our initial findings on the collapse of TMV-infected areas in symptomless inoculated tobacco leaves exposed to a heat treatment (6), other workers have used methods for detecting the initial TMV infection sites in tobacco leaves by heating (14, 15, 17, 22, 25), by freezing (15, 22), or by allowing the infected leaf to absorb actinomycin D (16), chromomycin A_3 (16), $^{14}CO_2$ (4, 5), or ultraviolet light (24).

MATERIALS AND METHODS.—*Nicotiana glutinosa* L., *N. sylvestris* Speg. & Comes, and the cultivars Turkish and Samsun NN of *N. tabacum* L. were grown in the greenhouse by seeding in sterilized vermiculite, transplanting the seedlings 2 weeks later into steam-sterilized composted soil in flats, and finally transferring each plant to a 10.2-cm diameter pot containing similar soil about 4 weeks after the original seeding. Seedlings of *Datura stramonium* L., *Lycopersicon esculentum* Mill., or *Beta vulgaris* L. developed for the first 2 weeks in sterilized vermiculite and were then individually transferred to separate 10.2-cm diameter pots containing steam-sterilized composted soil. The fertilization program, which began 1 week after

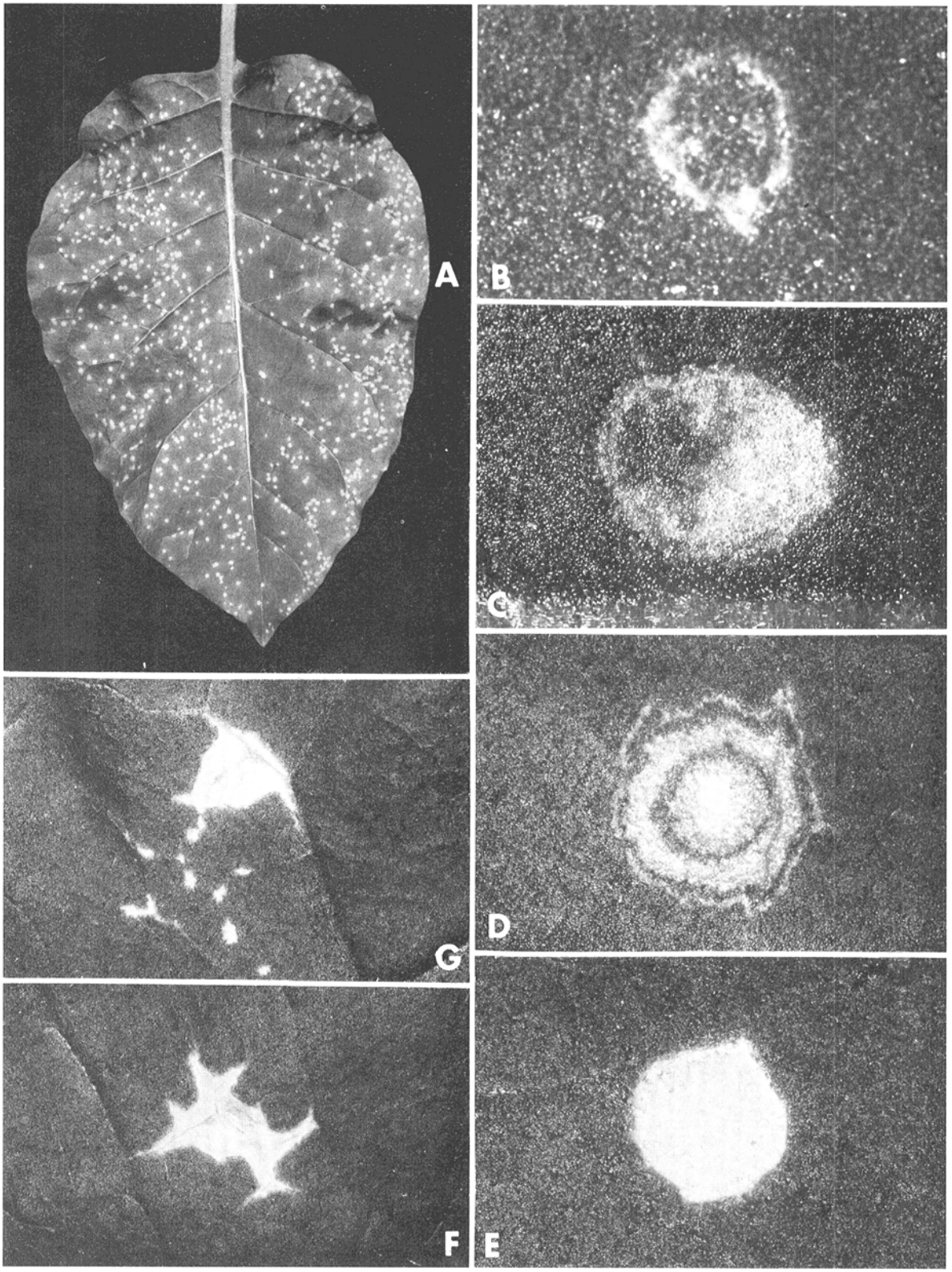

Fig. 1-(A to G). Necrotic lesions induced in Turkish tobacco leaves by heating the leaves in hot water at 50 C for 40 seconds 2-5 days after inoculation with the common strain of tobacco mosaic virus. **A)** Obvious lesions that developed in all parts of the heated inoculated leaf without injury. **B)** Necrotic ring representing the minimal amount of tissue collapsed by a heat treatment (× 8). **C,D)** Different patterns resulting from partial collapse of tissue within the heat-sensitive ring (× 12). **E)** Solid spot resulting when all tissue delimited by the heat-sensitive ring collapsed (× 9). **F)** Irregularly shaped lesion near a large vein of a leaf heated 3-5 days after inoculation (× 6). **G)** Small necrotic spots sometines developed along veins in the vicinity of a large collapsing lesion in a leaf heated 3-5 days after inoculation (× 6).

the last transplanting, consisted of filling the space above the soil in each pot once a day for 5 days each week with tap water containing 1.0 g of NH_4NO_3, 0.5 g of $NH_4H_2PO_4$, and 0.5 g of KCl per liter.

The virus isolates and hosts used were selected because infection normally caused no symptoms in inoculated leaves for at least 5 days following inoculation. With the exceptions of the beet mosaic virus (BMV) isolate and the chrysanthemum strain of tomato aspermy virus (TAV), these viruses were obtained from A. F. Ross. The TAV was isolated from an infected chrysanthemum by R. K. Horst, and BMV was recovered by C. G. Summers (23) from sugar beet leaves with mosaic symptoms. Cucumber mosaic virus (CMV) was isolated from a weed sample within 1 year of its use. Sugar beet plants systemically infected with BMV, Turkish tobacco plants systemically infected with TMV, and Samsun NN tobacco plants systemically infected with potato virus X (PVX), potato virus Y (PVY), CMV, or TAV were maintained in the greenhouse as sources for virus inoculum.

Most reported experiments were conducted with 6- to 9-week-old tobacco plants in a glass greenhouse at temperatures ranging from 20 C to 30 C, except during summer when temperatures sometimes exceeded 30 C. Warm-white VHO fluorescent lights supplemented the natural light during cloudy days. Each treatment within most experiments involved 10 leaves, two leaves on each of five plants. Inoculations were made by rubbing the youngest fully expanded leaves of each plant with a gauze pad previously dipped into a suspension of 38 μm (400-mesh) Carborundum in a diluted solution of virus extract prepared by grinding systemically infected leaves in a mortar with a pestle. The inoculated leaves were rinsed with tap water unless the virus inoculum contained less than one part of plant extract per hundred parts of water.

Heat treatment involved immersing the infected leaves of each plant for precisely timed intervals in a water bath containing 454 liters of tap water. The water temperature was thermostatically controlled to within 0.1 C of each desired temperature. Immediately after heating, each group of five-to-seven plants was placed in a 81.3×81.3×7.6-cm pan and caged with a 68.6×68.6×61-cm wooden frame covered on all but the bottom face by a layer of 0.1-mm clear plastic and a layer of 0.04-mm black plastic.

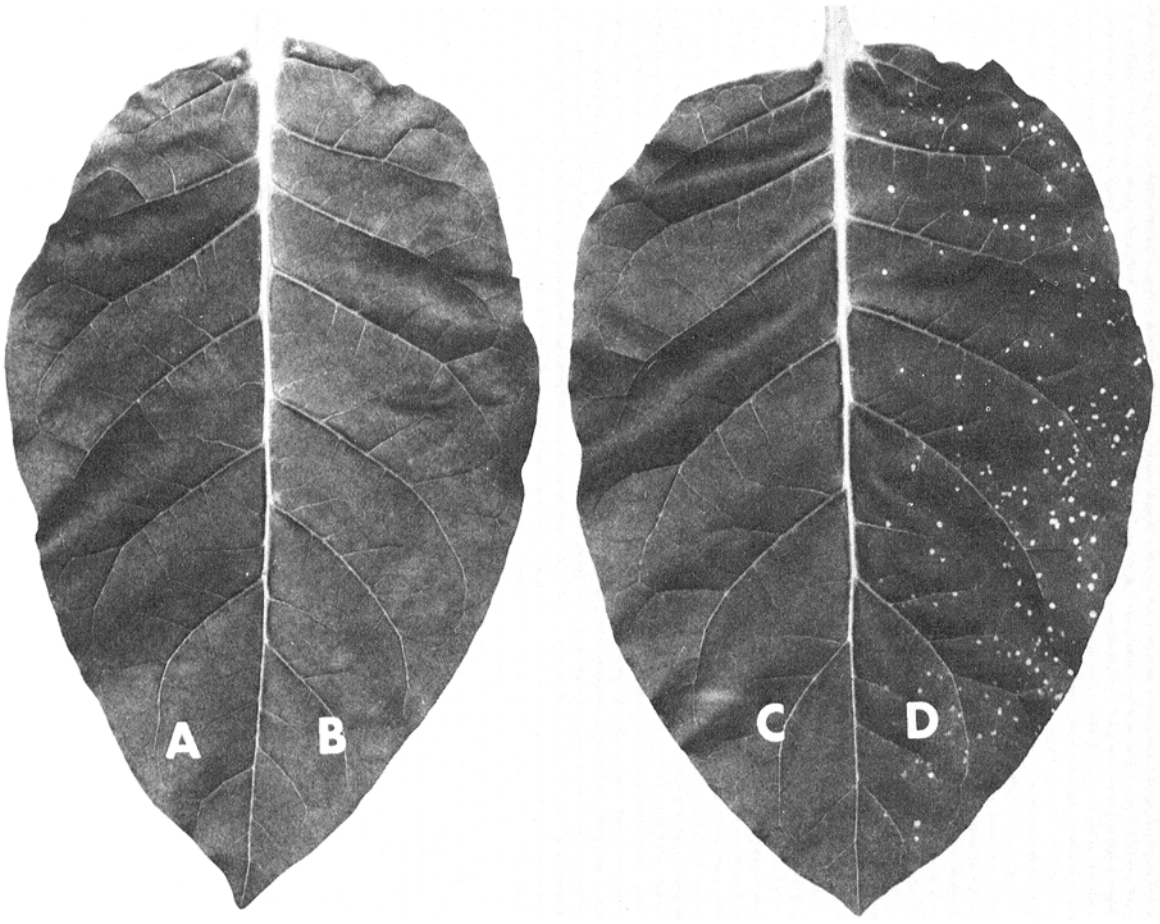

Fig. 2-(A to D). The extent of lesion formation induced in Turkish tobacco half-leaves that were uninoculated and not heated **A)**, inoculated and not heated **B)**, uninoculated and heated **C)**, or inoculated and heated **D)**. Inoculation was with tobacco mosaic virus 2 days before heat treatment in hot water at 50 C for 40 seconds.

Then enough cool tap water was added to fill each pan one-fourth full. These structures, which will be referred to as recovery chambers, were located in a controlled-environment chamber set at 21 C and at a 16-hour day with a light intensity of 8,611 lux provided by warm-white VHO fluorescent lights and incandescent bulbs. The relative humidity in this growth chamber was uncontrolled, but generally ranged between 50% and 60%. The conditions in these recovery chambers minimized injury in the heated leaves, presumably because the plants were exposed to darkness, high relative humidity, and cool temperatures following the treatment. After the frame of the cage was removed 2 days later, the treated plants were maintained in the 21 C growth chamber until tissue collapse in the lesion areas was complete.

Inoculated leaves were cold-treated by exposure to air or fluids maintained at low temperatures. In some tests, this treatment was preceded by the exposure of the plants to high temperature in a chamber maintained at 50-54 C. A freezer was used to treat inoculated plants in air at −2 to −6 C. In order to test subzero temperatures, 20% ethanol was selected as the liquid to be used in the cold treatment of inoculated leaves. Solidly frozen 20% ethanol was stored in the freezer at −20 C and thawed at room temperature before use. The temperature of the solution in a basin was maintained at −4 C by periodically adding melting 20% ethanol to the liquid during the treatment of inoculated leaves. These precautions insured that the 3-5 liters of 20% ethanol in the basin did not vary in temperature by more than 0.3 C. Injury was minimized by transferring the plants immediately after treatment to the recovery chambers previously described. The treated plants were removed from the recovery chambers 1-2 days later and subsequently maintained in the 21 C growth chamber.

Uptake of chemicals by cuttings was selected as the method of testing chemicals for their ability to induce lesions in exposed symptomless TMV-inoculated leaves, because this method insured the constant supply of chemical to the infected cells, and also permitted the detection of systemic virus movement into developing axillary buds during chemical uptake. Turkish tobacco plants were maintained in the greenhouse before virus inoculation and during the first few days of virus infection, but not after the initiation of chemical uptake. The growing tip and all but two partially expanded leaves were removed from each plant just before the remaining leaves were rubbed with TMV inoculum. On the second or third day after inoculation, each infected plant was cut 10-15 cm below the petiole of the oldest inoculated leaf, and the basal stem portion of each resulting cutting was immediately immersed in a chemical solution that had been prepared from reagent-grade crystals or powders on the same day. Since maintenance of the cuttings in the recovery chambers did not noticeably reduce ammonium-ion injury below the level usually obtained with treated tissue exposed to low light and low temperature in the 21 C growth chamber, all treated cuttings were kept in this growth chamber during chemical uptake and until lesion formation.

Experimental results were usually recorded 3-6 days after the initiation of a treatment. The mildest exposure which induced obvious lesions in all areas of the treated leaf was designated as the standard procedure. The lesion number per leaf was determined with a magnifying glass (× 1.2) and a lighted background. Lesion size was recorded as the average diameter of 100 randomly selected lesions distributed as 10 lesions in each of 10 leaves and measured by means of a stereoscopic microscope (× 10) equipped with an ocular micrometer. Each experiment was repeated during different times of the year.

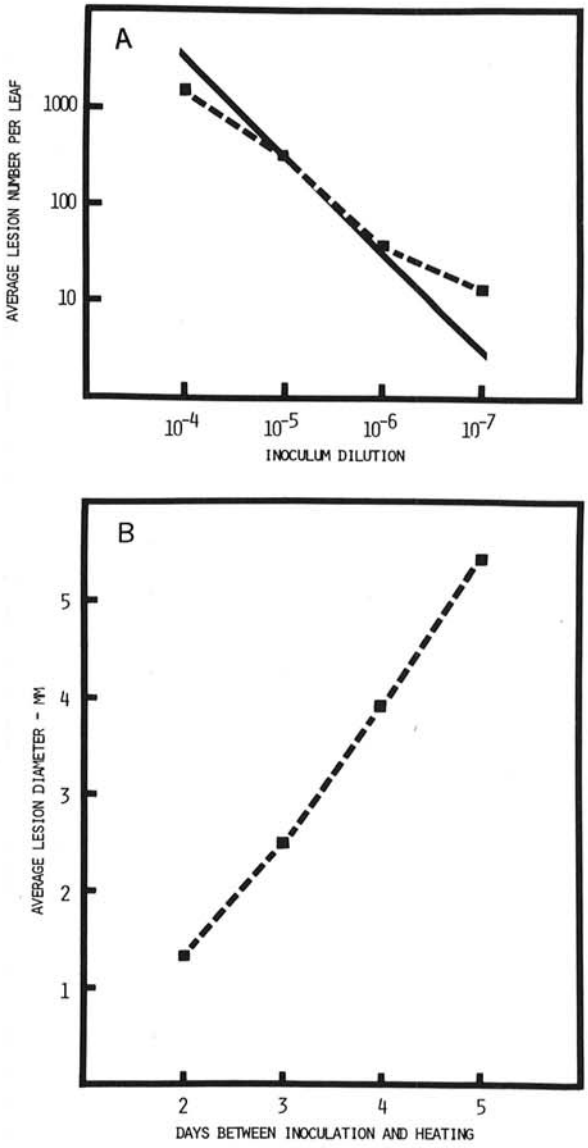

Fig. 3-(A, B). Changes in heat-induced lesion number with inoculum dilution, and in the size of the heat-sensitive area with the time between inoculation and treatment. **A)** Dilution curve (dotted line) plotted from the average number of heat-induced lesions in Turkish tobacco leaves inoculated with each indicated inoculum dilution of crude juice from plants systemically infected with tobacco mosaic virus. Solid line is a theoretical curve with a slope of unity. **B)** Curve plotted from the average diameter of the heat-sensitive areas in Turkish tobacco leaves heated at 50 C for 40 seconds on each indicated day following inoculation with tobacco mosaic virus.

RESULTS.—*Heat-induced lesion formation in Turkish tobacco leaves previously inoculated with TMV.*—Our initial attempts to kill only the infected cells in recently inoculated leaves involved exposing these leaves to treatments of extreme environmental conditions. The discovery of heat-induced lesion production in Turkish tobacco leaves previously inoculated with TMV (6) was made during an effort to determine the effect in nonhypersensitive tobacco of a hot water treatment that collapsed cells in a band around local lesions in hypersensitive tobacco leaves (3, 18, 19). A subsequent literature review revealed Yarwood's short description (26) of lesions induced by heating symptomless inoculated bean leaves infected with peach ringspot virus [Prunus necrotic ringspot virus (8)]. Yarwood reported an increase in the size of the collapsed areas as the time from inoculation to treatment lengthened, and he also noted that variations in the ability to obtain lesions occurred with different plant cultivars or virus strains. After our report (6) correlating heat-induced TMV lesions with the initial sites of infection in the inoculated tobacco leaf, Ohashi and Shimomura demonstrated that heat-induced lesion formation was possible in inoculated leaves of tobacco of different ages (14), after heat treatments in water at a temperature other than 50 C (14, 22), in hypersensitive tobacco maintained at 30 C (15, 22), and with other host-virus combinations (15). By heating inoculated tobacco leaves containing ringspots caused by potato mop-top virus infection, Jones (11) collapsed symptomless initial infection sites as well as living tissue at the boundary of or within the ringspots. He also induced ringspots consisting of the same number of concentric rings as the number of days between inoculation and treatment by heating symptomless inoculated Xanthi-nc tobacco (*N. tabacum*) leaves at 55 C for 10-15 seconds during maintenance at 22 C.

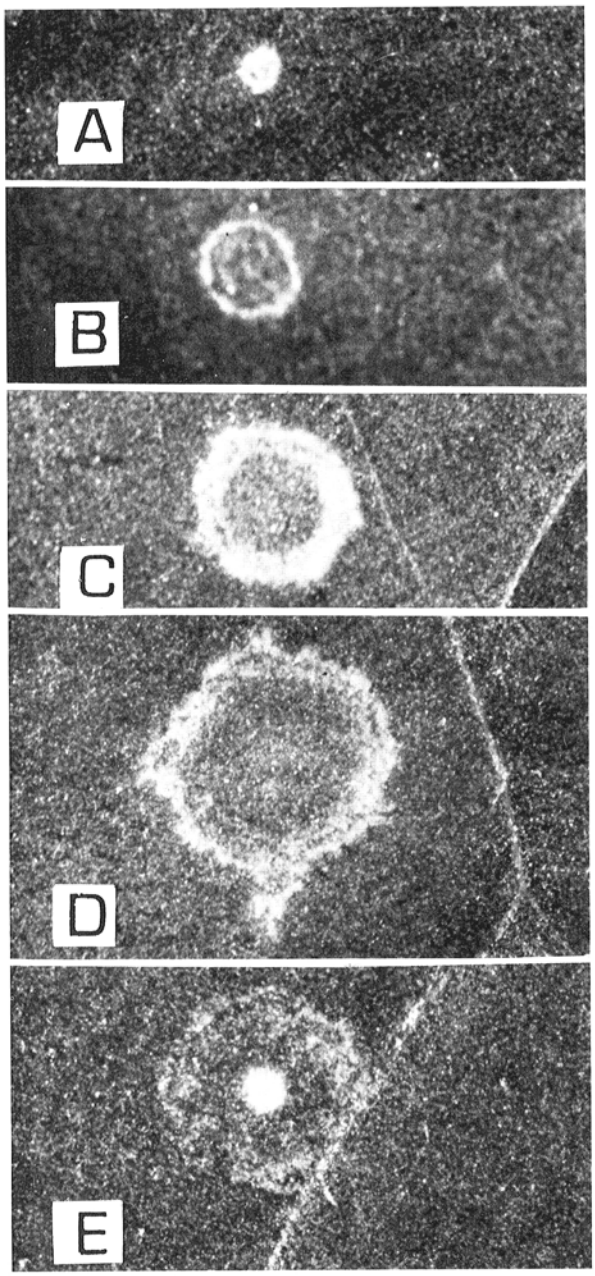

Fig. 4-(A to E). Size and appearance of lesions induced by heating inoculated Turkish tobacco leaves at 50 C for 40 seconds 2 days **A**), 3 days **B**), 4 days **C**), 5 days **D**), or 2 and 5 days **E**) after inoculation with tobacco mosaic virus (× 4.8).

In our experiments, the heat treatment of Turkish tobacco leaves at 50 C for 40 seconds 2-5 days after inoculation with the common strain of TMV, induced necrotic lesions in these leaves 24-48 hours later (Fig. 1-A). Dark-green, water-soaked areas in the heated leaf were usually the first indication of locations where heat-induced necrosis would occur during the next few hours. The extent of tissue collapse in these areas determined the final appearance of the lesions (Fig. 1-B, C, D, E). Necrotic rings, solid spots, and rings with partially collapsed centers were often induced in the same leaf. More rings than solid spots occurred in leaves of plants maintained around 30 C during infection periods longer than 2 days. Lesions adjacent to veins were usually irregular in shape (Fig. 1-F). The occurrence of a number of small spots along a vein near an irregular lesion (Fig. 1-G) became more probable as the elapsed time between inoculation and treatment was increased. Lesions also developed when Turkish tobacco leaves previously inoculated with TMV were heated at 55 C for 5 seconds or at 45 C for 10 minutes. There was no increase in lesion size following the initial necrotic reaction. In no case did the heat treatment prevent systemic movement of TMV.

The standard procedure for TMV lesion production throughout the year in leaves of 7- to 9-week-old Turkish tobacco plants grown in the greenhouse involved heating the infected leaves at 50 C for 40 seconds any time between 1000 and 2200 hours 2 days after leaves measuring 50-350 cm^2 in area had been inoculated with the common strain of TMV. Sublethal exposures of these inoculated leaves for longer than 40 seconds at 50 C were usually not any more effective at causing lesion formation than were 40-second treatments. Poorly collapsed or faint lesions occasionally occurred in some heat-treated, inoculated leaves previously exposed to extremely hot, bright conditions in the greenhouse during TMV infection in summer. The treatment induced lesion

formation in both attached and detached leaves. The ability to obtain lesions was not substantially affected if the growing point and all uninfected leaves were removed just before heating, or 2-4 days earlier. Lesion development in an inoculated leaf attached to a short stem section resulted after the newly prepared cutting was heated at 50 C for 40 seconds, positioned with the base of the stem section in distilled water, and maintained for 2 days in a recovery chamber. Lesions also were produced in inoculated leaves that were excised 2 days after inoculation, heated, and kept in a recovery chamber for 2 days with the petioles immersed in water. Heat-induced lesions developed when leaves excised 2 days after inoculation were heated, placed on wet paper in pans previously and subsequently covered with plastic, and kept at room temperature. The minimal exposure at 50 C necessary for the complete collapse of heat-induced TMV lesions was not noticeably altered if the inoculated Turkish tobacco leaves were systemically infected with a mild strain of PVX. During the above manipulations, this standard procedure caused no heat injury and resulted in obvious necrotic lesions measuring 1-2 mm in diameter in all parts of the inoculated leaf.

Correlation of heat-induced TMV lesions with the initial sites of virus infection.—Before heat-induced lesions can be used in research procedures which normally require local lesion hosts, evidence must be obtained demonstrating that this heat-induced necrosis only occurs at all or most initial infection sites in the inoculated leaf. Experiments evaluating this possible correlation were conducted with Turkish tobacco plants grown and maintained in the greenhouse. Two fully expanded leaves on each plant were inoculated with the common strain of TMV 1 day after the growing point and all unwanted leaves had been removed. A preliminary report of the results has been published (6).

Half-leaf tests demonstrated that necrotic lesions occurred only in those leaf areas which had been both inoculated and heated. A half of each leaf was inoculated with a dilution of crude virus extract in distilled water, and the opposite halves were rubbed with a solution containing a similar concentration of crude extract from healthy Turkish tobacco leaves. Two leaves on each of five plants inoculated in this manner were given the standard treatment for the production of heat-induced TMV lesions. Ten leaves on five similar plants were handled in the same way as the treated plants except that they were not heated. Necrotic spots developed only in the heated inoculated leaf tissue (Fig. 2).

Within the range of dilutions between one part of crude virus extract in 10^4 and 10^7 parts of distilled water, the average number of heat-induced lesions per leaf decreased with increased inoculum dilution (Fig. 3-A). When the average lesion number per leaf was plotted against the logarithm of the dilution, a typical dilution curve resulted. The numbers of heat-sensitive areas were approximately inversely proportional to the inoculum dilution in the range around dilutions of 10^{-5} and 10^{-6}.

The average lesion diameter was progressively greater as the elapsed time between inoculation and heat treatment increased from 2 to 5 days (Fig. 3-B, Fig. 4-A,B,C,D). The heat treatment of inoculated leaves 1 day after inoculation resulted in no visible lesions, and a similar treatment after the fourth day following inoculation induced large irregular lesions that were difficult to measure accurately. This expansion of the heat-sensitive area 2-5 days after inoculation was linear (Fig. 3-B) and was in agreement with the anticipated enlargement of the infected area during this time period (10).

Additional evidence that the factor responsible for heat sensitivity, presumably the virus, was spreading in the

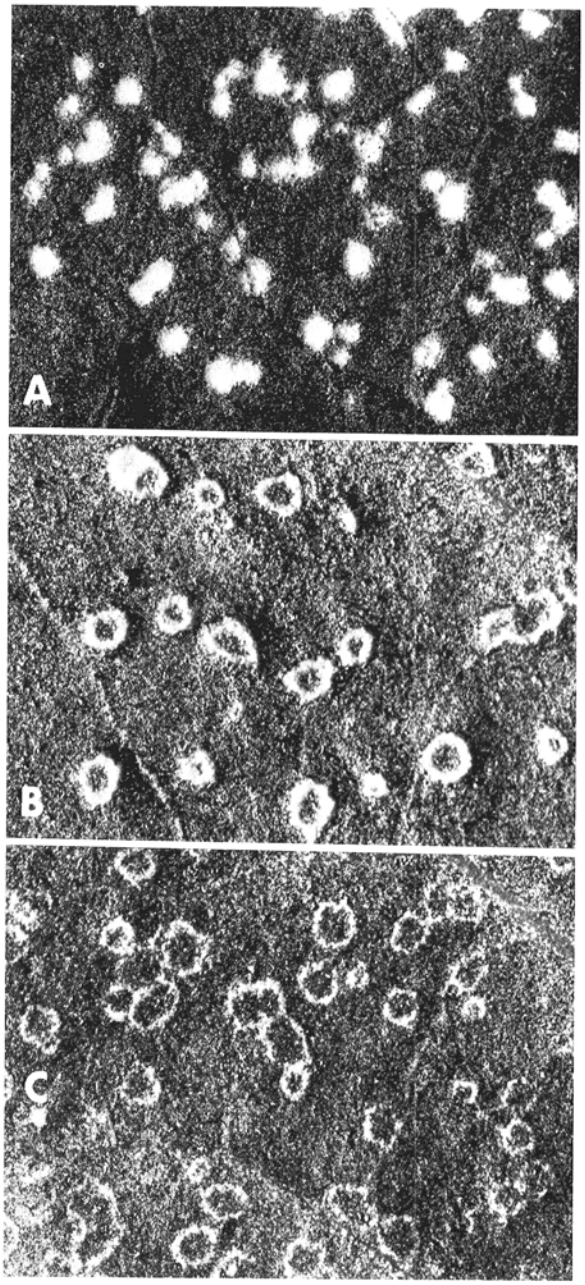

Fig. 5-(A to C. Lesions in Turkish tobacco leaves of plants grown for 2 weeks in the 20 C growth chamber and then inoculated with tobacco mosaic virus **A)**, potato virus X **B)**, or cucumber mosaic virus **C)** 2 days before heat treatment at 50 C for 30 seconds, 50 seconds, and 50 seconds, respectively (× 7).

inoculated leaf during this time interval was obtained when leaves containing lesions originally produced by heating 2 days after inoculation were reheated in the standard way 5 days following inoculation. The second heating often induced a faint necrotic ring around each original lesion, resulting in a ringspot-type symptom (Fig. 4-E).

Evidence associating heat-induced lesions with the sites of virus multiplication was obtained in attempts to recover TMV from lesion areas and from green tissue between lesions in inoculated leaves. A tobacco leaf with 10-20 lesions induced by heating 2 days after inoculation was dipped (2-3 days after heat treatment) in 0.05 M NaOH for a few minutes to remove any residual virus. After a distilled water rinse, a group of leaf disks each containing a lesion, and a group of disks from green areas at least 1 cm away from the nearest lesion, were removed from the leaf with separate flamed No. 3 cork borers. Each group was ground in a different glass homogenizer containing distilled water, and each extract was diluted to a concentration equivalent to one disk per 10 ml of distilled water. The infectivity of the two extracts was compared by rubbing two half-leaves on each of five trimmed Samsun NN tobacco plants with one extract, and then rubbing the opposite half-leaves with the other extract. In three experiments, extracts from green areas induced no lesions in the rubbed half-leaves whereas extracts from heat-induced lesions initiated an average of 288 lesions per half-leaf. These data provide evidence that all, or almost all, virus multiplication occurred in the region of the heat-induced necrosis.

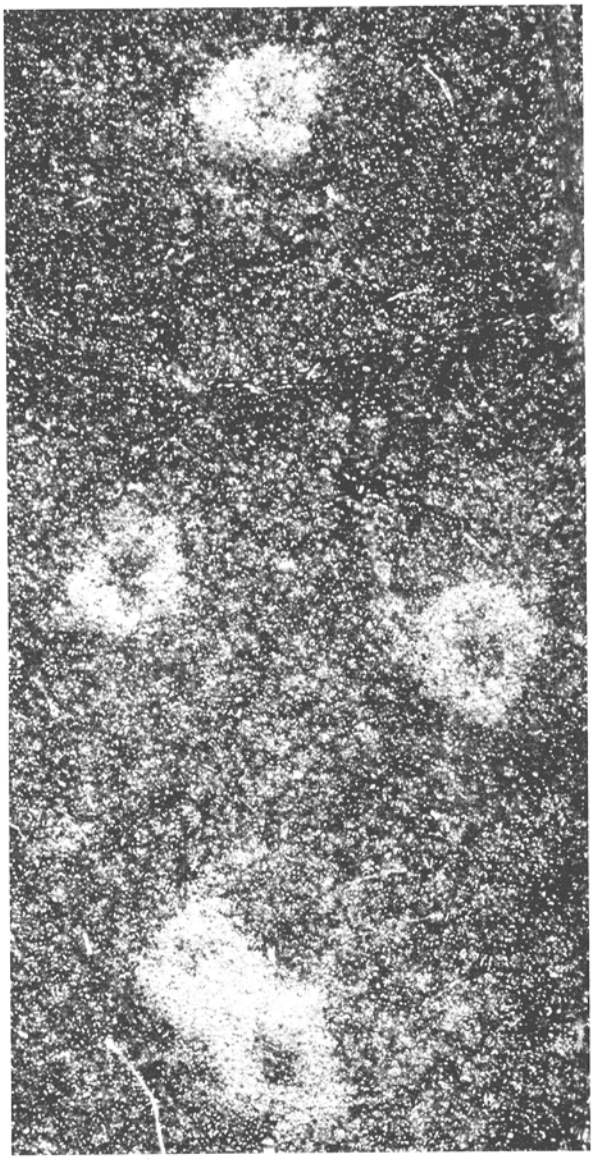

Fig. 6. Lesions induced in Turkish tobacco leaves that were inoculated with tobacco mosaic virus and 2 days later exposed to air at 50-54 C for 9 minutes just before being submerged in 20% ethanol at −4 C for 30 seconds (× 24).

Heat-induced lesions in tobacco leaves inoculated with other viruses.—When Turkish tobacco plants grown in the greenhouse were inoculated with viruses other than the common strain of TMV, consistent lesion induction by a standard heat treatment was not possible throughout the year. Seasonal variations in the ability to induce lesions in leaves of *Nicotiana tabacum* (Samsun NN or Turkish) inoculated with PVX, CMV, PVY, or TAV; in leaves of *Beta vulgaris* (sugar beet) inoculated with BMV; in leaves of *N. sylvestris* or *Lycopersicon esculentum* inoculated with PVX or TMV; and in leaves of *N. glutinosa* or *Datura stramonium* inoculated with PVX, were revealed by heating these leaves for 20-80 seconds at 50 C 2-4 days after inoculation at various times of the year. When plants were grown in the greenhouse during the cool cloudy days of winter, lesions and sometimes injury occurred in the relatively sensitive leaves after short hot-water exposures. Plants grown during hot sunny summer months were more resistant to heat injury, but lesion induction (if it was obtainable) required longer heating intervals at 50 C than was necessary during the winter. Therefore, in order to develop procedures for the consistent production of lesions throughout the year, Turkish tobacco plants preconditioned for 2 weeks in an environment similar to greenhouse conditions in winter were inoculated with certain viruses and exposed to different heat treatments.

Turkish tobacco plants grown for 6 weeks in the greenhouse were maintained for the next 2 weeks in a growth chamber set at 20 C, 75-85% relative humidity, and 10,764 lux light intensity for 13 hours a day. The light in this chamber was provided by warm-white VHO fluorescent lights and incandescent bulbs. During this 2-week period, the regular fertilization program was continued. The two youngest fully expanded leaves on each plant were inoculated with a diluted crude virus extract containing either PVX, CMV, or the common strain of TMV, immediately after the plant was decapitated and all unwanted leaves had been removed. These inoculated plants were kept in the 20 C chamber for 2 days. On the second day after inoculation, the leaves were immersed in hot water at 50 C for different intervals, and the treated plants were then maintained in recovery chambers for the next 2 days. In general, plants conditioned in this manner withstood hot water exposures at 50 C for up to 60 seconds without injury.

Necrotic rings measuring approximately 1 mm in diameter and distributed throughout all portions of the treated leaf, appeared 2-3 days after tobacco leaves previously inoculated with PVX or CMV had been heated for 50 seconds at 50 C (Fig. 5). Half-leaf tests confirmed that these lesions occurred only in leaf tissue that had been inoculated and heated. Under these conditions, the minimal heat treatment required for optimal lesion induction in tobacco leaves previously inoculated with the common strain of TMV was 30 seconds at 50 C (Fig. 5). Similar results were obtained with plants grown in the greenhouse during summer or winter before being conditioned in the 20 C chamber.

Lesion production following cold treatment of symptomless tobacco leaves inoculated with TMV.—If TMV-inoculated hypersensitive tobacco leaves are maintained at high temperatures after inoculation, infected tissue remains non-necrotic (2, 12, 13, 20, 21) but can be collapsed by exposing the leaf to low air (2, 12, 21) or water (15, 22) temperatures. However, lesions formed only occasionally in TMV-inoculated leaves of young nonhypersensitive tobacco plants after Ohashi and Shimomura (15, 22) immersed the detached leaves in ice water for 60 seconds on the second day of maintenance at 30 C. The reported induction of even a few lesions in cold-treated nonhypersensitive TMV-inoculated leaves prompted a reevaluation of some of our earlier experiments.

Turkish tobacco plants grown in the greenhouse at different times of the year possessed different degrees of resistance to cold treatment. Therefore, in an effort to insure uniform plants for the determination of the factors critical to TMV lesion induction, 6-week-old Turkish tobacco plants were transferred to the 20 C growth chamber and grown for 2 weeks more with the usual fertilization program, a practice that during previous experiments resulted in rapid succulent growth and optimal sensitivity to the induction of heat-induced TMV lesions. The growing point, and all but the two youngest fully expanded leaves, were removed from each plant just before the remaining attached leaves were inoculated with the common strain of TMV. Each attached leaf was inoculated on one half with a dilution of TMV crude extract and was rubbed on the opposite half with a similar dilution of extract from healthy leaves. These plants were kept in the 20 C chamber for the next 2-3 days, treated, and then maintained in recovery chambers for 2 days after treatment.

The ability of cold air or cold liquids to induce lesions without otherwise injuring TMV-inoculated leaves was first determined. Collapse of infected areas did not occur when inoculated leaves on plants grown in the 20 C chamber were exposed 2 days after TMV inoculation to cold air at −2 C to −6 C for 3 minutes, to 20% ethanol at 0 C for 1 minute, or to 20% ethanol at −4 C for 30 seconds. Treatments for longer durations at the indicated temperatures resulted in injury of both the inoculated and rubbed tissues of the treated leaf. In a similar study, Jones (11) failed to induce lesions by immersing Xanthi-nc tobacco (*N. tabacum*) leaves inoculated with potato mop-top virus in ice-cold water for 2 minutes during maintenance in a chamber at 22 C, growing conditions which insured the sensitivity of the infected tissue to heating.

These treatments differed from the procedure reported by Shimomura and Ohashi (22) in that our plants were not maintained at 30 C both before and after the cold treatment. The possibility that more than exposure to a cold liquid is necessary for lesion induction, prompted studies of techniques involving both hot and cold treatments. Characteristic lesions were induced in the inoculated tissue, but not in the healthy tissue, when leaves were subjected to hot air at 50-54 C for 9 minutes just

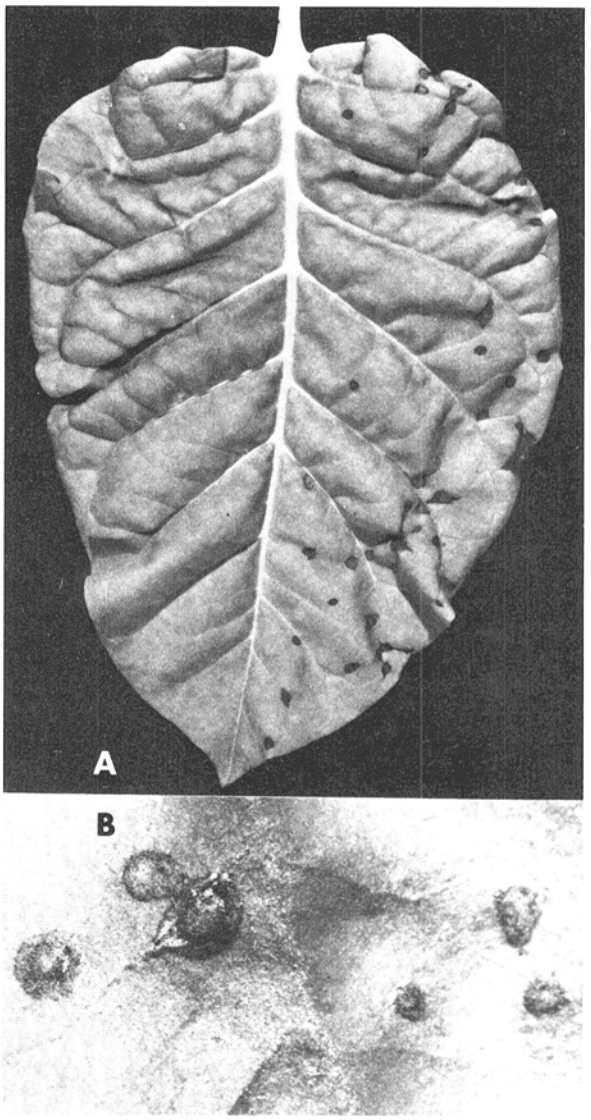

Fig. 7-(A, B). Necrotic lesions that developed within 1-3 days after uptake of 0.5 M NH_4Cl by Turkish tobacco leaves was initiated by placing the lower stem portion of cuttings in test solutions on the second day after the leaves were inoculated with tobacco mosaic virus. **A)** Treated leaf inoculated on the right half-leaf with tobacco mosaic virus from systemically infected leaves and rubbed on the left half-leaf with a similar dilution of extract from healthy tissue. **B)** Early appearance of sensitive areas as dark collapsed spots which later dried out and turned tan in color (× 4).

before being immersed in 20% ethanol at −4 C for 30 seconds on the second day after inoculation (Fig. 6). Of the limited number of similar treatments tried, this procedure was the most dependable for inducing lesion formation. The development of these small spots 1-2 days after treatment, did not prevent systemic virus movement. Unfortunately, the manipulations of this procedure were difficult to coordinate, and injury was not consistently prevented by placing the treated plants in the recovery chambers. Thus, further attempts to correlate these lesions with the initial sites of virus infection were not practical.

The possibility that the hot air treatment alone caused lesion induction was checked. Inoculated leaves kept in hot air at 50-54 C for 9 minutes 2 days after inoculation did not develop necrotic symptoms. When exposed for longer durations at these temperatures, the leaves were injured along the edges, but characteristic necrotic spots located exclusively in the inoculated half-leaves did not develop.

Lesion development during ammonium chloride uptake by tobacco leaves previously inoculated with TMV.—Bawden and Kassanis (1) were the first to report the development of necrosis in the symptomless TMV-infected tissue of inoculated tobacco leaves previously exposed to a chemical. Necrotic spots or rings that were associated with the initial sites of virus infection appeared 3-4 days after the inoculated leaves were sprayed with 2-thiouracil. These authors concluded that thiouracil-induced necrosis in the TMV-inoculated leaf was "a response of recently infected cells in which the virus is multiplying rapidly". Francki (7) disagreed with this conclusion after observing lesions induced by spray applications of thiouracil in both inoculated leaves and healthy leaves rubbed 1 day before the treatment. Recently Ohashi and Shimomura (16) induced lesions at the initial infection sites in symptomless inoculated tobacco leaves by immersing the leaf petioles in a solution of actinomycin D or chromomycin A_3 for less than an hour 1-2 days after inoculation of the leaves with TMV. In our preliminary studies, selective killing of the TMV-infected tissue was not observed when inoculated tobacco leaves were allowed to take up either thiouracil or actinomycin D through the vascular tissue. The reason for our failure is not known; the reports of successful lesion induction did not specify which conditions during plant growth, virus multiplication, or chemical uptake, were favorable or unfavorable to lesion production.

Our preliminary experiments with other chemicals gave promising results with some ammonium salts. However, the development of local lesions during the uptake of 0.5 M ammonium chloride by Turkish tobacco leaves previously inoculated with TMV was not consistent in plants grown in the greenhouse throughout the year. Between late May and late September, the hot sunny conditions in the greenhouse stimulated rapid development of the light-green leaves and increased the sensitivity of the presumed infected areas to ammonium ions. During winter, lesion induction was possible only following a succession of bright sunny days. With plants grown in the greenhouse during the normally cold cloudy days of winter, uptake of ammonium salts usually caused severe wilting and injury before any lesions were visible in the dark-green inoculated leaves of the slowly growing plants. Therefore, the following experiments were conducted with plants grown in the greenhouse during the summer when consistent lesion induction was possible.

Necrotic spots, and sometimes thick rings, developed in inoculated Turkish tobacco leaves 1-3 days after the basal stem portion of the cutting was immersed in 0.5 M NH_4Cl on the second day after inoculation with the common strain of TMV (Fig. 7). The collapsed areas first appeared as dark water-soaked areas in an otherwise yellowish-green leaf. After a few days, the lesions usually dried out and became lighter in color. Lesion formation did not prevent systemic virus movement in the cutting.

The induction of lesions at the initial sites of TMV infection in inoculated tobacco leaves was attributed to the ammonium ion, because lesions occurred only in inoculated tissue exposed to this ion and because virus was recovered only from these lesions. Cuttings with leaves inoculated on one half with TMV and rubbed on the opposite half with a similar dilution of extract from healthy leaves were exposed to either 0.5 M NH_4Cl or 0.5 M NaCl 2 days after inoculation. Characteristic lesions developed only in the inoculated portions of the leaves that absorbed NH_4Cl. The faint chlorotic spots that became visible 2-3 days after inoculation of some of the darkest-green leaves collapsed during NH_4Cl uptake by these leaves. In the leaves exposed to NaCl, veinal necrosis occurred in both healthy and inoculated areas a few days after lesions were produced in leaves treated with the ammonium ion. When extracts made from lesion areas or green areas punched out of the same decontaminated half-leaf were rubbed on opposite half-leaves of Samsun NN tobacco, necrotic lesions developed only in those half-leaves rubbed with the lesion extract.

The ability of various ammonium salts to induce lesions in TMV-inoculated leaves was also tested. Cuttings containing leaves inoculated on one half-leaf and rubbed with noninfectious extract on the opposite half-leaf were used to determine whether the necrosis induced by the tested salts occurred in infected or in all rubbed tissue. On the second day after inoculation, similar concentrations of several ammonium salts were prepared and evaluated. A ranking of these salts in the order of decreasing ability to cause lesions exclusively in optimally sensitive TMV-inoculated tissue would list NH_4CL, NH_4NO_3, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, and $(NH_4)_2CO_3$. When NH_4NO_3 or $(NH_4)_2SO_4$ were tested under a variety of conditions, neither salt induced lesion formation in plants grown under as many different suboptimal conditions as did NH_4Cl.

DISCUSSION.—The collapse of the infected areas in inoculated leaves exposed to the described treatments was detectable, because certain precautions minimized injury of uninfected cells. For example, maintenance of cuttings from trimmed inoculated plants at low temperature and low light intensity during uptake of ammonium ions reduced injury to healthy tissue. Also, exposure of inoculated leaves on trimmed plants to cool temperatures, darkness, and high relative humidity prevented injury from heat treatments. The failure of these measures to eliminate injury completely in leaves subjected to high air temperatures and then a cold liquid, prevented further research with this procedure. Perhaps future improvements in preserving treated tissue will

facilitate the use of cold treatments or unrecognized procedures presently not considered because they cause excessive tissue injury.

Data from several kinds of tests indicated that the heat treatment and the ammonium ion exposure were detecting the infected tissue in the inoculated leaf. Heat-induced TMV lesions appeared only in the treated inoculated half-leaves, lesion number was decreased by inoculum dilution, lesion size increased with longer infection periods before treatment, virus could be recovered only from the lesion area, and a necrotic ring developed around the initial lesion when leaves were reheated 3 days after the initial treatment. Exposure to 0.5 M NH_4Cl induced lesion formation only in the virus-containing areas of the inoculated tissue and collapsed the faint chlorotic spots which identified the initially infected areas in some inoculated leaves.

The induction of lesions by NH_4Cl only during the warm sunny months of the year discouraged the routine use of this technique as a research tool. Further work under different conditions, or with other host-virus combinations, might prove the procedure useful. With the exception of extremely hot periods in summer, the heat treatment consistently induced lesions throughout the year in TMV-inoculated leaves of greenhouse-grown plants. Of the three techniques investigated, the hot water treatment of TMV-inoculated tobacco leaves at 50 C for 40 seconds proved to be the simplest and most dependable.

Experiments with Turkish tobacco plants grown in controlled environments or in the greenhouse provided a possible explanation for lesion induction. Under growing conditions where virus-infected cells were optimally distinguishable from healthy cells by exposure of the inoculated leaf to water at 50 C for 30 seconds, the hot air treatment at 50 C of similar leaves did not detect any differences between these two types of cells. These results suggest that treatment at 50 C is not critically important to the collapse of virus-infected tissue. The fact that water is a better conductor of heat than air suggests that the rapidity of the temperature change, and not the final temperature of the leaf, may be the cause of necrosis. It is possible, however, that the actual final temperature of the infected areas exposed to hot air was not as high as that produced in similar tissues by the treatment with hot water. The hypothesis that necrosis results from a rapid temperature change is supported by the generalization that lesions were induced when inoculated leaves maintained at one temperature extreme were subsequently subjected to liquid at the opposite temperature extreme. For example, treatments with cold 20% ethanol at −4 C were successful when the TMV-inoculated leaves were kept at high temperature before treatment, but not successful when similar leaves were treated after removal from the 20 C growth chamber. Also, optimal lesion induction by hot-water treatments at 50 C was obtained with TMV-inoculated leaves of plants grown at 20 C, but poor lesion collapse sometimes occurred in inoculated leaves exposed to greenhouse conditions during hot summer days before heat treatment at 50 C. Similar trends were also observed when tobacco leaves inoculated with PVX or CMV were heated.

The experience gained during this research provides certain suggestions that may aid future attempts to induce lesions in other plants infected with other viruses. In order to maximize the effect of the treatment to be used, the plants to be inoculated should be grown under conditions optimal for rapid succulent growth. Use of a virus solution capable of inducing initial infection sites is essential. In order that collapsed virus-infected areas can later be distinguished from injured tissue, each leaf to be treated should contain an inoculated section and another section rubbed with a similar concentration of extract from healthy plants. During the time between inoculation and treatment, the plants should be exposed to an environment favorable for the rapid synthesis and maximum accumulation of the virus in the leaf. With host-virus combinations where virus multiplication is optimal in the 20 C to 30 C range, heating would probably be the treatment most likely to induce collapse of virus-infected tissue. Where leaves must be subjected to higher temperatures in order to maximize virus multiplication, the best method might be exposure to a cold liquid, or to ammonium ions. A program designed to expose a series of infected leaves to progressively more intensive treatments during each day after inoculation should determine whether lesion induction by a particular treatment is possible.

LITERATURE CITED

1. BAWDEN, F. C., and B. KASSANIS. 1954. Some effects of thiouracil on virus-infected plants. J. Gen. Microbiol. 10:160-173.
2. CARROLL, T. W., and T. KOSUGE. 1966. Effect of temperature on the response of systemically susceptible and hypersensitive Xanthi tobacco plants to tobacco mosaic virus infection. Phytopathology 56:873 (Abstr.).
3. DAVIS, R. E., and A. F. ROSS. 1968. Increased hypersensitivity induced in tobacco by systemic infection by potato virus Y. Virology 34:509-520.
4. DOKE, N., and T. HIRAI. 1970. Effects of tobacco mosaic virus infection on photosynthetic CO_2 fixation and $^{14}CO_2$ incorporation into protein in tobacco leaves. Virology 42:68-77.
5. DOKE, N., and T. HIRAI. 1970. Radioautographic studies on the photosynthetic CO_2 fixation in virus-infected leaves. Phytopathology 60:988-991.
6. FOSTER, J. A., and A. F. ROSS. 1968. Local lesion development in Turkish tobacco leaves heated after inoculation with tobacco mosaic virus. Phytopathology 58:1050 (Abstr.).
7. FRANCKI, R. I. B. 1962. The inhibition of plant virus multiplication in two host species by 2-thiouracil. Virology 17:1-8.
8. FULTON, R. W. 1970. Prunus necrotic ringspot virus. No. 5 *in* Descriptions of plant viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
9. HOLMES, F. O. 1929. Local lesions in tobacco mosaic. Bot. Gaz. 87:39-55.
10. HOLMES, F. O. 1931. Local lesions of mosaic in Nicotiana tabacum L. Contrib. Boyce Thompson Inst. 3:163-172.
11. JONES, R. A. C. 1973. Effects of heat shock treatment on symptom development in tobacco leaves inoculated with potato mop-top virus. Ann. Appl. Biol. 74:349-358.
12. KASSANIS, B. 1952. Some effects of high temperature on the susceptibility of plants to infection with viruses. Ann. Appl. Biol. 39:358-369.
13. MC KINNEY, H. H., and E. E. CLAYTON. 1945. Genotype and temperature in relation to symptoms caused in Nicotiana by the mosaic virus. J. Hered. 36:323-331.
14. OHASHI, Y., and T. SHIMOMURA. 1971. Necrotic lesion

induced by heat treatment on leaves of systemic host infected with tobacco mosaic virus. Ann. Phytopathol. Soc. Jap. 37:22-28.

15. OHASHI, Y., and T. SHIMOMURA. 1971. Induction of local lesion formation on leaves systemically infected with virus by a brief heat or cold treatment. Ann. Phytopathol. Soc. Jap. 37:211-214.
16. OHASHI, Y., and T. SHIMOMURA. 1972. Induction of localized necrotic lesions by actinomycin D on leaves systemically infected with tobacco mosaic virus. Virology 48:601-603.
17. OTSUKI, Y., T. SHIMOMURA, and I. TAKEBE. 1972. Tobacco mosaic virus multiplication and expression of the N gene in necrotic responding tobacco varieties. Virology 50:45-50.
18. ROSS, A. F. 1968. Brief heat treatment stops virus increase in tobacco mosaic virus lesions. Phytopathology 58:402 (Abstr.).
19. ROSS, A. F., and H. W. ISRAEL. 1970. Use of heat treatments in the study of acquired resistance to tobacco mosaic virus in hypersensitive tobacco. Phytopathology 60:755-770.
20. SAMUEL, G. 1931. Some experiments on inoculating methods with plant viruses, and on local lesions. Ann. Appl. Biol. 18:494-507.
21. SHIMOMURA, T. 1971. Necrosis and localization of infection in local lesion hosts. Phytopathol. Z. 70:185-196.
22. SHIMOMURA, T., and Y. OHASHI. 1971. Conditioning of local lesion formation by a brief heat or cold treatment of leaves systemically infected with TMV. Virology 43:531-532.
23. SUMMERS, C. G. 1970. Epidemiology of beet mosaic in central New York. Ph.D. Thesis. Cornell University, Ithaca. 90 p.
24. TAKAGI, Y., and Y. SUGIMURA. 1973. Local lesion formation by ultraviolet irradiation on leaves systemically infected with TMV. Can. J. Bot. 51:825-826.
25. TAKAHASHI, T. 1972. Studies on viral pathogenesis in plant hosts. III. Leaf age-dependent susceptibility to tobacco mosaic virus infection in 'Samsun NN' and 'Samsun' tobacco plants. Phytopathol. Z. 75:140-155.
26. YARWOOD, C. E. 1958. Heat activation of virus infections. Phytopathology 48:39-46.