TMV in 1930: Francis O. Holmes and the Local Lesion Assay

His development of a quantitative viral assay enabled him to identify the cause of tobacco mosaic disease and led to many additional insights

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In the early 20th century, viral infections were diagnosed by the process of elimination—if a disease was not caused by fungi or bacteria, the agent was likely a virus. Similarly, viruses were then being described by their shortcomings: too small to view with a light microscope, not culturable, and too small to be captured by filters.

In 1898, Dutch microbiologist Martinus W. Beijerinck attributed the cause of mosaic disease of tobacco to a virus, which he defined as a small, filterable, infectious entity (Fig. 1). Some plant viruses were then being named based on the visible effects on their hosts, such as Tobacco mosaic virus (TMV) and Tomato bushy stunt virus. In other cases, they were merely catalogued based on their host plant, such as Potato virus X and Potato virus Y.

Because of the economic importance of tobacco, TMV became a primary focus for early viral research. Then-current vogues had many scientists describing viruses as physiological problems, enzymes, or toxins. The crucial challenges of determining the nature of viruses and how to quantify them in terms of viral infectious units were not realized until the late 1920s.

Early Viral Research Focused on TMV

The local lesion assay, which Francis O. Holmes developed by about 1928, provided a means for monitoring virus titers during experiments involving TMV. From this assay and experiments that it enabled, Holmes made the imaginative leap to demonstrate that a single dominant gene in tobacco plants is associated with their susceptibility to TMV and, further, that TMV-susceptible tobacco plants could be made resistant to TMV infections through genetics. That assay thus made it possible to “do” plant virology research, much in the same manner that bacterial dilution plating made it possible to count and make pure cultures of bacteria.

What else enabled Holmes to reveal the nature of viruses? From his published articles in the Botanical Gazette, the Contributions of the Boyce Thompson Institute, and his correspondence at the Rockefeller Archives Center, one can construct a timeline of his discoveries and develop a sense of how Holmes conducted biological research. By analyzing what he observed, we can also examine how closely the progress
he described in his published papers depended on how deftly he used host plants to study viruses.

In 1923, L. O. Kunkel of the Boyce Thompson Institute for Plant Research (BTI) in Yonkers, N.Y., hired Holmes to study the etiology of TMV and other virus-like diseases of plants, a move that appeared to recognize among other talents his expertise in protozoology, the subject he studied for his dissertation. Indeed, some investigators then suspected that a protozoan caused tobacco mosaic disease. Holmes completed his D.Sc. in 1925 for work he did at Johns Hopkins University in Baltimore, Md. His dissertation research focused on *Phytomonas elmassiani*, a flagellated protozoan transmitted by the milkweed bug, *Oncopeltus fasciatus*, to milkweed plants, *Asclepias syriaca*.

In 1932, Holmes and others in Kunkel’s virology group moved to the newly founded division of plant pathology of the Rockefeller Institute for Medical Research (RIMR) in Princeton, N.J. Holmes retained this appointment until 1960. When he arrived at BTI, Holmes was asked to determine if a protozoan organism might be detected in tobacco and other plants with mosaic symptoms. Although he spent in excess of two years looking at more than 600 preparations via ultraviolet light microscopy, he was unable to identify such an agent. The only microbes he observed were detected in control samples, in which known plant pathogenic bacteria were purposefully added.

**Novel Analytic Approaches Enabled Holmes To Identify, Analyze TMV**

Holmes, although confident that the agent responsible for tobacco mosaic disease was not a protozoan, was confronted with a fundamental challenge: How could he determine if a viral agent was in the samples? At the time, he wrote: “[H]ow many units of virus, if such exist, were to be found in the small volumes of material?” Since the disease agent was apparently submicroscopic and the tobacco plant samples that he studied were very dilute, was it possible that there was no infectious agent in the sap extracts? These questions led him to design new analytic techniques and, soon, to his discovering the viral nature of the agent that causes mosaic disease in tobacco plants.

Holmes dipped fine insect pins into sap extracts from plants with mosaic disease, preparing that sap in the same way he had for his microscopy samples, and pushed these minute samples into the lower leaves of healthy tobacco plants. He observed that the upper, uninoculated leaves of those same plants developed yellowing and other mosaic symptoms within 7–10 days, providing evidence that the infections were systemic.

These experiments required significant amounts of greenhouse space, as Holmes and other virus workers tested many plants with sap extracts at different dilutions and at various temperatures to determine the nature of the disease-causing agent. As they conducted those experiments, they also followed the then-current consensus that a virus was either an enzyme or some form of physiological aberration.

In his 1928 *Botanical Gazette* paper, “Accuracy in Quantitative Work with Tobacco Mosaic Virus,” Holmes’s approach was empirical. He described how he inoculated hundreds of tobacco plants while looking for symptoms of mosaic disease. The state of the art in the late 1920s was to inspect the infected plants and decide if one “virus source contained the larger proportion of virus in

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**Figure 1**

*Tobacco mosaic virus* infection of a *Nicotiana sylvestris* plant (right) compared to healthy plant (left) as shown by H. A. Allard in 1914. Holmes also tested this tobacco species in his local lesion assays. [H. A. Allard. 1914. Bulletin of the U. S. Department of Agriculture, No. 40, The mosaic disease of tobacco. 33 pages.]
a given volume.” Holmes showed that the minute wounds produced with insect pins alone were not significant and did not affect the experimental outcome. Importantly, he showed that this method allowed for “uniformity of the dose” and, by inoculating a plant with “about five pin pricks,” it was possible to obtain “a fair percentage of infection.”

Holmes soon realized that he could use small, immature plants instead of mature plants to determine infectivity levels in samples of plant sap. In practical terms, 50 young tobacco plants in wooden flats required much less greenhouse space than did a comparable number of mature plants, particularly in experiments requiring many samples, for example, to evaluate dilution assays or the effect of various physicochemical manipulations on plant sap infectivity. To inoculate hundreds of small plants at once, Holmes developed a simple tool—five insect pins sandwiched between wooden pot labels (Fig. 2A and B).

In his now-classic 1929 Botanical Gazette paper, “Local Lesions on Tobacco,” Holmes provided a step-by-step logical explanation describing how local lesions represent an infection. Contrary to previously published observations, he now acknowledged the effects associated with pin inoculation, pointing to “pale yellow areas [that] sometimes develop around one or more of the pin pricks made in each plant,” even with very dilute concentrations of virus on the pins. Holmes wrote: “every plant which showed such local changes around one or more pin pricks developed mottling within a few days” and “most of the plants which did not show pale yellow areas near the inoculation punctures remained healthy.” These findings suggested to him that the “yellow areas might represent localized symptoms of a primary infection, since they were consistently followed by the familiar systemic symptoms.” Yet, he also noted, “the pale yellow areas were very inconspicuous.” These observations led him to search for another species of tobacco with which to correlate the oc-

**FIGURE 2**

Pin inoculation. This experiment was set up to recapitulate Holmes’ local lesion assay. (A) Five fine 00 insect pins were placed between wooden sticks and tied in place with twine. The heads of the pins in both instances were clipped off prior to assembly of the tools. I later modified the tool to use plastic pot labels and duct tape. (B) *Nicotiana tabacum* plants were inoculated with pins using extract of TMV-infected leaves and water (1:1 v/v). Plants were inspected often between 3 and 21 days post-inoculation to monitor for systemic infection. The white box on the left panel outlines the location of the 25 pin inoculations on a leaf. The right panel is an upper non-inoculated leaf of the same plant showing systemic infection following pin inoculation. (C) *Nicotiana glutinosa* leaf displaying local necrotic lesions following pin inoculation. No systemic infection developed, as was also described by Holmes. This assay was crucial in Holmes’ demonstration (and idea) that a single infection point was equivalent to a whole plant assay.
Studying Different Tobacco Species Yields Additional Insights

Holmes experimented with 17 species of tobacco, settling on *Nicotiana glutinosa* since it produced conspicuous, small, dark-brown lesions within 5 days of being inoculated with TMV (Fig. 2C). Those necrotic lesions ensured that the virus did not spread to other plants, reducing the potential for inadvertently contaminating other experiments.

With these changes, he could now inspect hundreds of plants per experiment. By observing symptoms on these small plants, he soon noticed that some portion of the leaves developed chlorotic or necrotic lesions at the point of the pin prick. “[A] few references in the literature indicated that local lesions have been observed, although their real nature has not been understood,” he reported in 1929. Holmes referred, in part, to observations by Carl A. Priode, who joined the BTI in 1927. He found that Tobacco ringspot virus (TRSV) formed ringspots first on inoculated leaves, then on upper noninoculated tobacco leaves. Priode thus provided solid evidence that early symptoms were predictive of subsequent systemic infection by TRSV.

Holmes came to believe that chlorotic or necrotic lesions were the point of primary infection. Despite the important role they played earlier, Holmes abandoned insect pins, when he found that rubbing plant leaves with TMV-inoculated cheesecloth or fingers dipped in TMV-containing plant sap worked as well. Subsequently, he began to use such lesions as a source of pure virus, used *N. glutinosa* to compare TMV titers from different plant sources, and also transferred a single dominant gene from *N. glutinosa* (the *N*-gene) to previously susceptible tobacco species, providing resistance to TMV infection.

Holmes used *N. glutinosa* and *N. rustica* to recapitulate his earlier efforts to develop a method for accurately and quantitatively measuring TMV (Fig. 3). This time, however, because local necrotic lesions could be scored “before systemic symptoms appear, economy of time is secured,” he explained. Moreover, testing one *N. glutinosa* plant led to the same accuracy on average as would testing “at least several hundred *N. tabacum* plants,” he noted with enthusiasm. His discovery made it possible to recognize very large numbers of successful viral transmissions on single plants by simple monitoring of the accumulation of necrotic local lesions.

In the three decades following Beijerinck’s assertion that a virus causes tobacco mosaic disease, many researchers used techniques similar to those developed by Holmes to inoculate other plant species as a way of studying viral diseases. Yet, the TMV and *N. glutinosa* local lesion assay became standard for researchers investigating host-virus interactions.

Although the commercial importance of tobacco drove the early studies of TMV, it soon also became the “right tool” for delving into fundamental questions of virus biology. Unlike TMV, many of those other viruses were not easy to work with for a variety of reasons, including that they were not filterable, could not infect plants via inoculation, or failed to induce local lesions. TMV, however, proved useful in many other labs. Further, the Holmes assay led to its use analytically to standardize virology assays, while it also was used widely to develop other tools for studying and understanding viruses. For example, Wendell Stanley, who was in the same virology group as Holmes at the Rockefeller Institute for Medical Research in Princeton, used the local lesion assay to learn more about the chemical nature of viruses.

Holmes had compared his local lesion technique with bacterial dilution plating. “In theory
Taking TMV from Archived Descriptions back to the Lab Bench

To gain a more personal sense of the 1929 Holmes experiments, I recently repeated parts of them, and began by grinding TMV-infected leaves in water and then pressing this mixture through cheesecloth. Next, I dipped the tines of 00-insect pins into my TMV-sap extract and pushed the tines through the surface of plant leaves from several kinds of tobacco plants, inoculating TMV onto \textit{N. tabacum} and \textit{N. glutinosa}, much as Holmes described in his papers (Fig. 2A). The point of inoculation is where minor damage appears relatively soon on the inoculated \textit{N. tabacum} leaves (Fig. 2B). Several days later, however, some of the upper, noninoculated leaves became mottled and puckered, thus demonstrating that the virus had moved from the point of inoculation to establish a systemic infection in the plants.

Using the pin-prick method, I applied TMV sap onto \textit{N. glutinosa} (Fig. 2C) leaves, but only a few points of inoculation resulted in infection, revealed by necrotic lesions, and none of the plants developed systemic lesions on other leaves. Then I recapitulated Holmes’ rub-inoculation experiments on \textit{N. glutinosa} with diluted TMV sap-samples (Fig. 3). As was reported by Holmes, the dilutions can be used to determine relative titers of virus from infected plants, although the dilution effect is not linear. The necrotic local lesions, are a result of a hypersensitive response triggered by the interaction of TMV with the plant \textit{N}–gene product, which Holmes also described.

From these experiments, which recapitulate parts of those described by Holmes in his 1928 and 1929 publications, it becomes evident that the pin-inoculation method affirmatively answered one of his early questions: Was there enough virus in the sap extract, which he had used for microscopy, to initiate a TMV infection on inoculated plants? Holmes had correctly reasoned that if a local lesion could be correlated with a systemic infection, then each local lesion represented one infection.

the two methods are similar, but in practice they have important differences,” pointed out his contemporary, F. C. Bawden in \textit{Plant Viruses and Virus Diseases} (1939). Bacterial plating involves inoculations on a uniform, solid medium. Plants are not nearly so uniform, and they are subject to a wider variety of environmental and host factors. Moreover, the necrotic lesion is the outcome of a host-pathogen interaction, whereas bacterial colonies appearing on solid agar merely indicates the viability of the culture in the inoculant. Further, plating provides a true count of bacteria, whereas a “virus is able to produce lesions only at suitable entry points and not equally over the whole surface of rubbed leaves,” Bawden noted. Therefore the local lesion assay revealed “relative infectivities” of TMV or other plant viruses, not their precise concentrations.

Nonetheless, Holmes showed that viruses can be observed and their relative titers determined. Because he made viruses “visible” on plant leaf surfaces, he effectively brought TMV into the realm of other infectious disease agents.

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SUGGESTED READING

\begin{itemize}
\item \textbf{Holmes, F. O.} 1929. Local lesions in tobacco mosaic. Botanical Gazette 87:39–70.
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