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Making a Virus Visible: Francis O. Holmes and a Biological Assay for *Tobacco mosaic virus*

KAREN-BETH G. SCHOLTHOF
Department of Plant Pathology and Microbiology
Texas A&M University
College Station, TX 77843-2132
USA
E-mail: kbgs@tamu.edu

Abstract. In the early twentieth century, viruses had yet to be defined in a material way. Instead, they were known better by what they were not – not bacteria, not culturable, and not visible with a light microscope. As with the ill-defined "gene" of genetics, viruses were microbes whose nature had not been revealed. Some clarity arrived in 1929 when Francis O. Holmes, a scientist at the Boyce Thompson Institute for Plant Research (Yonkers, NY) reported that *Tobacco mosaic virus* (TMV) could produce local necrotic lesions on tobacco plants and that these lesions were in proportion to dilutions of the inoculum. Holmes' method, the local lesion assay, provided the first evidence that viruses were discrete infectious particles, thus setting the stage for physicochemical studies of plant viruses. In a field where there are few eponymous methods or diseases, Holmes' assay continues to be a useful tool for the study of plant viruses. TMV was a success because the local lesion assay "made the virus visible" and standardized the work of virology towards determining the nature of the virus.

Keywords: *Tobacco mosaic virus*, Virology, Tobacco, Genetics, Boyce Thompson Institute for Plant Research, Scientific institutions, Plant pathology, Twentieth century biology, Bacteriophage, Rockefeller Institute for Medical Research

> How do we know that viruses exist? In the light of our present knowledge, this may seem to be a naïve question. But suppose we knew nothing about viruses and only suspected that there were such things; what chance would we have of finding one? (Stanley and Valens, 1961, p. 28).

In 1934, Wendell Stanley, a young scientist at the Rockefeller Institute for Medical Research (RIMR) "methodically and doggedly" (Williams, 1959, p. 98) worked to purify *Tobacco mosaic virus* (TMV) from infected

tobacco plants, relying on a technique that allowed for the quantitative determination of the infectious agent. Stanley was using a biological assay developed 5 years earlier by a co-worker, Francis O. Holmes, to monitor the efficacy of TMV purification schemes and the infectivity of the crystallized virus samples (Creager, 2002; van Helvoort, 1991; Lederman and Tolin, 1993; Norrby, 2008; Kay, 1986; van Helvoort, 1996). Holmes' research at the Boyce Thompson Institute for Plant Research (BTI), and later at the RIMR, provided a means by which it became possible to visualize the (disease) effects of TMV by biological assay on plant leaves. Holmes' assay allowed the researcher to both determine the presence and the accumulation of a virus, even as they were unable to physically see and quantify the number of particles. Prior to Holmes' discovery, as recalled by F. C. Bawden, "working with viruses was like trying to find a black cat in a dark cellar, with no certain knowledge that the cat was there." Holmes' assay not only allowed for the detection of the cat, but within a few years it was possible to "report a good deal about the shape, size and constitution of the cat" (Bawden, 1966, p. 2). Holmes' local lesion assay has been hailed as "a seminal assay" and "the simplest of them all" (Tooze, 1979), revealing that virus titer was "proportional to the dilution of the extracts. So even if [the virus] could not be visualized the infectious particles could be counted" (Tooze, 1979), thus allowing researchers to "see" the virus. Holmes' local lesion assay continues to be used by plant virologists studying virus-host interactions and in introductory plant pathology laboratory classrooms worldwide.

Holmes made it possible to "do" virology – by developing a technique to assay and make pure cultures of plant viruses – a novelty for the nascent field of virology. In this paper, I will show how Holmes developed the method, the pivotal role of the Institution in promoting the work, and how the innovative assay was rapidly absorbed into the canon of plant pathology. Finally, I will discuss how this assay stimulated new work in plant viruses, animal viruses, and bacteriophage.

An Institute for Plant Research

The Boyce Thompson Institute for Plant Research (BTI) opened in September 1924 in Yonkers, New York. It was founded by Col. Boyce Thompson with the intent of solving fundamental and practical problems of plant biology, in line with the role of the RIMR in improving human health. With an initial endowment estimated at $12 million

(Zimmerman, 1929, p. 1390) a corps of experts in chemistry, pathology, and microscopy were hired. The staff numbered 37 scientists and 50 other employees by 1929. The BTI had state-of-the-art growth rooms and greenhouses with controlled light intensity and spectra, temperature and humidity, carbon dioxide, refrigeration rooms, and laboratories for plant physiology, chemistry, and pathology.[1] The Institute welcomed collaborative research with universities, with especially close ties to Cornell University (Ithaca, NY).

The focus of the BTI on solving ‘‘practical plant problems in a fundamental way’’ (Zimmerman, 1929, pp. 1385–1386) was unique for the agricultural sciences – more in common to the Rockefeller Institute than the research programs of agricultural colleges or the USDA. By the early twentieth century, plant pathology was well-recognized discipline with degree-granting programs at land-grant colleges/universities and a professional society (Campbell et al., 1999; Peterson and Scholthof, 2010). The discipline was known for its problem-solving skills with several successes in plant disease control and identification with a focus on the ‘‘new botany,’’ bringing plants and their pathogens into the laboratory for microscopic, genetic, and taxonomic study (Kelman and Peterson, 2002; Campbell et al., 1999; Peterson and Scholthof, 2010). Plant pathology was strongest at the USDA with the greatest number of plant pathologists and the most influential research – including the nascent sub-discipline of plant virology.

Thompson’s plan for the Institute was expansive, with the intent of improving plant health towards reducing the potential for famine due to crop losses. This in turn, was intended to improve human health. The uniqueness of the BTI in the context of American science is that it was the first independently endowed institute dedicated to plant biology.[2]

In 1923, William Crocker, a plant physiologist at the University of Chicago, was appointed Managing Director. His first hire was

[1] As recalled by H. H. McKinney, a plant virologist with the USDA, the mission of the BTI was ‘‘high-level plant research under the leadership of Dr. Crocker… [which] was to become the center for the unfolding of a master program of plant virus research – others beware’’ (McKinney, 1972, p. 4).

[2] The lack of institutional history and biography of the BTI or seminal players (other than Wendell M. Stanley) requires the use of primary source materials, correspondence, and hagiography to piece together the intent and development of plant virus work from 1924 to 1930. Some examples include Hagedorn (1935), Crocker (1948), McNew (1956), Corner (1964), Peterson and Scholthof (2010), Scholthof (2004), Scholthof and Peterson (2006) and Creager (2002).

Louis Otto Kunkel, a plant pathologist. Following completion of a Ph.D. at Columbia University, Kunkel was hired by the USDA as a pathologist (1915–1920), which included a year studying in Germany and Sweden (1916). At the USDA Kunkel became familiar with virus problems on potato. Kunkel then joined the Hawaiian Sugar Planters' Association as an Associate Professor, where he discovered the specificity of insect transmission of plant viruses. At the BTI, Kunkel's charge was to determine the nature of the virus with a goal of advancing ''knowledge of these obscure and destructive diseases,'' by studying insect transmission, cataloging virus symptoms and host range, and controlling virus infections. This was to include determining the physical and chemical character of viruses and to ''attempts to isolate and grow the causal agent in pure culture'' (Kunkel, 1925, pp. 520–521).

When Kunkel established his research group in 1924, little progress had been made in understanding the nature of any virus in the three decades since Martinus W. Beijerinck had coined the modern use of ''virus'' as a small, filterable, infectious entity. Viruses remained so ill-defined that they were given a ''continual interchange of amenities sometimes metaphysical, sometimes almost mystical'' (Henderson Smith, 1938, p. 235). TMV was variously defined as a physiological anomaly, protozoan, enzyme, toxin, or novel infectious entity. This ''difference of opinion persisted because there was nothing known which was decisive one way or the other'' (Henderson Smith, 1938, p. 235), but the ''uncertainty as their nature continually attracted scientific interest'' (Bawden, 1939, p. 7). Crocker concurred, writing in 1927 that ''perhaps there is no problem in plant pathology more baffling than the actual nature of plant viruses. Are they a very primitive organism, are they mere chemical compounds of a type not yet understood and capable of reproducing themselves, or are some of them one and some of them the other?'' (McCallan, 1978, Report of the Managing Director, June 11, 1927, p. 7).

Kunkel hired Holmes in 1924 to work on mosaic and yellows problems towards resolving the ''nature'' of the virus – broadly encompassing a physical, biological and chemical definition of infectious filterable agents of disease. Holmes (1897–1990), born in Cambridge, Massachusetts, completed his B.S. in 1921 in the department of Biology and Public Health at the Massachusetts Institute of Technology (MIT) with an undergraduate thesis on the ''Mode of Entrance of Fusarium into Wheat Seedlings'' (Holmes, 1921). In 1925 Holmes

obtained his Sc.D. in protozoology at Johns Hopkins University, with minors in immunology and bacteriology. His dissertation was on a protozoan in milkweed plants (Holmes, 1923, 1925, 1928c). In October 1923, Holmes joined the BTI, where he remained until 1932 when he [and the other BTI plant virologists, with the exception of Helen Purdy Beale (Scholthof and Peterson, 2006)] moved with Kunkel to the newly founded RIMR Plant Pathology Division in Princeton, N.J. When the Princeton labs closed in 1950, Holmes moved to RIMR in New York City when he remained until his retirement in 1965 – the same year the Institute became Rockefeller University (Corner, 1964).

Holmes' job was to determine the etiology of "this mysterious and destructive group of plant diseases" using "an ultra-photographic microscope that photographs objects too small to see with the highest power of the microscope" (McCallan, 1978, Annual Meeting of the Board of Directors, September 14, 1926, p. 6).[3] Holmes, trained as a protozoologist, was to explore one hypothesis of the period: that viruses were protozoa, based on the appearance of the inclusion bodies in the infected cells. In the mid-1920s several researchers, including Kunkel, had reported these tell-tale microscopic effects associated with virus infections – namely dark structures, or inclusions, in the cytosol of infected plants (Goldstein, 1924, 1926, 1927; Eckerson, 1926; McKinney et al., 1923; Kunkel, 1922, 1925). Crocker realized "this question is one that may require long patient work for solution" (McCallan, 1978, Annual Meeting of the Board of Directors, September 14, 1926, p. 6), but after 3 years no progress had been made. By 1927, Holmes recognized that he must determine if the infected plant samples viewed under ultraviolet light microscope *even* contained the virus agent. This problem resulted in the development of the local lesion assay, which quickly became a generic tool for virus workers in 1929.

My particular interest here is how the method was realized by Holmes and became a standard for virologists, and by extension, how it may have influenced the thinking of animal and phage virologists. The

[3] This microscope with an ultraviolet light source was on loan from Columbia University. As described by Holmes (with a photograph of the apparatus), the light was emitted at a wavelength of 275 nm. The monochromatic light source, generated by a cadmium spark, in combination with quartz prisms, lenses, microscopy slides and coverslips, prevented chromatic aberration, thus enhancing resolution. This microscope allowed Holmes to detect objects with a diameter ca. 75–80 nm and ca. 150 nm (the range of the light microscope). If the virus was less than 75 nm in diameter, then it "would be hopeless to detect it" (Holmes, 1928d). A TMV particle is 15 nm $\times$ 300 nm, necessitating an electron microscope to visualize and physically count virus particles (Williams and Wyckoff, 1945; Creager, 2002).

intent is to look at ''the emergence of a broader 'generic' communal consensus or on how the specific becomes the generic,'' as described by Constant.[4] The development and acceptance of Holmes' assay provides a narrative for such a solution.

Developing an Assay

For Holmes, a quantitative biological assay (bioassay)[5] was key in determining the nature of the virus – was it a microörganism, an enzyme or a physiological abnormality? (Creager, 2002; van Helvoort, 1991; Lederman and Tolin, 1993; Norrby, 2008; Kay, 1986; van Helvoort, 1996). In 1927, in back-to-back papers in the *Journal of Agricultural Research* (McKinney, 1927a, b), McKinney, a career United States Department of Agriculture (USDA) scientist, pointed out that a ''quantitative method'' that could score for the ''occurrence of disease symptoms'' was essential (McKinney, 1927b, pp. 13–14). A successful strategy would mimic standardized bioassays, such as those used to test drugs, toxins, and vitamins.[6] From this, McKinney identified seven parameters for quantitative work on plant viruses (McKinney, 1927b):

1. The virus must lend itself to study in the expressed fluids without losing its potency in a short time.
2. The plant employed must be easily cultured under greenhouse conditions during as much of the year as possible.

[4] Edward W. Constant writes: ''Although some of the best recent work in the history of science focuses exactly on how specific pieces of science (theoretical or experimental results) come to be accepted by relevant communities, the emphasis for the most part has remained on the history and fate of specific solutions rather than on ''the emergence of a broader ''generic'' communal consensus or on how the specific becomes the generic'' (Constant, 1994, p. 448). For a recent example of looking at the generic, see García-Sancho's study of Frederick Sanger's techniques for protein and nucleic acid sequencing (García-Sancho, 2010).

[5] A biological assay (bioassay) for plant viruses is considered a modified form of Koch's postulates. To assay virus infectivity an extract of an infected plant sample is rubbed onto a leaf of a healthy plant. The inoculated plant is typically monitored for 7–14 days for the development of symptoms such as mosaic, stunting, and necrosis. For an example of typical TMV-associated symptoms, see www.apsnet.org/education/LessonsPlantPath/TMV/.

[6] Examples of these classic studies were performed for diphtheria toxin-serum treatment and niacin deficiency induced pellagra. For the historiography of these events, primary source material, and popular presentations, see Hammond (1999), Kraut (1996), Kraut (2003), Wells (1995), and Salisbury and Salisbury (2003).

3. It must be highly and uniformly susceptible to the disease, and must develop definite symptoms.
4. The plants used in a single experiment must be grown under the same conditions.
5. Growing conditions for all experiments must be standardized as far as possible.
6. Accidental infection through insect carriers, soil, contact with infected or infested materials, must be guarded against.
7. The inoculation technic [sic] employed must lend itself to the greatest possible uniformity and certainty.

McKinney monitored healthy, non-inoculated plants kept in proximity to the inoculated plants as an experimental control – they would not be expected to develop symptoms. Inoculations were made to plant stem, petiole, and ligule with a needle wrapped in cotton; after impaling the tissues, the swab was placed in the leaf axil (Figure 1). By monitoring symptoms and their intensity in the days post-inoculation, the percentage of infected plants for a given treatment or dilution could be quickly calculated. For example, McKinney showed it was possible to obtain 100% infection of inoculated plants from a 1:1000 dilution of TMV sap extract in water (McKinney, 1927b). Once this method was established more elaborate experiments could be performed including monitoring the effects of heat and chemical treatments, plant age, and growth conditions on the infectivity of TMV sap. The dilution endpoint assay – a method for determining the most dilute sap extract that results in infection of an inoculated plant – was used by McKinney to calculate the percentage of infection, instead of the traditional method of scoring for symptoms (+ or −). Yet, he was disappointed by its lack of consistency or accuracy due to "factors that could not be easily controlled," such as light and temperature. Because "the plant was the unit of infection" (Yarwood, 1957, p. 244), the utility of the assay was limited especially if greenhouse space was at a premium.[7]

[7] A comment by Robert W. Fulton, a plant virologist at the University of Wisconsin is instructive: "the accepted technique for inoculating plant viruses at that time was by needle pushed into leaves through drops of plant sap, a very inefficient method. Also, it was not realized how easily viruses such as TMV can be transmitted by casual contact. The inefficiency of needle pricking led to the use of large numbers of plants and an equally large number of controls. Later, in reminiscing, [James] Johnson [of the University of Wisconsin] remarked that any experiment then was considered a success if more inoculated plants developed symptoms than control plants" (Fulton, 1984, p. 29).

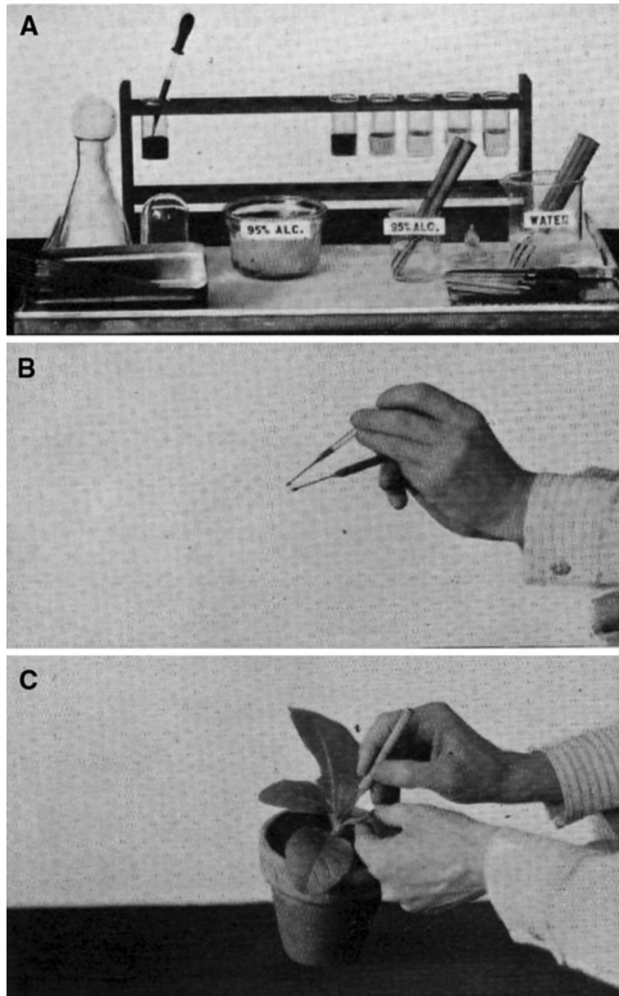

Figure 1. An experimental method and apparatus for impaling a tobacco plant with TMV sap extract. In 1927, McKinney described in detail how to inoculate plants for virus experiments. Panel A shows the test tubes with diluted sap extracts "and other items used for inoculating". Panel B shows how a sample (inoculum) would be absorbed to the "cotton on the tip of the needle". Panel C demonstrates how McKinney "pushes the cotton and inoculum into the tissues at the juncture of the stem and the leaf petiole" (McKinney, 1927b). The plants were scored for systemic symptoms ca. 10–14 days post-inoculation

These problems and lack of progress were not limited to TMV or other plant viruses. By the mid-1920s, dozens of plant viruses had been described based on their symptomology on crop plants or weeds, host range, dilution

end points and thermal inactivation, using sap extracts and very large numbers of plants that were scored for systemic symptoms. Yet little clarification of the chemical or physical nature of the virus was gained. Similar confounding issues were at hand for animal virus and bacteriophage workers. Review articles and textbooks show the level of conflict – what were these ultra-microbes and were they of a similar general nature?

Arguments were made that these agents were self-propagating proteins (or enzymes), that is, physiological aberrations instead of a filterable exogenous disease-causing entity. These discussions were occurring a decade after USDA scientist Henry A. Allard had elegantly demonstrated in 1916 that TMV-induced disease was not caused by aberrant host physiology, peroxidases or oxidases (enzymes), but by a virus – a small, filterable entity (Allard, 1916). These discussions continued for at least another decade, even after Stanley crystallized TMV and showed that those crystals were infectious. Similar contemporaneous arguments occurred on the nature of bacteriophage (Creager, 2010; d'Herelle, 1922; Duckworth, 1976; Summers, 1993, 1999; Twort, 1915).[8]

Setting the Stage

Holmes – having spent 3 years staring down the barrel of a microscope – was certain that TMV was not a protozoan. Yet he had a troublesome technical problem: did the small amounts of sap extract used for ultramicroscopy studies contain the active agent? (Holmes, 1928a).[9] His solution was to biologically assay the sap for infectivity. For this ''minute quantities of juice,'' collected on the tip of a fine insect pin, were poked through a healthy plant leaf. The development of systemic

[8] There is an extensive historiography of the early years of bacteriophage with a particular focus on d'Herelle and the later work of the American Phage Group (Summers, 1991, 1993, 1999, 2001; Creager, 2010; van Helvoort, 1992, 1994; Kay, 1993; Cairns et al., 1992; Stent, 1960; Luria, 1984), as well as the primary source literature and textbooks. More recently, Neeraja Sankaran has filled in the gaps of phage work between 1924 and 1927 with her study of F. M. Burnet (Sankaran, 2006, 2010). There also is a fairly extensive historiography of TMV, especially from 1930 to 1960, although there are some accounts of the early workers, with a focus on M. W. Beijerinck (Creager and Morgan, 2008; van Helvoort, 1991; Creager et al., 1999; Creager, 2002; Kay, 1986; Scholthof, 2004, 2011; Scholthof and Peterson, 2006; Scholthof et al., 1999; Wilkinson, 1976).

[9] The ''problem'' was presented in an abstract of the December 1927 annual meeting of the American Phytopathological Society (APS) in Nashville, Tennessee (Holmes, 1928a).

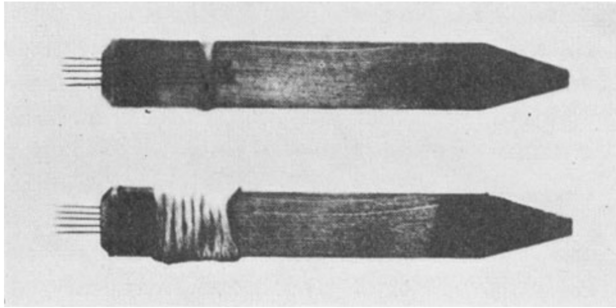

Figure 2. Inoculating pins for TMV assays used by Francis O. Holmes at the Boyce Thompson Institute for Plant Research. Five black enamel insect pins (size 00) were sandwiched between two wooden pot labels bound with string. This allowed for uniform and rapid inoculation of tobacco plants with plant sap for Holmes' local lesion assays (Holmes, 1928b). Reproduced with permission of the University of Chicago Press

symptoms on tobacco proved the microscopy samples had sufficient inoculum to establish an infection (Holmes, 1928a, p. 132).[10]

The novelty of this system was the tool – two wooden pot labels sandwiching five pins, held together with twine (Holmes, 1928b) (Figure 2) – and the rapidity of inoculation, making it possible to perform 500 pin-prick inoculations in an hour (Holmes, 1928b, p. 68). For these assays, Holmes used, as a stock solution, sap extracted from minced leaves of one hundred TMV-infected tobacco plants. Although the absolute concentration was unknown, it was a standard within the assay to determine a straightforward relationship between dilutions and number of plants infected. Holmes showed that the relationship of infectivity was not linear until more dilute samples were assayed. These assays required resources not available to other virus workers, of which McKinney had complained – to perform quantitative, meaningful assays it was necessary to grow several hundred or thousands of plants under controlled growth conditions. Holmes, at the BTI, had these resources. For example, he defined a "set" as trays of 50 plantlets (Figure 3) that were each inoculated with a serial dilutions of 1:2, 1:4, 1:8, 1:16, or 1:64, in addition to undiluted and mock-inoculated trays

[10] The use of "fine No. 00 black enamel insect pins" to transfer TMV (Holmes, 1928b, p. 67) may have originated with Francisco Seín, a Cornell-trained entomologist working at the Puerto Rico Insular Experiment Station. In an attempt to understand how aphids infected plant tissues with viruses, Seín mimicked their method by using a small needle. The collaborations, correspondence, visits, and reports between scientists at Cornell, Puerto Rico, and the BTI, suggests that Kunkel or Holmes were aware of Seín's findings (Seín, 1930, pp. 65–66) that were reported developed between 1925 and 1929 (Leonard, 1931, p. 144).

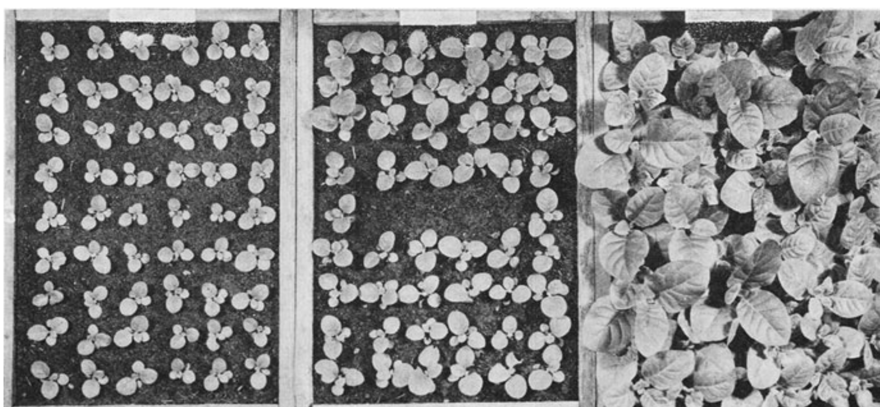

Figure 3. Flats of tobacco plantlets for TMV sap inoculation with insect pins, using the tool shown in Figure 1. A week after transplanting seedlings to flats, they were thinned to 50 plants per flat (center panel), inoculated, and scored for systemic symptoms (right panel). In the glossy photograph from the journal, it is possible on close inspection to see yellow (chlorotic) local lesions on some inoculated leaves (Holmes, 1928b). Holmes did not note this finding until the following year in his *Botanical Gazette* manuscript (Holmes, 1929) when he tested several species of tobacco for symptoms on inoculated leaves, as shown in Figure 4. Reproduced with permission of the University of Chicago Press

(ca. 350 plants). For an experiment, a set was repeated 8–24 times to calculate the number of plants infected, requiring more than 2500 plants (Holmes, 1928b, p. 70).

The use of trays decreased labor and space requirements and "the equipment needed for the experiments is simple, being new insect pins for each experiment and a few wooden pot labels" (Holmes, 1928b, p. 72). The sap dilutions allowed for an approximation of the number of plants required for comparative experiments if one sample differed in infectivity from another. By calculating the graphed data (Figure 4) it was possible for example, to determine how many plants should be inoculated to "differentiate" viruses of slightly different titers or to compare the infectivity of two different viruses (Holmes, 1928b, p. 71). Holmes calculated the relative reduction in the number of infected plants vs. those obtained with undiluted sap using the number of plants infected from the undiluted stock solution as the baseline (equivalent to 100%). Holmes suggested several types of experiments to measure certain parameters such as virus concentration, storage conditions (virus stability), and virus movement and distribution in the plant, but it

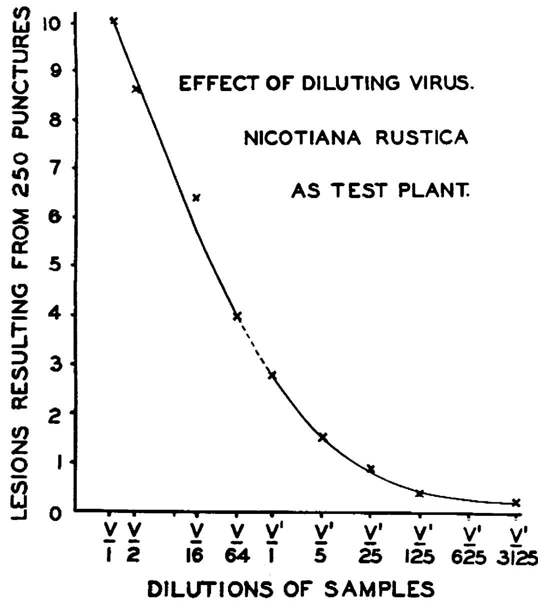

Figure 4. Graphical representation of the local lesion assay on *Nicotiana rustica*, showing the effect of sap dilutions of two samples of TMV (labeled V and V′), where V is more concentrated than V′. Holmes determined the titer of V was greater than V′ by counting "lesions resulting from 38,000 pin prick punctures," an example of the elaborate types of experiments that were possible at the state-of-the-art research facilities at the BTI and RIMR. Holmes provided the following interpretation of the data: "it will be observed that in the region in which four to ten lesions were obtained in each set of 250 punctures, the line appears nearly straight as drawn to a semilogarithmic scale. This part of the curve is similar to that known from earlier work with commercial tobacco [*Nicotiana tabacum*]. It is interesting to note the direction of the line when more dilute virus is used. With *N. rustica* it has been possible to study the lower range, because greater numbers of measurements can be made by the use of local lesions (such as those shown in Figure 5, panel 1) than by the use of the systemic disease as an indicator of successful transmission. The upper range is the portion most frequently dealt with, and the range in which the greatest accuracy can be obtained" (Holmes, 1929). Reproduced with permission of the University of Chicago Press

required far too many plants and inoculations for most workers to pursue these experimental protocols.

Seeing Local Lesions

While Holmes was methodically going about his research, two other young pathologists at Cornell University and Virginia Tech were testing the effects of other plant viruses on tobacco and reported observations

that contributed to Holmes development of the local lesion assay. In 1925, Karl Fernow[11] reported that TMV produced necrotic lesions on potato and *N. rustica* tobacco plants. And in 1927, Carl N. Priode[12] and co-workers described ringspot symptoms of *Tobacco ringspot virus* (TRSV) on five species of tobacco: *N. glutinosa*, *N. langsdorffii*, *N. paniculata*, *N. sylvestris*, and *N. tabacum* (Fromme et al., 1927). They showed TRSV infection initially was ''restricted'' to the inoculated leaf, but 5 days later ringspot symptoms appeared on the upper, non-inoculated leaves. The similarity of the symptoms on the inoculated and systemically infected leaves were important observations and, I suggest, Priode may have helped Holmes come to the realization that a local lesion was a means to quantitate TMV. This is a possibility because Priode joined the BTI in early 1927, where he continued his work on TRSV and, in 1929, published a paper showing that systemic infection consistently occurred within several days of observing local lesions on the inoculated leaf (Priode, 1928).

By 1929 Holmes had shown that minute amounts of sap from a TMV-infected plant was sufficient for a systemic infection. He then made a striking observation that the infection could be observed within days of pin-inoculation. He found that the inoculated leaves developed ''pale yellow areas'' around the pin-pricks and several days thereafter systemic mottling developed on the plant (Holmes, 1929). Holmes hypothesized that the ''very inconspicuous'' yellow areas were indicative of an early infection and that other tobacco species might produce a more easily observed lesions (Holmes, 1929, pp. 40–41). He reasoned that such a plant, would greatly speed up his experiments since the lesions could be used to score for infectivity in days instead of a week or more when systemic infection was the unit of infection. Local lesion assays would require much less greenhouse space, allowing for more repetitions of experiments in a shorter period of time.

Holmes tested 17 species of *Nicotiana* and identified five that ''showed pronounced necrotic local lesions instead of pale yellow areas''

[11] Fernow (1893–1983) received his B.S. (1916) and Ph.D. (1925) degrees at Cornell University staying on to establish the seed potato indexing program and working on potato diseases (Barrus and Smith, 1955; Fernow, 1925, 1983).

[12] Priode (1898–1985) completed a B.S. degree in Agronomy in 1925 at the Virginia Agricultural and Mechanical College Polytechnic Institute (VPI), followed by an appointment as an Assistant in Plant Pathology at the Virginia Experiment Station. He resigned his appointment on January 1, 1927 (Roane, 1992). For the experiments, Priode used needle inoculation of TRSV sap extracts on petunia and tobacco, resulting in local lesions. Dried TRSV-infected leaves also were used as inoculum for infectivity assays. In addition, extracts either kept at room temperature or frozen and thawed were used to demonstrate virulence was retained at colder temperatures.

following inoculation with TMV sap (Figure 5) (Holmes, 1929, p. 41). He quickly transitioned away from the cumbersome pin inoculation, finding that the most consistent results (accuracy) and fastest inoculations (hundreds of plants) were made by rubbing the leaves with a bit of cheesecloth or a fingertip dipped in plant sap. Holmes introduced another practice, first reported in Pirode's TRSV paper, to inoculate virus ''samples on opposite sides of the midvein of the same leaf or to use a large number of leaves for a single test… this is because leaves of different ages [on the same plant] have been found to differ somewhat in susceptibility'' (Holmes, 1929, p. 45). This ''half-leaf assay'' – used either to perform different dilutions on each side of a leaf or to duplicate the experimental assay using a single leaf, became a standard practice (Samuel and Bald, 1933; Samuel et al., 1935) and was statistically validated and improved upon by Helen Purdy Beale, also at BTI (Scholthof and Peterson, 2006; Youden and Beale, 1934; Youden et al., 1935).

Holmes stated that the method was intended ''to determine virus concentrations as accurately as bacterial numbers may be estimated or chemical concentrations may be calculated from quantitative analyses'' (Holmes, 1928b, p. 71). This connection to colony counting in bacteriology – making the local lesion test a way to ''microbiologize'' TMV – is important and interesting.[13] I have not found further elaboration by Holmes about the development of his ideas and how (or if) he was drawing on standard culturing techniques used by bacteriologists. Of course, he was familiar with, and exposed to, the cutting-edge bacteriological research of the day, having trained at MIT and Johns Hopkins, and through contact with (medical) researchers at Columbia University, perhaps obviating the need for such elaboration.[14] I suggest that Holmes' ability to imagine that a single virus particle could cause a local

[13] I am grateful to Angela Creager for ''microbiologicalize'' and urging elaboration on the parallels between the techniques.

[14] For example, William Sedgewick at MIT, was head of the Department of Biology and Public Health until his death in 1921, the same year that Holmes completed his undergraduate thesis on *Fusarium*. The scientific focus of Sedgwick's department was bacteriology, reflecting his status, with William Welch of Johns Hopkins, as a founder of the American Sanitarian movement (Benson, 1999). Sedgwick published ''Principles of Sanitary Science and the Public Health'' (1902) and in 1917 ''A Short History of Science''. Interestingly, an obituary noted that Sedgwick, ''was at his very best with a small group of students, following out in the experimental vein a line of thought which might lead from the structure of plant tissue to the domestic life of ancient Rome'' (Winslow, 1921, p. 260), allowing for speculation that he may have directly influenced Holmes' world view. Similar interactions would have occurred at Hopkins, when Holmes was at the School of Public Health and Hygiene (under Welch's direction from 1916 to 1927).

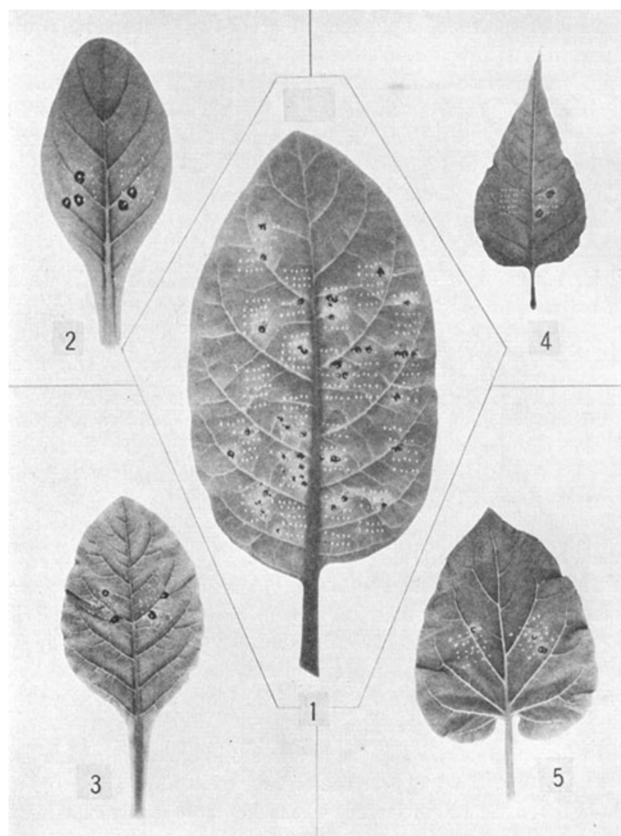

Figure 5. Local lesion response of selected tobacco species to insect pin inoculation with TMV sap. Panels 1–5 represent *Nicotiana rustica*, *N. langsdorfii*, *N. sanderae*, *N. acuminata*, and *N. glutinosa*, respectively. Note that the inoculation tool (Figure 1) was used to make 500 pin-pricks on a single *N. rustica* leaf, resulting in ca. 40 visible lesions. Each brown or chlorotic lesion represents a single point of infection visible on the photograph of each leaf. In contrast, the small *N. glutinosa* leaves are suitable for only 50 pin-pricks, which in this instance resulted in ca. 3 lesions described as "pale brown surrounded by rings of darker brown." Holmes preferred *N. glutinosa* plants for further TMV experimentation for several reasons: the small plant habit (size), making it an ideal greenhouse plant; the restriction of TMV to the inoculated leaf, thus reducing the possibility of cross-contamination between plants; and, of visible necrotic lesions within a few days following TMV rub inoculation (Holmes, 1929). Reproduced with permission of the University of Chicago Press

lesion, essentially representing the effect of a single infection, may have been influenced by his formative undergraduate and graduate research experiences at MIT and Johns Hopkins. For example, Holmes' undergraduate thesis included his careful drawings of germinating conidia (fungal spores) as they penetrated the leaf surface, showing typical yellow (chlorotic) or necrotic lesions (Holmes, 1921). His knowledge of

this response by the host plant to the fungal infection surely influenced his ‘‘preparedness’’ for the idea a local lesion could represent an infection by a single virus particle.[15]

By 1929, Holmes had conclusively demonstrated that local lesions are a necessary prelude to systemic infection (Holmes, 1929) and that until his work, when ‘‘local lesions have been observed… their real nature has not been understood’’ (Holmes, 1929, p. 39). His ideas were confirmed in a 1929 *Botanical Gazette* paper with several *Nicotiana* species that produced striking local lesions which were used for accurate, quantitative measurements of TMV infectivity (Holmes, 1929). When he used pinprick inoculations of TMV-sap extract, he scored lesions ‘‘before systemic symptoms appear’’ and the experiments were completed within one week (Table 1).[16] Holmes focused his work on two species: *N. glutinosa* with ‘‘necrotic lesions… appear[ing] very soon after inoculation’’ and *N. rustica* that could ‘‘accommodate 250 or 500 punctures’’ per leaf ‘‘sufficient to allow a fairly accurate reading of virus strength to be made with few plants’’ (Holmes, 1929, p. 45).[17]

Holmes then explains the utility of *N. glutinosa* for measuring virus concentrations: i) it has a low virus content when diseased, reducing the incidence of cross-contamination; ii) the lesions develop very rapidly, allowing for the plants to be scored and then discarded within a few days of inoculation; iii) large numbers of lesions can be observed on a single leaf; and, iv) rub inoculation can be used instead of pin pricks, allowing for rapid inoculation.[18] These adaptations really brought the local lesion assay to the forefront of virology.

[15] In 1929, although virus strains had not yet been carefully studied, Holmes also reasoned (and then showed) that the local lesion assay might be used to isolate pure lines of virus strains. The local lesion method to select pure strains of viruses continues to be used by plant virologists, especially to establish a pure preparation of a newly isolated virus.

[16] To clarify, TMV inoculation of *N. rustica* at room temperature results in systemic infection, in contrast to *N. glutinosa*.

[17] These experiments were confirmed, using similar plants and the tools described by Holmes (Scholthof, 2011). For more on interpreting historical experiments in a modern context, ‘‘Doing Biology’’ is a valuable guide (Hagen et al., 1997). This resource is updated as an online publication at doingbiology.net [accessed 28 February 2013].

[18] Holmes described the experimental conditions in detail, such as growing *N. glutinosa* plants ‘‘in 4-inch clay pots until flower buds begin to appear’’ at which time he ‘‘pinched off the remaining [young] leaves and the growing point… [leaving] a sturdy stem supporting five large leaves’’ (Holmes, 1929). A cheesecloth pad was used to wipe virus to the upper leaf surface with one plant of five leaves for each dilution. Following the inoculation ‘‘a full stream of tap water is used to wash away excess virus’’. In a few days the lesions were counted and an undiluted sap extract would ‘‘result in the production of about 300–600 lesions on each test plant’’.

Table 1. Tobacco mosaic virus assays on *Nicotiana* species

Species	Symptoms	Days	Outcome
N. rustica	Necrotic spots, brown irregular shapes	8–10	Spreading necrotic spots in inoculated leaves; leaf drops off after lesions coalesce
N. langsdorffii, *N. sanderae*	Blackish lesions with concentric rings of dead tissue	?[a] 8–10	Sometimes veins and stems develop spreading necrosis
N. acuminata	Lesions irregular in outline, brown in color	7–14	Increase in size of lesions more slowly than other species
N. glutinosa	Rapidly developing necrotic lesions; centers dry down to light brown color	2–3[b]	Dark rings form concentrically

[a] For *N. langsdorffii* the number of days post-inoculation when symptoms were not recorded. *N. langsdorffii* and *N. sanderae* results are combined since the symptoms and outcome were identical, based on data presented in the *Botanical Gazette* (Holmes, 1929). The question mark (?) indicates that Holmes did not record when symptoms were first observed.
[b] Holmes referred to this reaction as being as rapid as bacteria are counted by plating methods.

To exhibit these results, Holmes produced what would become an iconic figure for plant virology (Figure 6). A dilution series from 1:1 to 1:1000 of TMV-sap extract showed the easily visible (and countable) lesions on *N. glutinosa* where a "single test plant is sufficient to give an excellent idea of the strength of the virus sample in hand" (Holmes, 1929, p. 48). In the course of 1 year, Holmes had progressed from thinking about rapid assays using hundreds of plants, to determining that a single plant could produce sufficient lesions to stand in for dozens if not hundreds of plants. He had fulfilled the challenge set by McKinney to satisfy the seven points for being able to study plant viruses in the laboratory. Holmes realized the significance of his finding as early as 1929: "one test plant of *N. glutinosa* used as described serves the purpose for which at least several hundred *N. tabacum* plants are required" for typical virus assays of the day (Holmes, 1929, pp. 48, 50–51). As reported by Crocker to the BTI Board of Directors in 1931, "Doctor Holmes has worked out methods for the quantitative determination of the amount of virus in plants that are far more delicate and accurate than previous methods. These put new and important tools into the hand of investigators of virus diseases" (McCallan, 1978, Annual Report for the Year Ending December 31, 1930 at Board of Directors Meeting of June 8, 1931, pp. 14–15). In addition, the assay was quick and easy – instead of scoring systemic infections after several weeks, within a few days of inoculating *N. glutinosa* TMV local lesions could be counted and the data

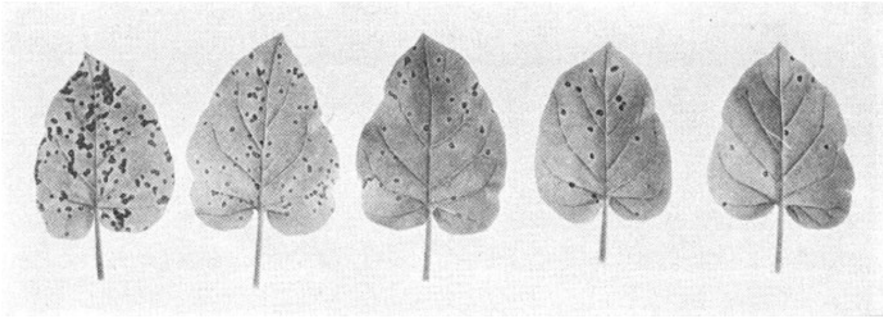

Figure 6. Dilution assay on *N. glutinosa* using TMV sap and rub inoculation. From left to right the sap was diluted 1:1, 1:3.16; 1:10; 1:100, 1:1000. The "decrease in lesions with serial dilution is plainly shown" and this simple and quick assay was a reliable and useful means to determine the concentration of virus for empirical experimentation, such as virus purification schemes, and obtaining "pure cultures" from a single lesion (Holmes, 1929). Holmes used cheesecloth pads or his finger to rub-inoculate the leaf, having abandoned the pin-prick method. Holmes showed a single *N. glutinosa* plant displaying local lesions substituted for a flat of 50 *N. tabacum* plants (Figure 3). A TMV experiment using *N. glutinosa* was complete in several days versus ca. two weeks for *N. tabacum*, which had had to be scored for symptoms of systemic infection (Holmes, 1929). Reproduced with permission of the University of Chicago Press

recorded. His manuscripts provide detailed written methodology, helpful photographs of virus symptoms on plants, and tables or graphs of the quantitative data. And, in communicating his results under the auspices of the prestigious Boyce Thompson Institute, Holmes insinuated himself as an important contributor to the plant virology literature.[19]

Following the local lesion assay, Holmes made a further advance in tracking the primary lesion responses. The scientific consensus before Holmes held that *N. tabacum* was a systemic host for TMV, but did not develop local lesions. In 1931, as an extension of his local lesion assay, Holmes showed that iodine made local lesions "more conspicuous" in Turkish tobacco, with abnormal coloration around the site of infection occurring as early as 36 hours post-inoculation (Holmes, 1931d, p. 163; 1932;

[19] Some established plant virologists, including Vinson and Petre in Kunkel's group at the BTI, apparently "did not use Holmes' local lesion test and continued the needle inoculation… because they simply paid no attention to the work of the young parasitologist and were well familiar with the [older] needle inoculation technique", as remembered by Karl Maramorosch in a letter to Angela N. H. Creager, Princeton University, Princeton, New Jersey, September 13, 1999.

Bawden, 1939, p. 28).[20] These observations were confirmed by Samuel (1931c), a plant pathologist in Australia.[21] Holmes (1932, p. 323) used iodine to trace the "location of the virus" and the progress of infection in thirteen plant species, some of which did not support TMV systemic infection. Once again, Holmes used photographs of the iodine assays to make the virus visible. The photographs in both instances were "not illustration, but argument" that communicated to virologists "both what we know and how we know" (Wise, 2006, pp. 81–82) – that is, local lesions were the evidence of TMV infection.[22] Photographs by Holmes and Samuel, which were subsequently reproduced in textbooks, were also intended as "visual cases to train the reader's (student's) eye" (Anderson, 2009, p. 120), specifically to see necrotic lesions that had earlier been ignored by virologists. Holmes' local lesion assay on *N. glutinosa* (Figure 6) is "materialized epistemology" – becoming a part of the material culture of research, towards determining the physicochemical nature of the virus.

Among the immediate uses of the local lesion assay were the studies of virus spread in the plant, isolation of pure strains, and evaluating the effects of virus purification methods on infectivity. The earlier push to define units of infection[23] became moot when Stanley reported the virus could form crystals, and soon thereafter that TMV was an infectious

[20] Starch accumulation as evidence of a virus infection had been described in 1913 by Hendrik M. Quanjer (de Bruyn, 1961) at the Agricultural University in Wageningen, the Netherlands. He showed that starch accumulated in the leaf lamina (blade) during an infection with *Potato leafroll virus*, an aphid-transmitted virus that replicates in the phloem. Quanjer suggested that disease was "due to disturbed transport on account of the necrosis of the phloem, which is the main downward channel for carbohydrates" (Barton-Wright, 1932). In 1930, plant physiologists in England showed starch was retained "at the points of infection" in tomato leaves infected with *Aucuba mosaic virus*, a strain of TMV (Bolas and Bewley, 1930). The method used by Holmes was to soak the leaves overnight in ethanol, stain them in a solution of iodine, wash the leaves in ethanol, and make a photographic record. If starch accumulated, the lesion "appeared as a gray area on a yellowish-brown background" (Holmes, 1931d).

[21] The relationship between Holmes and Samuel is discussed below.

[22] This use of photographs to show scientific data is described as "materialized epistemology – that is doing the work of knowledge production", and is elaborated on in recent articles by Nancy Anderson, Adam Mosley, Norton Wise, and *Objectivity* by Lorraine Daston and Peter Galison (Anderson, 2009; Daston and Galison, 2007; Mosley, 2007; Wise, 2006).

[23] McKinney (1927b) had suggested developing a "virus-unit" using whole plants and systemic infections. And Geoffrey Samuel told Holmes about his ideas for a "glutinosa unit" that would allow virus workers to compare data "some international unit of comparison" and he would "be only too glad to see you [Holmes] take up the measurement question yourself, for it needs the opportunity of working with thousands or tens of thousands of plants, such as you have" (Samuel, 1933a).

nucleoprotein (95% protein, 5% RNA) (Creager, 2002; Kay, 1986). These advances and the ability to purify and weigh milligram amounts of virus (to determine the extinction coefficient with a spectrophotometer) and to use serology to accurately determine virus concentrations and localize the virus in the plant, marked a transition of TMV and other viruses from plant pathology problems to laboratory objects for biochemical and molecular biology studies.[24]

Response to the Method

The adoption of new methods and scientific discoveries can be extraordinarily rapid or delayed – even by years or decades, within and across scientific boundaries, for inexplicable reasons (Becsei-Kilborn, 2010; Brakke, 1979; Hook 2002). The local lesion assay by Holmes was almost immediately absorbed as a standard tool for virus work.[25] There are several reasons for acceptance of the assay, including the scientific reputation of the science and scientists at the BTI and RIMR, the simplicity of the method, and the clarity with which Holmes presented his results.

BTI scientists were the first to confirm the utility of the local lesion assay. When sap from TMV-infected plants was exposed to UV light before inoculation infectivity on *N. glutinosa* was abolished; but, exposure to UV light after inoculation had no discernable effect on local lesion accumulation (Arthur and Newell, 1929). And under the direction of Holmes, W. C. Price showed that common bean (*Phaseolus vulgaris*) also could be used to test TMV infectivity by scoring for small necrotic lesions (Price, 1930).[26] Similarly, Kunkel, who had hired Holmes at

[24] For an example of this strategy, see Goldberg and Brakke (1987).

[25] The analysis from 1930 to 1935 was made using JSTOR www.jstor.org to track the citation record of Holmes papers (1928, 1929, 1930). Textbooks were identified from at the Mann Library (Cornell University), Sterling Evans Library (Texas A&M University), the Core Historical Literature of Agriculture http://chla.library.cornell.edu/, and Google Books (search term: ''plant pathology'' with limits of ''book'' and ''full text''. The tabulation of virus manuscripts in *Phytopathology*, the journal of record for the American Phytopathological Society, is shown in Table 2.

[26] Holmes in a letter to Samuel told of two reasons to use beans: ''(1) growth requires 10 days from seeding to inoculation; 5 more days before discarding; (2) some varieties are very sensitive to inoculation in the sense of producing numerous lesions, [and]... three difficulties; (1) no lesions appear at cool temperatures (70°F. or higher is necessary); (2) variations in pressure of inoculation are important; (3) transfer back to tobacco is very unsatisfactory, apparently because little virus develops''. Holmes was still using *N. glutinosa* ''because of the need for retransfer to tobacco to preserve strains, and because selection of bean strains and study of methods are still in progress here [at RIMR]'' (Holmes, 1933).

BTI, insisted on the use of the local lesion assay in a 1933 letter of support for T. E. Rawlins' application for funding from the American Academy of Arts & Sciences. Rawlins had shown that concentrated TMV was double refractive and it might provide a means to determine the concentration and shape of the virus. Kunkel was enthusiastic about this approach with the caveat that if Rawlins' work was to be of value it would have to be tested against "the local lesion method of Holmes and the serological methods of Purdy" (Kunkel, 1933).[27] Soon thereafter, using Holmes' local lesion assay, Rawlins and William Takahashi published a series of papers detailing the inactivation of TMV by exposure to high frequency sound radiation, correlating virus concentration with double stream refraction, and showing TMV was likely a submicroscopic rod-shaped particle (Takahashi and Christensen, 1934; Takahashi and Rawlins, 1934, 1935, 1937). Only the 1934 Takahashi and Christenson paper explicitly mentioned or cited Holmes' eponymous assay, reflecting that the *N. glutinosa* local lesion assay had become a commonplace method for virus workers.

In Great Britain, Caldwell was an early adherent of the method – to the extent that apparently he wanted to establish priority for introducing it to UK scientists.[28] In 1935 he claimed that he had used *N. glutinosa* as early as 1930 to show "that the number of lesions on the leaf was proportional to the concentration of virus in any infective juice" (Caldwell, 1935, p. 70), but this was not the case. Samuel had established priority among the UK workers.[29]

Bringing the Method to the General Virus Worker

Extensive correspondence between Holmes and Geoffrey Samuel provides a window on the development and promulgation of the art of the

[27] For more on the development of serological methods for plant virology and, by extension to plant pathology, see Scholthof and Peterson (2006) and references therein.

[28] Publishing by plant pathologists was country-centric in the early twentieth century. US plant pathologists published in *Phytopathology* and the *Botanical Gazette*; UK phytopathologists published in the *Annals of Applied Biology*.

[29] The relationship between John Caldwell and Geoffrey Samuel is unknown, although it may have been somewhat contentious, with hints both in the literature and a letter from Samuel to Holmes in May, 1935, writing: "I shall be very interested to hear Caldwell's account of your lab – provided he tells us anything" (Samuel, 1935). By mid-1936, F. C. Bawden had moved from Cambridge to Rothamsted, replacing Caldwell and continuing his TMV virus-purification work (Samuel, 1936). For more on Bawden, see Creager's *The Life of a Virus* (Creager, 2002).

local lesion assay. These scientists also illustrate a cultural divide between the research scientist and the practitioner plant pathologist, or more generally, the widening divide between basic and applied research. Samuel saw his "job" as building a "hybrid culture," as described by Kohler, bringing the state-of-the art advancement to less than adequate research facilities, thus "creating a border zone of practice" via his ability to "borrow, share, or collaborate" with Holmes (Kohler, 2002, p. 35).

In December 1922 Samuel was appointed plant pathology lecturer at the University of Adelaide; this was funded under the Waite bequest, which became the Waite Agricultural Research Institute in 1924. He took 8 months leave visiting plant pathologists in England for about 6 months, with brief travel to France, the Netherlands, and South Africa. In December 1926 he received his M.S. degree from the University of Adelaide and ca. 1929 was appointed head of plant pathology the Waite Institute, where he remained until 1934 (Garrett, 1972, p. 3; Edgeloe, 1984, p. 21).

In 1916 in Melbourne, Australia, "spotted wilt" – a new disease of tomatoes – was causing a general wilt and collapse of infected plants. By 1925 it had spread to all tomato growing regions in Australia, causing ever increasing economic losses, yet Samuel lacked a contained growth chamber for experiments to determine if there was an insect vector for what was thought to be a virus disease. This problem was serious enough that in 1926 the Council for Scientific and Industrial Research provided £1,600 to build a greenhouse at the Waite Institute. Samuel determined that thrips, a tiny insect, transmitted *Tomato spotted wilt virus*. This practical problem soon became a laboratory interest for Samuel and in June 1930 he took a sabbatical leave at the University of Wisconsin-Madison, funded by an Honorary Fellowship in Plant Pathology, awarded by the Regents of the University of Wisconsin, to work with James Johnson, a respected plant virologist (Fulton, 1984). At Wisconsin, Samuel working with TMV, confirmed the results Holmes had reported using the *N. glutinosa*-necrotic local lesion assay. During the sabbatical, Samuel visited Holmes at the BTI and, subsequently, they had an extensive, friendly correspondence, mostly about TMV, for several years (Samuel, 1931a, c; Holmes, 1931b). In 1934 Samuel took a position at the Rothamsted Experimental Station (Harpenden, England), as a mycologist in plant pathology "in order to get more into the centre of things for a few years" (Samuel, 1933b). He then transferred ca. 1937 to the Plant Pathology Laboratory within the Ministry of Agriculture and Fisheries at Harpenden.

Samuel's stated intent, as elaborated below, was to bring Holmes' method to plant pathologists who needed a straightforward method to

evaluate virus infections from plants collected in the field and lacked the elaborate facilities found at the BTI or RIMR. Samuel showed the usefulness of the local lesion assay for several virus and host plant combinations and explained that "local lesions, as Holmes has called them, are often very characteristic, and of considerable additional value in the total clinical or symptom picture of the disease" (Samuel, 1931c, p. 500).

Samuel continued to experience the frustrations of working in less than optimal greenhouse and laboratory conditions, telling Holmes that "quantitative work is almost hopeless" with ambient temperatures in the greenhouse soaring to 120°F (Samuel, 1930)[30] and later, an epidemic of blue mold that "swept through our green house & took every plant of tobacco & *N. glutinosa*" (Samuel, 1932b). The facilities at BTI and RIMR were to be envied. Holmes' papers reflected data from large numbers of plants per experiment and many replications of each parameter. Samuel was envious of Holmes "chance to work through 4000 plants in less than 3 months. Heavens alive! I feel like abandoning work altogether when I think of our one little unheated greenhouse in which we can scarcely grow 4000 plants in a year" (Samuel, 1933a).

Geoffrey Samuel considered it his job to confirm, "popularize" (especially for virologists in the United Kingdom), and extend Holmes' work, as exemplified by two papers published in 1931 and 1933 (Samuel, 1931c; Samuel and Bald, 1933). For this, Samuel used the "half-leaf assay," multiple hosts, multiple viruses, and most importantly, instead of tabulating the data, he illustrated the results with drawings and photographs. Although Samuel published only seven virus papers, all in the *Annals of Applied Biology* (Samuel, 1931c, 1934; Samuel and Bald 1933; Samuel et al., 1935; Best and Samuel, 1936a, b; Bald and Samuel 1934), he is well-known for his work on TMV and *Tomato spotted wilt virus*. Both Samuel and McKinney lacked a Ph.D. degree, but even with his successes in virology Samuel seems to have felt somewhat marginalized as a scientist.[31] In contrast, McKinney was very well recognized

[30] The *N*-gene protein is temperature sensitive, *i.e.*, it is not biologically functional at temperatures above 30°C (Erickson et al., 1999), which makes it impossible to perform the assay.

[31] Reading between the lines of a letter dated 26 April 1932 from Samuel to Holmes, there is some suggestion that RIMR, through Holmes, may have sent out a feeler about possible employment. Samuel writes: "The hint conveyed in your previous letter, which arrived about a fortnight ago but which I had not yet answered, was very cheering but I realize the difficulties which confront any fulfillment. No doctor's degree, which we don't worry about so much over here, not being an American, & not having much published work on viruses', are obstacles which governing councils find it rather hard to get over" (Samuel, 1932a).

among the US virus workers and made a career at the USDA. Samuel abandoned his virus research when he moved to Rothamsted, returning to general plant pathology and taking on more administrative duties. Virology was moving from the field to the laboratory – as plant pathology and its sister areas of biochemistry and virology experienced the tug of professionalization. For Samuel there was little likelihood of a faculty appointment the US or England without the Ph.D.

Even with delays, by 1931 both scientists were pleased with their results on virus movement and began to discuss the types of experiments that would increase their understanding of the biology of the host-virus interactions. For reasons that are unclear, Samuel deferred to Holmes, even to delay publication of a manuscript in *Annals of Applied Biology* showing that two virus strains could be distinguished by starch localization. In this instance Samuel asked the editor to delay publication until ''August at the earliest, or possibly better to keep till October since I wanted your [Holmes'] paper to be out well in the lead'' (Samuel, 1931a).

It is unclear why Samuel was so differential to Holmes, perhaps the reason is as simple as the status that Holmes had as a BTI (and RIMR-Princeton) scientist compared to Samuel's lack of a Ph.D. and his acknowledged isolation as a researcher in Australia, which may have led to this apparent imbalance. Samuel did feel at a disadvantage, writing in the same letter that he did not ''waste much breath'' in his forthcoming manuscript describing the starch effects ''assuming that everybody would know what I was talking about from having read your paper'' (Samuel, 1931a).

Holmes wanted his and Samuel's manuscripts on the local lesion starch assays to make sense ''when read together'' and felt ''they will both be acceptable contributions to the literature of the mosaics'' (Holmes, 1931c). Samuel received the reprints of Holmes' paper in October, confirming that rearrangement of the manuscript figures ''greatly improved your paper in the way of making things clearer for those not familiar with the ideas'' (Samuel, 1931b). And, a reply from Holmes in early December, indicated that the *Annals of Applied Biology* journal had arrived and ''the photographs reproduced very well, and are striking example of the clearness with which the effects of the primary infections with viruses can be shown.... I want to give you my heartfelt thanks for much needed confirmation of my results on the disturbances in the starch distribution'' (Holmes, 1931a).

Samuel regularly wrote to Holmes of his desire to bring the assay to the general virus worker. His comments, fully elaborated in the spring

of 1932, reveal his ultimate goals as well as frustration with applied plant pathologists:

> It is remarkable how people have not yet assimilated the local lesion idea & all its inferences. I am now finishing off my next virus paper, which is again to try & popularize your ideas.... It seemed to me, when you told me about your attentuation [sic] work, that you had left the quantitative aspect of primary lesion work, & since it is absolutely necessary – for practical use to be made of it – I started in on it as soon as I returned from America, & have been going at it for the whole year, more or less. [The] whole thing is an attempt to show the general virus worker how to use the local lesion method (Samuel, 1932a).[32]

And, again later in the year, Samuel lamented that the method was "being adopted by virus workers with painful slowness" (Samuel, 1932b). Two months later in a five page letter Samuel again tells Holmes that the intent of his work was "more with the idea of popularizing" the quantitative method "with viruses than anything else" (Samuel, 1933a). This was repeated in the *Annals of Applied Biology* in 1933, when Samuel again pushes for the use of the method, making the (false) claim that "nearly four years" had passed and "no papers have appeared" with Holmes' local lesion method (Samuel and Bald, 1933, p. 70).

As I will show below, the method was being used by those in the "labscape." However, Samuel's frustrations may have been with those in "the landscape" – the general virus worker – who had little need for the method or were slow to take on new methods.[33] Samuel remained keen on pursuing the quantitative assay, indicating:

> ... I think I shall now join the group of people who have been "pounding away at you" to do more of this [quantitative] work. It needs the facilities which are available at the Rockefeller Institute & few other places in the world, & it is really a public duty which you owe since you fathered the whole idea. Perhaps there are certain things which you would like to have done under completely different environmental conditions & if so we should be only too glad to do what we could. It might at least bring to light difficulties which the "ordinary virus worker" who must work with a limited number of plants might experience (Samuel, 1933a).

[32] Emphasis in the original handwritten letter of underline and double-underline.

[33] Inspection of correspondence, notes, and experiment station reports generated by applied plant pathologists may be a fruitful vein for investigation of the perceived gap between basic and applied research scientists, and how laboratory science arrived in the field. This is conversation (sometimes heated) that continues today between laboratory scientists and extension plant pathologists.

Holmes and others at the BTI and RIMR had moved away from quantitative assays. Instead, local lesions became a quick means to approximate virus titer on *N. glutinosa*. As a bioassay it allowed for fairly rapid determination of the efficacy of biochemical and physicochemical treatments with a goal of purification of TMV to determine the "nature" of the virus. The first wave of acceptance came from the BTI workers, followed by laboratory scientists. Holmes would "generally send out about 100 reprints" and keep on hand another "50 for later requests" (Holmes, 1934). From these numbers, it is reasonable to assume that his contemporaries in plant virology (and by default, plant pathology) would have access to the BTI papers, by reprint or journal subscription. Although Samuel was keen to have the local lesion assay method accepted by the "general virus worker," the "first wave" would be bench scientists.

The dissemination of Holmes' findings can also be inferred by inspection of several textbooks in the early 1930s (Barton-Wright, 1932). For example, in England, also a center for plant virology research, Kenneth Smith's *Recent Advances in the Study of Plant Viruses* in 1933 (an American imprint followed in 1934) summarized Holmes' method as a "useful technique for the investigator of the local movement of the virus in its host [and] it is also suitable for determining accurately the properties of a virus on a quantitative basis" (Smith, 1933b, p. 226). Smith also suggested the general utility of the method for several plant viruses and plant species by mentioning the work of Samuel, Price, and Caldwell (Smith, 1933b). Of course, there were lines of investigation for which quantitative measures of infectivity were not important or not required. And, Holmes introduced this in 1929 when he essentially abandoned the pin-prick method. For many virus workers, rub-inoculation and observing lesions was sufficient to assay infectivity.

From my study, what is most apparent is that Holmes developed a useful method and, probably as the result of the BTI and RIMR workers using it, the assay was quickly absorbed by plant virus workers in the laboratory environment (Table 2). Yet the rapid acceptance was not due only to the science – it was the means by which the results became public. To facilitate rapid publication, both institutes had a cozy arrangement with several journals to publish papers "out of order of receipt" for an agreed upon fee or donation to the associated scientific society. This ability to have paper published almost immediately after submission to the editor likely facilitated the rapid acceptance of the local lesion assay. Samuel also did his part by making the assay

transparent, or to use current parlance, "user-friendly," including a step-by-step account of how to perform the assay. The application of the local lesion method had utility beyond the quantitative needs of laboratory researchers. The *N. glutinosa* local lesion assay was rapidly taken up by general virus workers to detect TMV (and other virus infections) in crop plants, weeds, and natural settings. In fact, it became so commonplace that authors referred only to "Holmes' method" in their text, but did not provide a citation in the reference section of their manuscripts (Price, 1933; Hoggan, 1934; Jensen, 1933; Pierce, 1934; Grant, 1934; Kunkel, 1934a, 1934b; Wellman, 1934; Price, 1930; Samuel, 1931c; Stanley, 1934; Stanley, 1934b, Stanley, 1934a; Takahashi and Christensen, 1934; Takahashi and Rawlins, 1934; White, 1934). For example, in a paper on serological assays for virus purification, it was noted that the preparations "contained a high concentration of virus, as tested by Holmes's *Nicotiana glutinosa* method" without citing the pertinent *Botanical Gazette* papers (Birkeland, 1934, p. 430).

Both Holmes and Samuel brought the method forward to the laboratory and the field. Smith, in his 1933 textbook *Recent Advances in the Study of Plant Viruses*, also supports that "Holmes' method not only constitutes a useful technique for the investigator of the local movement of the virus in its host but is also suitable for determining accurately the properties of a virus on a quantitative basis" (Smith, 1933b, p. 430). In the same year, in *Biological Reviews* Smith again summarized plant virology with an emphasis on Holmes' results using the local lesion assay to determine virus movement, the use of the starch assay to visualize local lesions, Price's work on common bean for the local lesion assay, and Samuel's demonstrations that the local lesion and starch assays had broad utility for plant virologists. For the bench

Table 2. The use of Holmes' local lesion assay, *Phytopathology*, 1931–1936

Years	Total Papers[a]	Virus[b]	Local Lesion[c]	RIMR[d]
1931	91	7	0	0
1932	96	4	0	0
1933	107	19	1	1
1934	164	31	12	10
1935	105	20	9	10
1936	120	17	7	7

[a] Total number of papers published in *Phytopathology*, including full papers and notes, but excluding abstracts of the annual meeting.

[b] Of the papers published, the number of plant virus papers.

[c] Of the plant virus papers, the number using a local assay to detect plant viruses.

[d] Of the local lesion assay papers published, the number originating from RIMR researchers. In 1933, the source is the Boyce Thompson Institute for Plant Research (BTI), prior to the move of the virologists to RIMR.

scientist, these methods were quickly regarded as commonplace and necessary tools for plant virology.

Prior to Holmes, the majority of papers in the three decades following Beijerinck's work had used host plants and inoculations to identify and study virus diseases. With the local lesion assay, following from several extent observations, Holmes had a method that fulfilled criteria set forth by McKinney for rapid, accurate quantitative virus studies. The local lesion assay became an accepted technique to (indirectly) measure, count, and to make a physical record of a virus infection. This assay came to represent knowledge (epistemic) and, as a heuristic technique, took on deeper and more nuanced meanings as Holmes pursued his work on virus strains, resistance genes, and virus movement.

The Meaning of the Assay

Surprisingly, there is relatively little mention of the broader meaning or utility of the local lesion assay. Holmes' findings (for example Figures 4 and 6 herein) beg the question of parallels with phage biology and bacterial plating. As noted earlier, local lesions did (and do) have the effect of "microbiologizing" TMV. It helped to make the virus "visible," an especially critical need as the physicochemical analyses developed in the early 1930s. Yet, Bawden, an eminent plant virologist, pushed the differences. In his 1929 paper, Holmes indicated necrotic lesions were "as helpful in the study of TMV as Koch's plate method is in the study of bacterial cultures" providing an assay that could be "measured as readily and as rapidly as bacteria are counted by plating methods," that is, within 2–3 days post-inoculation (Holmes, 1929, p. 54, p. 47). Holmes also compared his local lesion technique with bacterial dilution plating (see Figure 6). Later Bawden pointed out significant differences in the meaning of the results (Bawden, 1939, p. 22): plants, as living organisms used for virus inoculation, could not be tested in the exquisitely controlled environment of plating bacteria on nutrient agar in an incubator. A virus is dependent on gaining entry into living cells and, presumably, not every cell on the leaf was amenable to infection. As Holmes had shown by dilution assay, there was not a direct relationship between sap dilution and the number of lesions produced. In contrast, an exact calculation of viable bacterial cells can be made by counting colonies. Therefore, the local lesion and the bacterial colony are not equivalent quantitative methods. Instead, with practice and controlled environmental conditions, Holmes' assay is "a method whereby relative virus concentrations can be estimated with a reasonable degree of accuracy" (Bawden, 1939, p. 27).

For cognoscenti today, phage may have appeared to be a better analogy than bacterial colonies, but there are problems with this assumption. First, the nature of phage was not better understood than that of TMV in 1929.[34] Both were described as filterable, non-culturable, and submicroscopic. For Holmes to describe his findings by analogy, and perhaps to bring his method to the general virus worker, bacteria were the exemplar. In the early twentieth century, the methodology to isolate, culture, and identify bacteria from humans, plants and animals was commonplace for microbiologists. By analogy to bacteria, TMV titers, as explained by Holmes, could be approximated by dilution assay, single lesions could be "picked" from *N. glutinosa* and used to establish a "pure" culture by inoculation of *N. rustica* or other plants supporting a systemic infection.

Much later, in a 1968 review article, Holmes modified his own perspective (Holmes, 1968). He considered the "primary lesion method of determining virus concentrations is comparable to plaque-counting for bacteriophages and to the counting of lesions on chorioallantoic membranes of developing chick embryos for some animal viruses" referencing the work of Samuel and Bald (1933), Kent (1937), and Goodpasture et al. (1932). Yet, Holmes never cited Felix d'Herelle's work on bacteriophage.[35] This was not an oversight – bacteriophage were not going to bring clarity to the explanation both because of the confusion of their nature and because of their narrow host range (or specificity).[36] Here, again, the nature of TMV was seemingly of a different kind. Holmes' virus had a broad host range, infecting dozens of species of plants (tobaccos, tomato, bean, eggplant, pepper, beets, phlox, etc.) and induced host responses ranging from localized necrosis to systemic yellowing (chlorosis) and stunting.

In 1928, Holmes' lack of discussion of bacteriophage was reasonable since the nature of viruses was being described as filterable or particulate – either enzyme or colloid – thus, there was nothing "visible" to refer to, other than bacterial colonies. As a single cell, a bacterium is not detected by the naked eye, yet plating them on nutrient agar allowed for the visible accumulation of colonies of cells; similarly, TMV was not visible as sap extract, but "culturing" it on a host plant that responded with local lesion made the virus detectable.

Although Holmes' method of isolating pure cultures or clones of plant viruses "was accomplished by a plaque technique long before a

[34] For example, F. M. Burnet wrote 1955 that bacteriophage "remained an isolated and bacteriological curiosity without influence on the development of biology until 1930" (Burnet, 1955, p. 3). For more on Burnet's phage work see Sankaran (2006).

[35] In the quotation, Holmes was referring to a bacteriophage associated with *Agrobacterium tumefaciens*, a phytopathogenic bacterium (Holmes, 1968).

[36] Bacteriophage were studied because of their therapeutic potential to combat bacterial infections in humans and livestock (Summers, 1999, 2001).

similar method was applied to animal viruses'' (Hirst, 1962), when Dulbecco (1952) developed a plaque assay for animal viruses, Holmes was not cited. In 1953, George Streisinger, working in Max Delbruck's lab at Cal Tech, tried to develop a plaque assay system with TMV in cell culture; he soon ''got tired of plant viruses not forming plaques'' (Streisinger, 2007). Stressinger matriculated to phage and, later, developed zebrafish as a model species (Endersby, 2007).

Holmes himself did not endeavor to use biochemical or physicochemical methods to decipher the meaning or nature of plant viruses. He came into virology with the need to solve a problem – to detect or visualize the virus as an agent. Following three frustrating years of microscopy, he still had not ''visualized'' the virus, but instead had determined its infectivity. The appearance of local lesions was an ''indicator'' – a biological assay – akin as other assays that measure activity without ever providing visual access the enzyme, toxin, or hormone. With this, Holmes did provide a solution to a fundamental problem that had immediate practical outcomes for both the biochemist and the general virus worker, a stated mission of the Boyce Thompson Institute for Plant Research.

Conclusion

Holmes bridged the scientific era of TMV research by facilitating the transition of TMV from a ''known unknown'' to a ''known known.'' His necrotic local lesion assay redefined TMV as a reagent and material for molecular and genetic study, well outside the domain of the traditional plant pathologist. By 1938, Holmes' local lesion assay had become a standard method for physicochemical study of plant viruses. The assay was used for virus studies meant for determining infectivity following physical (freeze–thaw, heat) and ultraviolet light and X-ray treatments; the effects of chemicals and enzymes on virus purification by (ultra) centrifugation, filtration, and electrophoresis; and serological specificity, first for TMV, but then for other plant viruses (Smith, 1933a, b).[37]

[37] Holmes also gets a brief mention in Greer Williams' *The Virus Hunters*, where Stanley, the hero, had selected TMV because it was ''so highly infectious'' that it was possible to ''dilute the solution a million or a billion times and it still can produce infection.'' But Stanley's success was in part predicated by ''Dr. Francis O. Holmes of the Rockefeller Institute [who] had established not long before Stanley came on the scene, the number of spots of infection are in direct proportion to the virus concentration of the solution, so the investigator can count the specks and estimate the quantity of viruses in the solution'' (Williams, 1959, pp. 96–97).

TMV and *N. glutinosa* became standard plant virology because the local lesion assay was a simple, easily learned method.[38] It was, of course, also chance that drove the use of TMV as a standard, resulting in it becoming a model system for virology (Creager, 2002; Scholthof, 2004). Many other viruses remained in the negative category – not filterable, not infectious by rub-inoculation, no known local lesion hosts, and not observable with the early electron microscopes. When TMV became "visible" via the local lesion assay it moved across many scientific boundaries, beyond the nascent field of virology. It also made possible the development of other tools for the job of understanding the nature of the virus.

Holmes developed a method to monitor and study TMV infections in plants – the local lesion assay. The appearance of lesions turned TMV into a visible entity, revealed as infection foci, suitable for quantitative bioassays. Holmes ushered in the era of the biochemical study of plant viruses (Creager, 2002) – a gift for those researchers looking to understand their nature.

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[38] This included popular use of the assay for classrooms and in "how to" books. For example, Paul Thompson's book *The Virus Realm* (1968) provided a chapter on experiments to do at home, and for this TMV was the experimental subject matter. It is a three part outline of Holmes' methods, from rub inoculation to systemic host, then local lesion assay, half-leaf assay, chemical and temperature effects, and host range (Thompson, 1968).

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