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Historical reminiscences of phytoplasma discovery

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Abstract

Many plant diseases, earlier described as virus diseases, have been recognized as phytoplasma or spiroplasma diseases during the past 44 years. The breakthrough discovery came in 1967, when Japanese plant pathologists and entomologists reported detection of mycoplasma-resembling microorganisms in diseased plants and insect vectors, and the temporary recovery of diseased plants, treated with tetracycline antibiotics. For many years no credit was given to the crucial role played by the veterinarian Kaoru Koshimizu, who first recognized phytoplasmas in electron micrographs of thin sections from mulberry dwarf diseased plants, prepared by Y. Doi. In 1967, at the same plant pathology conference in Japan, entomologists S. Nasu and associates reported the detection of phytoplasmas in rice yellow dwarf disease and in the insect vector, but this report was hardly mentioned. Attempts to culture the fastidious phytoplasmas did not succeed, while spiroplasmas, first recognized by R. Davis, have been cultured. Several careers have been made by phytoplasma researchers, but some were destroyed by erroneous reports and one ended tragically through political involvement. The striking progress in the study of phytoplasmas, demonstrated by the First, and the current, Second IPWG meeting, clearly illustrates the benefits derived from collaboration between experts working in different countries, from free exchanges of information, and from participating in symposia and congresses.

Key words: phytoplasma , tetracycline antibiotics, yellows-type diseases.

Introduction

The first phytoplasma disease has been described 1000 years ago in China (Wang and Maramorosch 1998). During the Song dynasty, 960-1227, the "Yao-yellow kind" peonies, hailed as the most beautiful tree peonies, with a delicate green color, were annually presented to the imperial court. The pale-green flowers were widely acclaimed in China for centuries, although the peony tress that produced them were less vigorous and the green flowers produced no seed. It took more than 800 years before the cause of tree peony greening and the beneficial effect of phytoplasmas could be documented. Is it proper to call these phytoplasma infected peony trees "diseased"? The phytoplasmas made the peony trees more desirable and the phytoplasma infection , in this instance, became beneficial to tree peonies. Currently, our interest in phytoplasmas is primarily directed to the serious diseases, caused by them all over the world. Phytoplasmas have destroyed pears and apples in Europe and in the United States, coconut and other palms in tropical and subtropical areas, food crops, lumber, shade trees and ornamental flowers all over the world. Since 2004, when the first phytoplasma genomic sequence was published, the genomic sequencing of phytoplasmas has progressed, promising the creation of novel measures to stop the devastating infections of crop and fiber plants.

In my historical recollection I shall focus not only on the published findings but also on the scientists involved in the early phytoplasma and spiroplasma research. Before 1967, many plant pathologists and entomologists, working with yellows-type diseased plants, tried to detect particles resembling known viruses of plants, animals, or bacteria. I shall describe my own failure to find the pathogens of aster yellows disease, the failed attempts to culture phytoplasmas, the background of the 1967 recognition of phytoplasmas in Japan, and the errors that occurred in my own and in other laboratories.

Early criteria, applied to viruses, were inadequate to distinguish between viruses and other filterable disease agents. When electron microscopy of thin sections was introduced, no virus-like particles could be detected in thin sections of many diseased plants or in purified plant or insect vector extracts. Errors made before and after 1967 by me and others demonstrated how failure to collaborate with colleagues, working in different fields, resulted in missed opportunities. I shall stress the concept that progress in phytoplasma research can best be achieved by collaboration with colleagues in other laboratories and in other fields.

Missed opportunities

In 1924 the mystery transmission of the aster yellows disease was solved by L. O. Kunkel at the Boyce Thompson Institute in Yonkers, New York, when he found that a leafhopper, *Macrosteles fascifrons*, transmitted the infectious agent from plant to plant (Kunkel, 1926). Since no bacteria or fungi were found in diseased plants, Kunkel concluded that the causative pathogen was a virus. He suspected that this virus multiplied in leafhopper vectors and this assumption was confirmed by my serial passage technique, using needle inoculation of leafhopper vectors (Maramorosch, 1952).

In the summer of 1957 I was working at the Cold Spring Harbor Laboratory on Long Island, New York, where Barbara McClintock, who 30 years later received the Nobel Prize for her discovery of "jumping genes", permitted me to use her greenhouses for maintaining leafhopper vectors of aster yellows. I prepared extracts from diseased plants and from leafhopper vectors, adding measured amounts of penicillin, streptomycin, and tetracycline, and injecting small amounts into the bodies of leafhoppers. I was convinced that the antibiotics

would have no effect and that the injected leafhoppers would continue to infect aster seedlings. As expected, this happened with the insects that were injected with penicillin and streptomycin. However, the tetracycline injected leafhoppers failed to infect plants. I was convinced that the failure to transmit was meaningless, because it was well known that viruses were not affected by tetracycline antibiotics. Instead of repeating the experiment during the fall, I published the results, concluding that the lack of transmission was, most likely, caused by the heat in the green houses (Maramorosch 1958). Had I repeated the tests, perhaps the correct conclusion would have been reached and I would have made the discovery of phytoplasmas 10 years before my Japanese colleagues announced their findings. I missed the opportunity because I was convinced that I was working with a plant virus.

In 1966, together with my associate Hiroyuki Hirumi, I visited in Philadelphia Werner Henle, the discoverer of the mononucleosis virus. Henle's electron microscopist Hummeler examined our electron micrographs and remarked: I see that your cultures are contaminated with mycoplasmas. I had never before heard the word mycoplasma, but, instead of inquiring what the word meant, I said that the pictures were not of cell cultures, but of thin sections of leafhopper salivary glands, made by Hirumi (1969). I was not familiar with the work of Chanock *et al*. (1962), who successfully cultured the infectious agent of "atypical virus pneumonia", named by Hayflick *Mycoplasma pneumoniae.* Apparently, Hummeler recognized phytoplasmas in our electron micrographs of leafhopper vector salivary glands. It was another missed opportunity.

The 1967 breakthrough

In November 1967 the Japanese Plant Pathology Society was holding their annual meeting in Sapporo. My former associate, Eishiro Shikata, at Hokkaido University, was the secretary of the society, receiving abstracts of papers a few weeks earlier. Shikata wrote to me, requesting 6 negatives of electron micrographs made by him in 1964. Several hundred glass negatives were stored in my laboratory and since Shikata mentioned that the requested plates contained the aster yellows pathogen, I checked the numbered negatives, but found no virus particles on the pictures and did not mail the requested plates. Several weeks later I found out, that Shikata wanted to take part in the discussion of Doi's paper and show phytoplasmas in his own electron micrographs, that he could not identify earlier. I wondered how Doi was able to recognize the "MLOs"? At the same meeting the entomologist S. Nasu from Tsukuba submitted an abstract, reporting MLOs in thin sections of rice yellow dwarf diseased plants, and in the leafhopper vector *Nephotettix apicalis*.(Nasu *et al*., 1967*)*. Why was this important contribution ignored in subsequent papers and review articles, not only in Europe, but, surprisingly, also in Japan? It took several years before I solved this puzzle. Japanese plant pathologists omitted Nasu *et al*. because they knew that Nasu submitted his abstract only after reading the tentative draft of the program. He rushed to his laboratory, prepared thin sections of diseased rice plants and leafhopper vectors, and submitted the results in time to be presented orally, and printed, in the same issue of the journal as the two reports from Asuyama's plant pathology department , by Doi *et al*. (1967) and Ishiie *et al*. (1967). Asuyama knew that Nasu found out about MLOs only after being tipped off by the abstracts of the Tokyo plant pathologists. Asuyama felt that Nasu's findings were not an original idea and the work was not worth mentioning. In the meantime I was told that the MLOs were not the original idea of Doi either and that there was someone, who tipped Doi off. Intrigued, I wrote to Asuyama, but he did not reply. After my third letter, Asuyama replied, that Doi was familiar with all earlier mycoplasma publications and that he was the sole discoverer. Was this really so?

In 1974, in Tokyo, I finally met the mysterious person who was responsible for recognizing mycoplasma resembling structures in electron micrographs made by Doi. It was Kaoru Koshimizu, a veterinarian from the Poutry Department of Tokyo University. In 1967 he saw the electron micrographs made by Doi, and asked whether Doi was working with mycoplasmas. Doi, who never heard about mycoplasmas, immediately noticed the similarity of the structures in Koshimizu's and his own electron micrographs. He replied that he was searching for virus particles in sections of mulberry dwarf diseased plants. Koshimizu then asked whether Doi tried to cure diseased plants with tetracyclines. Doi replied that tetracyclines have no effect on viruses. "Not on viruses, but they are used to cure turkeys suffering from mycoplasma infection" stated Koshimizu. Doi repeated the conversation to Asuyama, who then requested Ishii to obtain tetracycline samples from the poultry department. He requested Ishiie to apply tetracycline to leaves and the soil around potted mulberry seedlings,infected with mulberry dwarf "virus". When the treated mulberry seedlings began to recover, other diseased plants were similarly tested and the two seminal papers were submitted to the forthcoming annual meeting in Sapporo.

I felt that the crucial role of Koshimizu should have been acknowledged by Asuyama and his associates. This did not diminish their achievement, but it demonstrated the advantage of communicating with scientists from other departments, other universities and institutes, as well as other countries.

In January 1968, in the program of lectures, to be presented at the New York Academy of Sciences, appeared the title and short abstract of my lecture on "MLOs" in aster yellows and corn stunt diseased plants. The abstracts were distributed to more than 20,000 Academy members. Among them was C. Vago in St. Cristol les Ales, France. My abstract did not mention the three 1967 Japanese papers, but my paper, published a few weeks later, presented the complete story, including details of the Japanese breakthrough (1968). In May 1968 in the Compte Rendu appeared the first French paper on the detection of "MLOs", by Giannotti *et al*. (1968), submitted by C. Vago. Not only was there no mention

about the Japanese findings or work in the United States, but the work in France was presented as one of the greatest discoveries of the XX Century, comparable to Pasteur's work in the XIX century.

I was well acquainted with Vago. Years earlier, he was very helpful in my attempts to culture invertebrate cells, we communicated frequently, and jointly organized the First Invertebrate Cell Culture Conference in Montpellier in 1962. Therefore I send to Vago the published full text of my lecture, with the references of the Japanese papers. I asked him why the work, carried out by others, had not been mentioned. Vago replied that there were strikes at French universities and the library in Montpellier did not receive current literature. Despite this explanation, a whole series of papers by Giannotti *et al*. was published during the following months, constantly omitting the work published in 1967, and the subsequent extensive work, carried out by several Japanese experiment stations. The claim, that the finding of phytoplasmas was a French discovery, was repeated several times and its great importance for world's science repeated. Then came Giannotti's claim, that he succeeded in culturing phytoplasmas. Attempts to confirm this in other laboratories failed. Giannotti was invited to Bové's laboratory to demonstrate how he cultured phytoplasmas in cell-free media. He brought his material to Bordeaux, and presented his technique. When he was ready to leave for the airport, he wanted to take back his media and everything else that he brought from St. Christol. Robert Davis was spending his sabbatical in Bové's laboratory at that time. He and Bové wanted to repeat the experiments with Giannotti's original material. On the morning of his departure Giannotti was told that his material was locked in a greenhouse by a gardener, who became ill and could not come that morning. Giannotti had to depart, leaving his media and plants in Bordeaux. Bové and Davis, unable to confirm the phytoplasma cultivation, notified Giannotti. Nevertheless, he did not recant his results. He came to a meeting in Florida several months later and stated again that he successfully cultured phytoplasmas.

Failed cultivation attempts in my laboratory

Attempts to culture phytoplasmas were also made in my laboratory. One of my postdoctoral associates, a Fulbright scholar from Yugoslavia, Biljana Plavsic, used horse serum in her media and after a few days observed what appeared like colony growth. Fortunately, before rushing to submit the results to a scientific journal, I mailed the photographs of the presumptive colonies to Ruth G. Wittler, a mycoplasma expert at Walter Reed Army Institute in Washington, D.C. She immediately replied, identifying the growth as "pseudo colonies", that were known to appear when high concentrations of horse or rabbit serum were used in culture media. The reply saved me the embarrassment of publishing the presumptive successful cultivation of phytoplasmas. We published a short abstract about the pseudo colonies (1971).

Although cultivation of phytoplasmas has not yet been achieved, I hope that collaborations between phytoplasmologists and other microbiologists will eventually result in the cultivation of the fastidious microorganisms.

Incompatibility of phytoplasma research with politics

In 1972 my postdoctoral associate, Biljana Plavsic, made her most important discovery, recognizing phytoplasmas in inflorescences of coconut palms affected by lethal yellowing disease (1972). Earlier reports listed the palm disease as a virus disease. The devastation caused by it on several Caribbean islands and in southern Florida was of great concern. The findings by Plavsic *et al*. were soon confirmed in Great Britain and in Germany. Plavsic published 7 additional papers during her 18 months in my laboratory and she continued her phytoplasma research after returning to her university in Sarajevo, former Yugoslavia.

When Yugoslavia fell apart into 7 republics, she decided to become a politician. At first, she was very successful, becoming the only woman elected president of the newly created Republic of Bosnia. She was hailed as the ablest politician in former Yugoslavia, solving many problems and achieving great popularity. Unfortunately, when war broke out between Croats, Serbs, and Bosnian Muslims, Plavsic became the supporter of the campaign of persecution, and in 1992 tens of thousands of Bosnians were killed and ethnic killing was being carried out under her presidency. When Plavsic became vice-president under Radovan Karadzic, she inspired the Serbs to take up arms against their Croat and Muslim neighbors, proclaiming the cultural and racial superiority of Serbs over Muslims.

In 2002 Plavsic travelled voluntarily to The Hague, to face the United Nations International War Tribunal. She was promptly arrested and presented with the evidence of the horrendous war crimes. She pleaded guilty and was sentenced to 11 years in jail. Two years ago she was released. Biljana Plavsic lives now in retirement in Beograd. Very few people know that the discoverer of the cause of lethal yellowing is the same person who became first famous, and later infamous, as president of Bosnia. Had she remained a virologist and phytoplasmologist, instead of turning to politics, she would have been a very prominent scientist today.

Conclusions

Phytoplasma research has greatly progressed during the passed 44 years. Nearly 1,000 plant diseases and many insect vectors have been identified and collaboration between researchers from different countries and different disciplines accounted for the rapid progress achieved in recent years. Science recognizes no political, religious, ethnic, or geographic borders, and we, as scientists, speak the same language of science, collaborating with each other, irrespective of background and

political believes. Currently molecular biologists, plant pathologists entomologists and microbiologists from different countries collaborate, increasing and expanding our knowledge of phytoplasma pathogens, vectors, and phytoplasma diseases worldwide. Sequencing of phytoplasma genomes is yielding new knowledge, leading to novel approaches to the control of phytoplasma diseases and control of insect vectors. The historical events of the passed century provided the basis for the current molecular biology study of phytoplasmas.

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