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Hundred years of Koch's Postulates and the history of etiology in plant virus research[1]

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Abstract

The centenary of Koch's Postulates in plant pathology is a moment to look back at the history of etiology in the study of plant virus diseases and at the development of our knowledge of plant viruses.

Koch's Postulates were long considered inapplicable to viruses. These very postulates, however, together with the application of Chamberland filters allowed the discovery of viruses and their distinction from micro-organisms. But for some time, the true nature of these 'new' pathogens remained obscure and their identification was hard, of not impossible, since Koch's Postulates in their literal sense did not apply to viruses.

After 1935, techniques of virus isolation were elaborated, at first very gradually but with the advent of moleular biology since World War II booming. They made viruses accessible to experimental manipulation and research, and permitted the study of causal relationships between virus and disease by application of Koch's Postulates, though adapted and now redefined.

Such etiological studies and the gradual introduction of methods and philosophies, implicitly derived from Koch's Postulates, have allowed the description and classification of a rapidly increasing number of true viruses as disease incitants. They have also allowed the detection of a continuously increasing number of other pathogens previously mistaken for viruses, but differing in essence. This is helping us to purify the virus concept and to define it better.

Introduction

Plant virology originated as an agricultural discipline towards the end of the last century. Plant viruses were first detected because of their harmful effects on crops. Most plant virus research is still in agricultural research institutions. Such institutions first came into being around the middle of last century in Germany as a consequence of von Liebig's theory on the role of mineral elements in plant nutrition first published in 1840 (Von Liebig, 1840). Von Liebig continuously stressed the importance of experimentation. Adolf Mayer (Fig. 1), who later became known for his pioneering work on tobacco mosaic, had a thorough background in agricultural research in Germany and had even published a German textbook on agricultural chemistry a few years before coming to the Netherlands in 1876 (Mayer, 1870).

[1] Elaboration of an opening address at the virus session of an FBPP/SGM conference on 'Plant disease etiology, 100 years of Koch's Postulates' held in London, 17-19 December 1980.

Since the dawn of history, all sorts of ailments, often developing into disastrous epidemics, had already attracted attention and appealed to superstition. In the nineteenth century, micro-organisms inreasingly found in or on diseased plant tissues were considered a consequence rather than a cause of disease. But about the middle of the century, awareness of their involvement as disease incitants was gradually increasing. Such 'venomous' or 'slimy' causative agents were often called 'viruses', but this name is as vague as the term 'bug' for any insect or other creeping or crawling invertebrate or even for a disease-producing germ or other micro-organism. Study of their role in disease etiology was one of the early tasks of expanding agricultural research.

That was the scene when Koch and his 'Postulates' entered. Up to now, very little attention has been paid to them in plant virology. Hardly any textbook on plant viruses has ever referred to them. Are they really inapplicable to true plant viruses? Why have they been neglected, and to what effect? To answer these questions better, we must look to the philosophy behind the Postulates and we will have to do so in historical perspective.

Koch and the origin of microbiology

Research on fermentation by Pasteur (1860) had shown that under present conditions life never originates spontaneously, that it needs germs, however small, to develop from. But what about infectious diseases? Where do they come from and how do they get about? These were questions increasingly haunting the young country doctor Robert Koch in the small Prussian town of Wollstein during the early 1870s.

It is tempting to narrate Koch's life history as interestingly recorded by Paul de Kruif in 'Microbe hunters' (1926). Infectious diseases were haphazardly levying their toll without Koch being able to control them. Anthrax was such a strange disease, worrying farmers all over Europe. 'Here and there, it ruined the prosperous owner of a thousand sheep or killed the only cow of a widow and unpredictably spread to further victims including farmer, shepherd or a hide dealer'.

During snatched moments between calls to sick people, Koch studied with the microscope blood samples from animals that had died of anthrax and found peculiar threads and rods. They were always absent from blood from healthy animals. White mice inoculated with carefully cleaned and oven-heated slivers of wood were then found to serve as inexpensive means for growing the microbes. For one month, the mice were serially inoculated from one to another. Under the microscope the microbes could afterwards be directly observed multiplying in the watery liquid from the eye of an ox in Koch's ingeniously developed hanging-drop preparations. After propagating microbes under such conditions for eight generations pure from any other microbe and away from the animal, he back-inoculated to white mice. So he finally succeeded in artificially reproducing the disease in these animals with characteristic symptoms and swarms of the same microbe. In 1876, Koch had been able without modern aids such as syringes, test tubes and Petri dishes to prove that one kind of microbe caused one definite kind of disease.

However, this epoch-making achievement did not yet revolutionize scientific thinking until Koch's appointment at the Imperial Health Office in Berlin in 1880

Fig. 1. Adolf Mayer (1843-1942); photograph taken when he was teacher in agricultural chemistry from 1876 to 1904 at Agricultural College and director of Agricultural Experiment Station, both at Wageningen.
(From album presented to Mayer at his retirement in 1904).

Fig. 1 Adolf Mayer (1843-1942); foto genomen toen hij van 1876 tot 1904 leraar landbouwscheikunde was aan de Rijkslandbouwschool en tevens directeur van het Rijkslandbouwproefstation, beide te Wageningen.
(Uit album aan Mayer aangeboden bij zijn afscheid in 1904).

provided him with the facilities to further improve the techniques of 'microbe hunting'. The story of the half of a boiled potato left on a table of Koch's laboratory, found covered with differently coloured colonies of bacteria is well known. It led to the development of beef broth culture media solidified with gelatin. They allowed single germ colononies to develop separately from one another, led to pure cultures and really got microbiology off the ground. After Koch's Nobel prize winning discovery of *Mycobacterium tuberculosis* soon afterwards in 1882, the game of 'microbe hunting' was really on.

Koch's Postulates

Before long, many researchers joined the study of infectious diseases by following Koch's example and his approach. These rules soon became known as 'Koch's rules' (Erwin F. Smith, 1905) and later as 'Koch's Postulates'. Table 1 lists them, as summarized by the Terminology Sub-Committee of the Federation of British Plant Pathologists (1973). It is not clear when they were formulated and by whom. As

Table 1. Koch's Postulates for proving the pathogenicity of an organism.

1. The suspected causal organism must be constantly associated with the disease.
2. It must be isolated and grown in pure culture.
3. When inoculated into the healthy host, it must reproduce the disease.
4. The same organism must be re-isolated from the experimentally infected host.

Tabel 1 De Postulaten van Koch voor het bewijzen van het ziekmakende vermogen van een organisme.

early as 1840, before the specific relation of micro-organisms to disease was accepted, Henle stated the conditions that should be met before an agent could be considered the proved cause of an infectious malady (Rivers, 1935). The rules were first convincingly put into practice by Koch to prove the pathogenicity of *Bacillus anthracis* in 1876. However, the second postulate could only be widely applied after introduction of solid media in 1880 or soon after. In fact, the first three postulates were discussed by Koch in 1883 in a paper on the transmissibility of anthrax (Koch, 1883). The last criterion, though already practised by Koch himself, was added by Erwin Smith (1905) when redefining and interpreting Koch's rules in his textbook on 'Bacteria in relation to plant disease'.

The Postulates emphasize isolation of the causal factors of disease from their natural victim and their separation from accidental contaminants, allow their reliable identification, and artificial reproduction of the natural disease under controlled conditions. So the Postulates have permitted the transfer of the natural problem of disease to the experimental environment, as earlier vigorously advocated by Von Liebig to allow exclusion of all factors but one and to allow experimental manipulation of this single factor. It is *the* basis of the modern analytical approach to nature. Postulate 2 required culture of the pathogen in vitro and unfortunately has this literal application prevented a more liberal approach to Koch's principles.

Detection of plant viruses

The 'germ theory' soon boomed and was greatly stimulated by Koch's isolation of the incitants of tuberculosis (1882) and of cholera (1883). These discoveries were rapidly followed by the detection of several other pathogenic bacteria. Among these were also bacteria pathogenic to plants such as 'Wakker's hyacinth germ', *Xanthomonas hyacinthi,* the cause of yellow disease of hyacinth (Wakker, 1883-1885), and of *Erwinia amylovora* the incitant of fire blight of apple and pear (Burrill, 1886) and so on. The germ theory and Koch's Postulates also boosted the study of fungal diseases. However it soon became clear that not all contagious plant diseases met Koch's requirements.

First there was the mosaic disease of tobacco which Adolf Mayer started to study in 1879, a few years after coming to Wageningen. He soon proved its infectious nature despite the absence of a visible cause and in 1882 he speculated on the existence of a 'soluble possibly enzyme-like contagium, although almost any analogy for such a supposition is failing in science' (Mayer, 1882). In his classical paper published in 1886, he unfortunately gave up this idea and finally concluded that the mosaic disease 'is bacterial, but that the infectious forms have not yet been isolated, nor are their forms and mode of life known' (Mayer, 1886).

Meanwhile porcelain filters had been developed that hold up bacteria and could be used to produce 'physiologically pure water' (Chamberland, 1884). Dmitrij Ivanovskij (1892) in Russia was the first to demonstrate that infectivity of sap from mosaic-diseased tobacco plants was retained after passage through Chamberland filter 'candles', thus further improving Mayer's results. Did this mark *the* beginning of virology and was Ivanovskij really the 'first discoverer of viruses' as suggested on the postage stamp issued to commemorate the centenary of his birth (Fig. 2)? Like Mayer, Ivanovskij remained hypnotized by Koch's way of thinking. He kept

Fig. 2. Postage stamp issued in Russia in 1964 on the occasion of Dmitrij Ivanovskij's 100th birthday.

Fig. 2. Postzegel in 1964 in Rusland uitgegeven ter herdenking van Dmitrii Iwanowskii's 100ste verjaardag.

insisting that he was dealing with a microbe that might have passed the filter pores or have produced a filtrable toxin.

At that time, Martinus Willem Beijerinck (Fig. 3) had already left Wageningen, where he taught botany, had got to know Mayer, and had become familiar with the peculiar mosaic disease of tobacco. In 1885, he became microbiologist of the Netherlands Yeasts and Alcohol Factory at Delft. There he kept an interest in the mosaic disease, or in the spot disease as he called it. As early as 1887, he unsuccess-

Fig. 3. Martinus Willem Beijerinck (1851-1931), teacher of botany at the Agricultural College at Wageningen from 1876 to 1885 and later professor of microbiology at the Polytechnical School, later Technical University of Delft (1895-1921). (From album presented to professor Mayer at his retirement in 1904).

Fig. 3. Martinus Willem Beijerinck (1851-1931), van 1876 tot 1885 leraar plantkunde aan de Rijkslandbouwschool te Wageningen en van 1895 tot 1921 hoogleraar mirobiologie aan de Polytechnische School, later Technische Hogeschool, te Delft.
(Uit album aangeboden aan professor Mayer bij diens afscheid in 1904).

fully tried to isolate a micro-organism. When he became Professor of Microbiology at the Technical University of Delft in 1895, one of his first scientific endeavours was to look more seriously for the incitant of the intriguing tobacco disease. Ignorant of Ivanovskij's work, he did identical filter experiments and found that filtrates after three months of storage were still free from bacteria but were infectious. He was then the first to realize that he was dealing with something special, different from a corpuscular agent or a 'contagium fixum'. It was something in liquid or soluble state. 'Without being able to grow independently, it is drawn into the growth of the dividing cells and here increased to a great degree without losing in any way its own individuality in the process'. He called it a *'contagium vivum fluidum'* or just *'virus'* (Beijerinck, 1898).

A few years later, Ivanovskij criticized Beijerinck's conclusions and reemphasized the mirobial nature of the pathogen of tobacco mosaic. He then stressed it to be a *contagium fixum* and claimed that it could even multiply in agar. A special section of his dissertation published in Warsaw in 1903 deals with 'die Kultur des Mikroben der Mosaikkrankheit' (Ivanovskij, 1903). It demonstrates the outcome when theory fossilizes into dogma.

Bauer (1904) also used the word dogma a year later in his classical paper 'on the etiology of infectious variegation', when refuting the possibility of the causal involvement of a living organisms. He also referred to the mosaic disease of tobacco and, at the end of his famous paper, he wrote: 'For a further insight into the etiology of these diseases, the old dogma of the unconditionally parasitic nature of all infectious diseases seems to me to be only an obstruction'.

The other example of diseases that did not meet Koch's rules is peach yellows and some related diseases characterized by yellowing and growth abnormalities consisting of stunting and witches' broom growth, and virescence and phyllody of flower organs. Peach yellows had been intensively studied by Erwin F. Smith (1888) for a possible micro-organism by analogy with the bacterium *Erwinia amylovora,* some years earlier detected by Burrill as the incitant of fire blight of apple and pear (Burrill, 1880 and 1881). No such incitant could be revealed for peach yellows, although Smith could transmit the disease from plant to plant by budding and grafting.

The famous Dutch botanist and geneticist Hugo de Vries (1896) must have been dealing with a related pathogen in 1893 and 1894 when studying an epidemic of virescences in some 27 species in his garden in Amsterdam (Bos, 1966). This disease in turn must undoubtedly have been related to aster yellows later studied in much detail in the United States by Kunkel (1924 and 1926) and others. De Vries could not discern a cause either macroscopically or microscopically and therefore hesitated to publish the results of his observations. However, he finally did so, being 'convinced of the infectious nature of the disease and of its spread by flying insects, in the hope that others may later be more succesful in finding the parasite' (De Vries, 1896).

Koch's Postulates and the filter experiments had thus helped to distinguish mosaic diseases, since Ivanovskij and Beijerinck often ascribed to 'filtrable viruses', and yellows diseases from those caused by micro-organisms. But how clear was the distinction and why did it still take 37 years since Beijerinck's paper before the veil covering the real nature of true viruses was first hesitantly lifted by Stanley (1935)? And why did it take 72 years from De Vries's publication, before the 'yellows-type' diseases could be associated with filtrable micro-organisms, such as mycoplasmas

(Doi et al., 1976)? This is because Koch's Postulates could not be applied to such disease incitants themselves to permit their direct handling for experiment. At least, the requirement of culture in vitro was impossible. That is why they could not be studied for their intrinsic properties, and why their true nature as well as their proper identity remained obscure. Meanwhile, plant virology although increasingly recognized as a new discipline within the domain of plant pathology was left in its dark age with much room for speculation and without proper communication.

Characteristic of that period is a short citation from Heald's Manual of Plant Diseases (1926): 'Virus diseases: This is a group of somewhat related diseases in which the disturbed condition is the result of an infectious principle, a so-called 'virus' which can be transmitted from diseased to healthy plants and communicate the disease. These troubles agree with the parasitic diseases due to bacteria, slime molds and fungi in being infectious, but no visible organisms or causal agents are known. The infective principle, whatever it may be, is present in the juice or cell sap of a diseased plant, the different diseases showing various degrees of infectiousness' . . . 'The virus diseases behave so much like germ diseases that there has been a common theory that they are due to invisible micro-organisms much smaller than the smallest bacteria'.

Nevertheless, Koch's Postulates kept haunting virology, as is obvious from the persistent dark-age definition of a plant virus. Virus was considered to be a submicroscopic (invisible) agent of infectious disease (it was 'beyond the microscope': K.M. Smith, 1943) that could not be cultured on artificial media. It caused systemic infection in its plant hosts and could at least be transmitted artificially by grafting. In practice, a virus was something that Koch's Postulates did not apply to. Consequently, virology remained saddled with all contagious diseases of unknown and invisible cause, the virus concept remained the waste-paper basket of a great variety of obscure pathogens, and the plant virologist was often left with the unpleasant task of diagnosis where mycologists and bacteriologists failed to pinpoint an incitant.

Plant virology as a science

From 1905 to 1920 and more rapidly during the 1920s, it became increasingly clear that there were a considerable number of different entities behind virus or virus-like diseases. Illustrative is the title of a paper by Van der Meulen (1928) from Quanjer's laboratory at Wageningen on 'the specialization and infection sources of mosaic diseases of agricultural crops'. The different entities could and still can be studied, and to some extent described and distinguished for a series of biological characteristics, thanks to their infectivity (Table 2: Biological Properties 1 to 4).

Soon after 1920, an urgent need was felt for facts about the 'viruses' themselves. That is how so-called 'physico-chemical properties' (dilution end-point, thermal inactivation point, ageing in vitro, and resistance to certain chemicals) were introduced to assist in differentiation and classification of viruses (Johnson, 1927; further elaborated by Johnson and Grant, 1932). In fact, the term physico-chemical properties was misleading, since what they meant is the persistence of virus infectivity in vitro (Biological Property 5).

There have been endeavours to standardize techniques for the study of biological properties (as for legume viruses by Bos et al., 1960). But these properties remain

Table 2. Biological properties used for virus characterization.

1.	host range
2.	symptoms (sometimes in special test or indicator plants)
3.	(cross-protection)
4.	manner of transmission
	– with sap and through contact
	– in seed and pollen
	– in soil (by nematodes or fungi)
	– by insects or mites
	– through grafting (and by dodder)
5.	persistence of infectivity in expressed sap after
	– storage in vitro
	– dilution
	– heating for 10 minutes

Tabel 2. Biologische eigenschappen gebruikt voor viruskarakterisering.

very variable, final data greatly depending on virus source (and virus concentration), on manner of transmission and on test plant, and all are influenced by external conditions (Bos, 1976). This also holds for the persistence of infectivity in plant sap (Bos, 1976; recently further documented by Francki, 1980).

With a rapidly increasing number of known virus diseases since about 1930, information on the viruses themselves and on their intrinsic properties became ever more pressing. The epoch-making isolation of tobacco mosaic in pure form as paracrystalline material by Stanley (1935) and its characterization as a nucleoprotein by Bawden & Pirie (1937), but also its first visual perception by Kausche et al. (1939) with their 'Übermikroskop' opened up a new era.

Molecular biology rapidly developed since World War II, as did the number of techniques derived from this new field of study for isolating viruses from multicomponent host sap and for separating them from one another while preserving their integrity, including their infectivity and disease-generating capacity. Viruses could then be manipulated outside their natural hosts and studied in the experimental environment in the spirit of Liebig and Koch. So Stanley's and Bawden and Pirie's work really marked the beginning of virology as a science.

The viruses thus isolated and studied were open to detailed description on their intrinsic properties (Table 3). Electron microscopy opened up an entirely new field of direct observation of virus particles themselves. The advent of techniques of tissue embedding and ultrathin sectioning and staining for study with the electron microscope, and direct staining of crude sap preparations have greatly facilitated the rapid detection of particles, already known from purified preparations, in crude plant sap and in host tissues. These methods thus allowed Koch's first postulate to be directly put into practice. No wonder electron microscopy has boomed!

Last but not least, the particles studied in detail by physico-chemistry, electron microscopy and serology, could be reintroduced into the original natural hosts to be examined for their pathogenicity. At long last, Koch's 3rd and 4th postulates were applicable.

Tabel 3. Intrinsic properties used for virus characterization.

1. physico-chemical properties
   - components (multiparticulate viruses)
   - particle size (see also 2, below)
   - sedimentation coefficient
   - molecular weight
   - diffusion coefficient
   - isoelectric point
   - partial specific volume
   - electrophoretic mobility
   - buoyant density
   - protein coat: contents, structure and amino-acid composition; additional proteins in infected host cells
   - nucleic acid: content, type, components, nucleotide composition
2. electron microscopic properties
   - particle morphology
   - particle sizes
   - site in the cell and accumulation
3. serological specificity

Tabel 3. Intrinsieke eigenschappen gebruikt voor viruskarakterisering.

An important question is whether the particles reintroduced into test plants are indeed identical with those studied in vitro. Inoculations are always with suspensions, of which only few particles anonymously initiate infection. Absolute homogeneity of a purified suspension is hard to demonstrate although characterization of the virions may facilitate recognition of contaminants and has in the past often done so. Multipartite viruses and satellites pose special problems. But even there, separation of components has achieved a high degree of perfection. Virologists must still constantly watch out for contaminations by unknown viruses or viroids.

Another point is that the study of physico-chemical properties does not (yet) provide a complete solution to virus identification. Viruses are genetically polycistronic and not more than two genes or cistrons determine the configuration of amino acids in the protein coat, and thus size, shape and serological affinities of the particles. The intrinsic properties cover only part of the total genetic information. Viruses are more than mere macromolecules. Hence biological properties such as symptoms, host ranges and ways of transmission remain necessary in identifying viruses and in studying their ecological and pathogenic relationships with plants, including crops.

The viruses that are not normally sap-transmissible because they are phloem-limited in plants are worth mention. They have long resisted efforts to study their intrinsic properties, and the true nature of several of these incitants long remained obscure. Even if particles reminiscent of viruses were observed, Koch's 3rd postulate was inapplicable until two methods were developed to reintroduce such particles or preparations into the phloem of assay plants with the help of their natural vectors (Fig. 4). In a number of instances, research workers could make the vectors viruliferous before test feeding either by feeding them on the virus-containing suspension mostly through membranes, as first developed for leafhoppers by Carter (1927;

Fig. 4. Re-introduction into test plants of non sap-transmissible phloem-limited viruses after study in vitro (C) with help of vectors artificially made viruliferous: (A) by injection into haemocoele, (B) by feeding via membrane. Test plant (D) reacts if virus was present in suspension tested. (E) Healthy control plant.

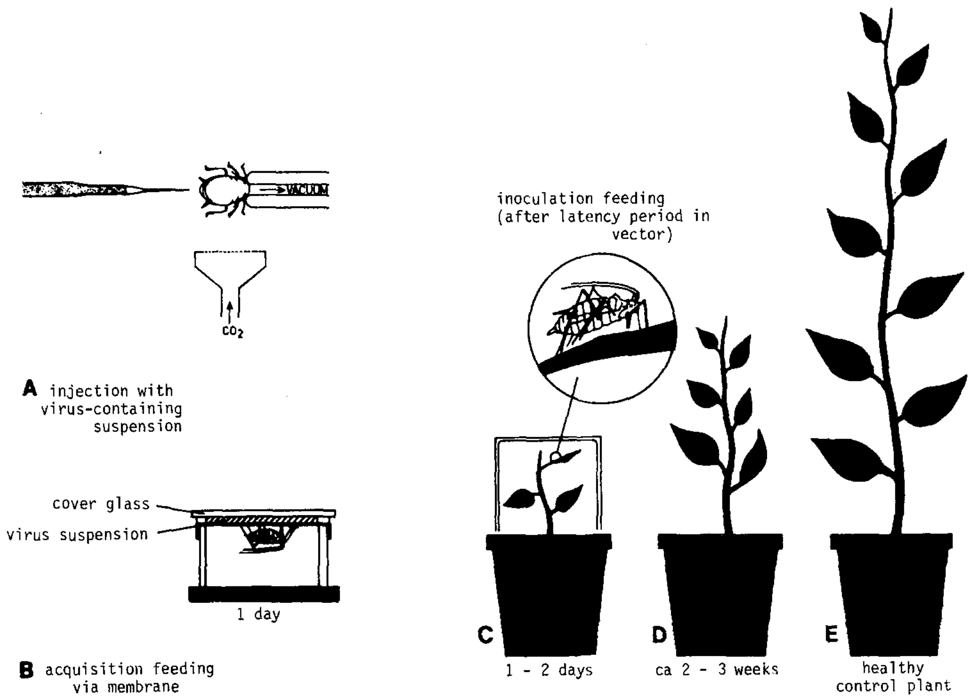

Fig. 4. Herinbreng in toetsplanten van niet-met-sap-over-te-brengen aan-het-floëem-gebonden virussen na bestudering in vitro (C) met behulp van vectoren die kunstmatig virus-dragend gemaakt zijn (A) door injectie in lichaamsholte, of (B) door voeding via membraan. De toetsplant (D) reageert indien in getoetste suspensie virus aanwezig was. (E) Gezonde controleplant.

1928) and later applied to aphids by Rochow (1960), or by injecting them with the suspension, as first for leafhoppers by Storey (1932) and Black and Brakke (1952) and later for aphids by Stegwee and Ponsen (1958). In fact, the technique of membrane feeding was first developed by Storey (1932) for demonstrating the filterability of maize streak virus which is not mechanically transmissible from plant to plant. The techniques of membrane feeding and injection have more recently greatly assisted in the isolation and characterization of several viruses which are insect transmitted in the persistent manner such as aphid-borne luteoviruses.

There is another group of virus-like pathogens that have long escaped detection because of extremely small size and lack of protein. Advanced physico-chemical techniques have allowed their isolation from infected plants and characterization in vitro. The first of this group was the incitant of potato spindle tuber by Diener (1971). Such 'miniviruses' only consist of ribonucleic acid of 75 000-100 000 daltons and are now considered the smallest known agents of infectious disease. They are subviral in size, hence called viroids (Diener, 1974 and 1979), and are of utmost interest as tools for the study of the moleculair biology of disease.

Redefinition of Koch's Postulates for virology

Since viruses in the original sense of Koch's Postulates cannot be grown on artificial nutrient media but can now be isolated and studied in vitro, time is overdue to redefine the original postulates to virological needs.

Table 4 lists the redefined postulates for proving virus pathogenicity. They also serve to prove the virus nature of a pathogen and hardly need further explanation. Postulate 2 replaces the requirement of pure microbial culture, although selective isolation of a virus through differential hosts, and its multiplication on differential hosts in part resembles the isolation and pure culture of microbes. However absolute purity will remain hard to guarantee with viruses. The virus nature of a pathogen is not well established without physico-chemical characterization.

With the increasing opportunity to apply the postulates to virus diseases, there is no need to rediscuss Rivers' (1937) endeavour for viruses in general to get away from all but the first postulate of Koch. He compared viruses with obligate parasites, which could not be grown in pure culture. That is why Koch (1891) himself stated that 'if the regular and exclusive occurrence of the parasite is demonstrated, the causal relationship between parasite and disease is validly established' (translation by Rivers, 1937). The pinpointing of an agent as a virus absolutely requires the above postulate 2, however.

Table 4. Redefined Postulates for proving the pathogenicity of a virus and the virus nature a pathogen.

1. The virus must be concomitant with the disease.
2. It must be isolated from the diseased plant:
   - separated from contaminating pathogens,
   - multiplied in a propagation host,
   - isolated from plant sap and purified physico-chemically, and
   - identified for its intrinsic properties.
3. When inoculated into a healthy host plant, it must reproduce the disease.
4. The same virus must be demonstrated to occur in and must be re-isolated from the experimental host.

Tabel 4. Opnieuw gedefinieerde Postulaten voor het bewijzen van het ziekmakende vermogen van een virus en de virusaard van een ziekteverwekker.

Koch's Postulates and virus identification

The redefined Postulates are the ultimate backbone of reliable diagnosis of plant virus diseases, as summarized by Bos (1976) in 'Ten commandments', to make diagnosis really etiological. Because of the sophisticated techniques still required, most diagnostic work continues to belong to the domain of the scientific laboratory. Many viruses have not yet been characterized or have been inadequately described.

Hence, diagnosis often ends with the description of a new virus: diagnosis and characterization are two inseparable aspects of identification (Bos, 1976).

Routine diagnosis is often based on limited application of the commandments, while neglecting Koch's requirement to reproduce the disease artificially with the isolated pathogen. Too many plant virologists are still unaware of Koch's Postulates and even good horses have stumbled in too readily attributing the disease to a virus isolated from diseased plants. One reason is the high incidence of latent infections in plants and the chance that a latent component if easily detected is mistaken for the cause of disease in multiple infection. In plants, multiple infections often occur and each component may contribute to the final syndrome, either independently or in synergistic or antagonistic interaction. The same holds for multiple infections by viruses and other pathogens.

Erroneous conclusions have also resulted from contamination by a virus entering the 'culture' with the seed of test plants used. Seed-borne viruses may be easily overlooked because they are often symptomless in seedlings. There are of course several other risks of contamination , such as in soil for tobacco necrosis virus. Research requires strict hygiene.

A pathogen should not be called a virus merely on the basis of symptoms in natural host or test plants. In my book on symptoms (Bos, 1978), I discussed a number of disorders resembling virus diseases, that can easily be misinterpreted as due to virus. Notorious are some local and systemic insect toxaemias characterized by chlorosis, vein-banding, veinal chlorosis, chlorotic streaks and stripes, and even wilting and malformation, including enations.

Finally, reference should be made to several pathogens denoted as viruses because they are covered by the 'dark-age' definition of a plant virus, but which have not yet been isolated nor characterized by physico-chemical means as 'true' viruses. They are nothing but virus-like in their relationships to plant and often to vector. Application of modern techniques of virus research is increasingly revealing the true nature of such pathogens as viruses or as something else.

Detection of 'virus-like' micro-organisms

The significance of the redefined Postulates of Koch has been best demonstrated by the discovery of mycoplasma-like organisms as incitants of witches' broom or yellows-type diseases, already studied by Erwin Smith (1888) and De Vries (1896). The incitants beautifully fitted the dark-age definition of virus, which was based on the inapplicability of Koch's Postulates to virus diseases. There was no doubt at all about their virus nature after the classical proof of infectivity of aster yellows by grafting and by leafhopper transmission (Kunkel, 1924 and 1926) and after demonstration of multiplication in the vector (Black, 1941). In fact, in their relationships to plant hosts (systemic infection, limitation to phloem, production of phloem degeneration and resulting secondary symptoms like stunting and yellowing), and in their relationships to the vectors (leafhoppers, and sometimes psyllids) they still behave like viruses, notably luteoviruses.

However, later endeavours to isolate a virus in the sense of Koch's redefined Postulates (Table 4) continually failed, and it turned out to be impossible to detect the incitant visually and to characterize it physico-chemically. In 1967, a breakthrough resulted from the application of techniques of ultrathin sectioning of diseased tissues and their study with the electron microscope, as initiated in Japan by Doi et al. (1967). Since then the presence of wall-less pleiomorphic mycoplasma-like organisms (MLO) and disease have been found to be concurrent phenomena in the host, and both concomitantly reacted to tetracycline antibiotics. Their presence within the vector was also found to be associated with transmission of the disease. Hence, little doubt was left about their causative involvement in the diseases concerned, although the mycoplasmas have still not been convincingly cultured on artificial media and reinoculated into susceptible plant hosts. For a detailed review see Grunewaldt-Stöcker and Nienhaus (1977).

Soon after the discovery of mycoplasmas, spiral motile forms were detected in association with corn (maize) stunt disease (Davis et al., 1972) and the genus name *Spiroplasma* was introduced (Davis and Worley, 1973). Koch's Postulates in their original sense were fulfilled for *Spiroplasma citri* to prove its causality of citrus strubborn disease. The broth-cultured agent was injected into the leafhopper *Euscelis plebejus* which introduced it into sweet orange seedlings, which later developed typical symptoms. The presence of the agent was then confirmed by electron microscopy and reisolation of the organism (Markham et al., 1974).

The detection of the widespread occurrence of Rickettsia-like organisms (RLO) or Rickettsia-like bacteria (RLB) in homopterous insects (Maillet, 1971) and in dodder (Giannotti et al., 1970) made Davis and Whitcomb (1971) suggest that these small and fastidious bacteria, which have a rigid cell wall and, as a rule, cannot be cultured in cell-free media, may gain considerable interest in plant pathology. Quite a number have been already associated with major diseases transmitted by leafhoppers or psyllids and long ascribed to viruses, as was Pierce's disease of grapevine (xylem-limited) and clover club leaf (phloem-limited). For a survey of the literature and further examples see Hopkins (1977). Pierce (1892) had already observed bacteria in diseased grapevines but could not culture them. After the late 1930s, the disease was ascribed to virus because of graft transmission. The 'Rickettsia' now associated with Pierce's disease was recently grown on a special medium and vacuum-inoculated into grapevine cuttings, which reproduced the disease after being rooted. The micro-organism was later consistently reisolated and is now considered a bacterium rather than a Rickettsia (Davis et al., 1978). Likewise, the xylem-limited ratoon stunt of sugar-cane, formerly associated with 'Rickettsia' is now attributed to a small coryneform bacterium (Teakle et al., 1973).

Since 1931, various reports have appeared on the concomitant association of flagellates *(Phytomonas* spp.*)* with phloem disorders of coffee (phloem necrosis, zeefvatenziekte, Stahel, 1954; Vermeulen, 1968), of coconut and oil palm in Surinam (heartrot or fatal wilt; Parthasarathy et al., 1976; Parthasarathy and Van Slobbe, 1978; Van Slobbe et al., 1978), and of oil palm in Peru where the flagellates apparently passed through sieve-plate pores and promote progressive infection (Dollet et al., 1977). However, causality of these organisms for these diseases awaits proof.

The detection of these virus-like incitants of infectious diseases and their differentiation had opened new chapters in plant pathology.

Some of the micro-organisms concerned are so small that they pass filters holding up bacteria, as proved for mycoplasmas of man and animals (Andrews, 1972). So the criterion of filtrability has lost its value to distinguish between viruses and micro-organisms. This has further stressed the necessity of Koch's Postulates, in whatever form, for the study of any type of infectious disease.

Evolution of the virus concept

Fig. 5 diagrammatically recapitulates the history of etiology in the study of plant virus diseases from the time that an infectious plant disease was first tackled scientifically by De Bary (1853). This history cannot be dealt with without discussing the nature of viruses and it simultaneously represents the evolution of the virus concept.

In old times viruses were considered poisonous, venomous, and mysterious agents associated with disease and the term also covered bacteria and other micro-organisms. The word 'virus' in its general meaning still persists in the adjective 'virulent' for poisonous, venomous or malignent pathogens.

Koch's Postulates have first enabled the isolation of micro-organisms on artificial media, and helped the study of diseases caused by bacteria and fungi to really get off the ground. Soon thereafter Ivanovskij and Beyerinck's filter experiments proved the existence of 'filtrable viruses' separable from micro-organisms by their extremely small size. Since they were 'beyond the microscope' and could not be cultured according to Koch's rules they remained shrouded in mystery and speculation. Meanwhile an increasing number of virus diseases was described pointing to the existence of many different viruses.

The techniques of virus isolation and purification from sap of diseased plants, gradually developing since Stanley's and Bawden and Pirie's pioneering work but

Fig. 5. Diagram representing the history of etiology in the study of plant virus diseases.

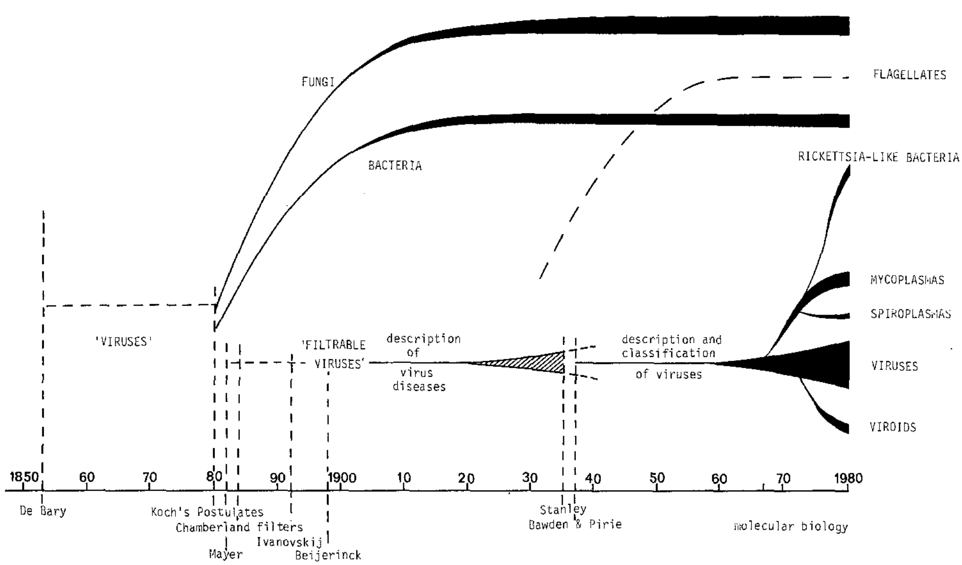

Fig. 5. Diagram, dat de geschiedenis weergeeft van de etiologie bij de bestudering van planteviruszíekten.

now booming thanks to molecular biology, have allowed the isolation of viruses in pure form and their study outside the original hosts in the spirit of Koch. Numerous viruses have already been characterized physico-chemically and there is an emerging order of viruses studied for their intrinsic properties.

These studies have permitted redefinition of Koch's Postulates to virological needs. Methods based on these postulates, although redefined just now, have already during the last 13 years revealed the existence of an increasing number of minute pathogens that resemble viruses in their relationships to plant hosts and vectors but are larger and more complex in structure so that they escape mere physico-chemical characterization. In the end, they are micro-organisms, but most of them resist culture outside living host cells. Also 'miniviruses' or viroids, almost too small to contain one gene, have been described as a separate group but they still fit the prevailing definition of virus.

The remaining 'true' viruses can now be described in terms of physico-chemistry. They have no metabolism of their own and multiply or are being multiplied in living host cells only, at least they require host ribosomes and building blocks. They are submicroscopic infectious agents either consisting of genomic RNA or DNA with or without protective protein and in a few instances with an extra envelope and some additional components. In fact, they are small packages of genetic material, alien to their hosts.

Thus, a better understanding of the real nature of true viruses and a better definition of the virus concept have resulted. A pathogen should not be called a virus unless seen with the electron microscope and characterized physico-chemically. Its pathogenic involvement may be inferred from concomitant occurrence with disease, but proof is not complete unless it is isolated in pure form and thereafter tested for pathogenicity. Since there still are many diseases tentatively ascribed to viruses, nature may have several more surprises in store.

Concluding remarks

The vicissitudes of Koch's Postulates in plant virology represent the history of etiology in this discipline. The special nature of viruses made progress laborious but now spectacular with the manipulation of viruses themselves as demanded by the spirit of Koch's Postulates. Their redeployment has allowed a better comprehension of the true nature and identity of plant viruses.

In essence, we are dealing with man's fascination with the reality surrounding him, with the immense complexity of nature. The history of plant virology is a lesson: The theories and the ensuing procedures needed to study multifactorial reality should never fossilize into 'graven images' or rigid 'postulates', blocking further advance. They will have to be continuously and critically adapted to the ever increasing range of information.

Finally, it is worth recalling the embryonic beginning of plant virology and Mayer's inference as to the existence of a 'soluble possibly enzyme-like contagium', as illustrated by Raemakers' cartoon from the turn of last century (Fig. 6). The drawing indicates appreciation by Mayer's colleagues that he was dealing with something really special 'at the threshold of life' (as expressed in the title of a book by Fraenkel-Conrat, 1962). In the background appears the magician of Goethe's Faust,

Fig. 6. Cartoon of Professor Adolf Mayer by Louis Raemakers, at the time of Mayer teacher of handdrawing at the Agricultural College, later wellknown for his political cartoons. (From Historical Collection, Agricultural University, Wageningen).

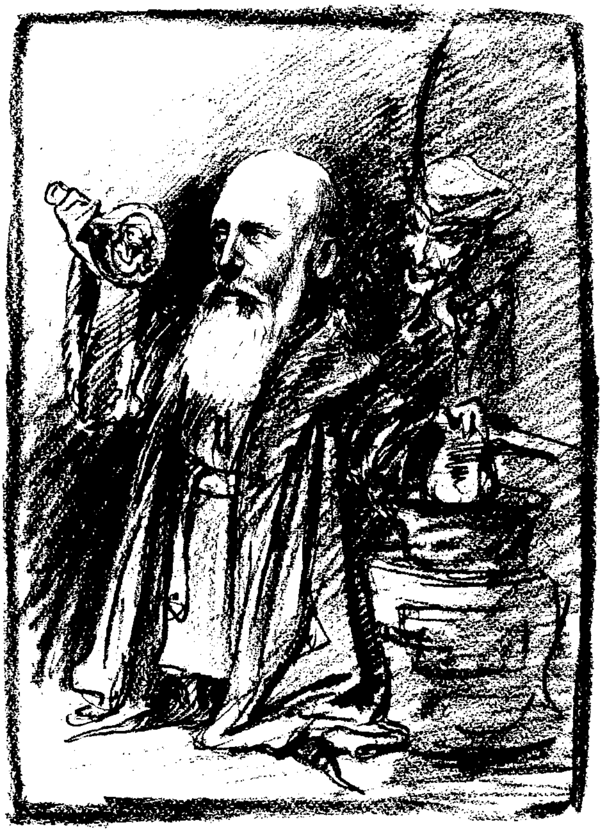

Fig. 6. Karikatuur van professor Adolf Mayer door Louis Raemakers, ten tijde van Mayer leraar handtekenen aan de Rijks Land-, Tuin- en Bosbouwschool in Wageningen, later bekend om zijn spotprenten.
(Uit: Historische Verzameling, Landbouwhogeschool, Wageningen).

lost to the devil due to his arrogant curiosity, but in spite of numerous mistakes finally to be saved through his incessant urge for perfection. May this urge never abandon the virologists since much work is still lying ahead.

Samenvatting

Honderd jaar Postulaten van Koch en de geschiedenis van de etiologie bij het plantevirusonderzoek

De herdenking van de honderdjarige toepassing van de Postulaten van Koch in de planteziektenkunde geeft de gelegenheid om terug te blikken op de geschiedenis van

de etiologie van plantevirusziekten en op de ontwikkeling van onze kennis van plantevirussen.

De Postulaten van Koch werden lange tijd niet toepasselijk geacht op virussen en virusziekten. Toch blijken juist deze postulaten tezamen met het in gebruik komen van Chamberland bacteriefilters de ontdekking van virussen als ziekteverwekkers van geheel andere aard dan micro-organismen te hebben mogelijk gemaakt. Wel bleef de werkelijke aard van deze 'nieuwe' pathogenen lange tijd onduidelijk en de identificeerbaarheid uiterst moeilijk tot onmogelijk door het niet op hen toepasbaar zijn van de Postulaten van Koch in de letterlijke betekenis ervan.

Sinds 1935 aanvankelijk slechts zeer langzaam tot ontwikkeling gekomen technieken van virusisolatie, vooral die ontleend aan de moleculaire biologie, hebben het mogelijk gemaakt virussen toch toegankelijk te maken voor bestudering buiten de plant en voor onderzoek van de causale relatie tussen virus en virusziekte door toepassing van de Postulaten van Koch, zij het in aangepaste en nu opnieuw gedefinieerde zin.

Tijdens dit onderzoek en door de geleidelijk weer meer en meer aan de Postulaten van Koch ontleende werk- en denkwijzen is het reeds mogelijk gebleken om niet alleen een snel toenemend aantal echte virussen als ziekteverwekkers te beschrijven en te onderscheiden, maar tevens een aantal wezenlijk andere ziekteverwekkers op het spoor te komen, die voorheen voor virussen werden aangezien. Gedoeld wordt hier op de ontdekking in planten van mycoplasma's in 1967, van spiroplasma's in 1971, van rickettsia-achtige bacteriën in 1970 en mogelijk ook van flagellaten al in 1931 en hun verband met planteziekten. Deze in de vaatbundels voorkomende micro-organismen gedragen zich in hun relatie tot plant en overbrenger in sterke mate als virussen. Door hun ontdekking is het mogelijk geworden om het virusbegrip geleidelijk aan te 'zuiveren' en het allengs beter te definiëren, vooral in termen ontleend aan de moleculaire biologie. Vraagpunt hierbij is of de sinds 1971 bekend geworden 'minivirussen' nog tot de virussen mogen worden gerekend of dat het daarbij gaat om speciale viroïden.

De geschiedenis van de etiologie bij de bestudering van virussen en virusziekten heeft tenslotte geleerd dat theorieën en de erop gebaseerde werkwijzen ter bestudering van de multifactoriële werkelijkheid nooit mogen verstarren tot 'gesneden beelden' en versteende 'postulaten' die verdere vooruitgang blokkeren. Ze dienen voortdurend kritisch te worden aangepast aan de gestaag toenemende kennis.

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