A COMPILATION OF THE TEN YEARS WORK CONDUCTED IN THE LABORATORY OF MYCOPLASMAS AT I.N.R.A. DIJON TO SOLVE THE RIDDLE OF GRAPEVINE FLAVESCENCE DORÉE AND OTHER YELLOW'S DISEASES OF PLANTS (1969-1981)

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INTRODUCTION

The current abundance of scientific bibliography has led to omit the original references if a former compilation does exist. That commodity soon opened way to various slips when such a compilation did not exist.

Thus, the various handicaps occurring for the pathologist in the study of grapevine-Flavescence dorée (FD) or of other yellow's phytoplasma diseases became completely forgotten, even in very important handbooks and synthesis which in turn are used as "valuable" compilations.

The problem was that the pathogen agent of that kind of diseases was unable to grow in a broth, according to bacteriology and was too heterogen to be purified as a virus. It was then impossible at that time either to characterize or to diagnose these pathogens.

A compilation of the ten years research conducted in our laboratory in order to find a valid solution to those diseases then seems essential. Such is the purpose of this paper.

To start with, a short survey of the previous state of the research. Then the successive stages of the studies:

− Obtention of a working cycle *Vicia faba* and vector *Euscelidius variegatus* for FD.

− Development of an infectivity test and its use to improve the extract concentration in infectious units.

− The world's first serological result and first visualization of a phytoplasma pathogen in a liquid medium.

As an epilogue, the further researchs developments conducted in our laboratory in the same cycle of research : development of immunoenzymatic methods (ELISA, DOT BLOT) ; introduction and use of the immunocytologic methods ; purification of the phytoplasma on affinity column; obtaining monoclonal antibodies ; DNA cloning and elaboration of molecular biology tests.

NB : That compilation is a recasting, slightly shorter, of a historic review wich appeared in french in the "Progrès agricole et viticole" N° 5, 2008, pp. 67-79 under the title: La Flavescence dorée de la Vigne et les jaunisses à phytoplasmes : ni bactérioses, ni viroses. Comment a-t-on pu les aborder ?

A SURVEY OF THE PREVIOUS STATE OF THE RESEARCH

The history of the yellow's diseases of plant goes back to the research of KUNKEL on *Aster yellow's* in United States (1927-1930), then to the *Stolbur*, in Eastern Europe from 1945 onwards. Those diseases were characterized by their symptoms, their transmission by grafting and by leafhoppers. But the pathogen, assumed to be a virus, was never found either in extracts, or in ultra thin sections by electronmicroscopy examination.

The studies of those diseases then remained confined to epidemiology, methods of transmission by grafting, by leafhopper or eventually by dodder for possible transfer to Periwinkle *Catharantus roseus* which reacted by relatively specific symptoms.

In France, the yellow's diseases make a spectacular entry in the fifties with the outbreak of the grapevine Flavescence dorée (FD) in South West France. The characteristics of that disease, its symptoms, its transmission by grafting [2] and by a leafhopper rapidly discovered [31] enabled us to connect it to the yellow's group. The epidemy appeared disquieting, the number of infected stock plants being often multiplied by ten in one year [2, 5] .

However a phenomenon of spontaneous recovery, definitive if the plant is not reinoculated [4, 5] allowed to fight the disease only by breaking up the vector cycle : insecticide sprays during the hatching period [33], winter ovicid treatments [17, 18].

In 1966, we found that a hot water treatment of the cuttings, 72 hours at 30° Centigrade reduced the proportions of diseased plants by 80 % [6]. The idea was adaptated by GOHEEN in 1973 to Pierces disease in California. More precise researches on FD showed that the treatment in hot water at 50°C for 35 to 60 minutes resulted in a definitive cure for 100 % of the cuttings without any mortality [26]. Those results were largely used in France and throughout the world for imported wood and nurseries.

Finally the epidemiology and the specificity of the leafhopper *Scaphoideus littoralis/titanus* for FD made it possible to distinguish FD from *Bois noir* which spread in Burgundy [3] and from the PHY disease. Therefore the plurality of the grapevine yellow's was already shown at that time [16].

The discovery in 1967 by Japanese virologists of wallless prokaryotes called *Mycoplasma like organisms* (MLO) in ultra thin sections of the phloem of the plants affected by yellow's diseases [27], rapidly changed the orientation of the research all over the world: MLO were discovered for every disease of that type.

For FD they were not found in diseased stock plants but in young plants artificially inoculated by *S. titanus* that were to show symptoms two weeks later [15]. On the other hand, MLO were regularly found in FD infected broadbeans (see below).

The discovery of the MLO soon raised the hope of cultivating those procaryotes in the artificial media containing serum used for *in vitro* culture of the animal mycoplasma. But all those attempts remained everywhere unsuccessful. Those cultures only resulted in cultivation of the ubiquist saprophytic *Acheloplasma*. The MLO were not cultivable.

We then come to the long trudge of our laboratory to try again the path of virology : the attempts at extracting, purifying and concentrating the pathogen, with the additional handicap of dealing with pathogen agents extremely heterogeneous.

THE TEN YEARS WORK FOR DEVELOPING LABORATORY DIAGNOSIS METHODS FOR FLAVESCENCE DORÉE AND YELLOW'S DISEASES

As a result of the former researchs the specific difficulties of the MLO, now called phytoplasma, lie on the facts that they are not cultivable *in vitro* like bacteria or animal mycoplasmas and that they are not purifiable like viruses. Indeed, generally speaking, the viruses consist of identical particles whereas phytoplasms are extremely heterogeneous in size, shape and density. Thus the obtention of purified and concentrated phytoplasms was made impossible by the fact that the methods of bacteriology (cultivation) and of virology (purification) were both impracticable. We have then to discard the idea that the good antibodies have simply been "obtained". They were in fact the result of ten years of laborious work. We are now going to follow its successive stages.

1) Transmission of Flavescence dorée to herbaceous plants and choice of broadbean (Vicia faba) as a test plant

FD was difficult to study in its host, the grapevine. In that woody plant the incubation *over the winter* is long and irregular [4, 5]. We also have to remember the difficulties occurring for extracting viruses from grapevine tissues which contain large amounts of tannins and acidity. Virologists had to use extracting solutions containing nicotine, a poison for the phytoplasms. It then appeared necessary to find a more suitable host plant.

We first tried to transmit FD from grapevine to other species by dodder but without success. The possibility remained to use the leafhopper *S. titanus*. But that vector was specific to the grapevine [32]. We were then obliged to test its survival on other botanic species.

It soon appeared that *S. titanus* was only able to survive on a very small number of botanic species. It became necessary to test its survival on numerous species throughout the year [12, 13].

But *S. Titanus* has only one generation in a year ! Its eggs are laid at the end of summer in the phloem of two or three years old branches. We had to collect that wood in the fields and to keep it in the best conditions for the eggs as long as possible. Low temperatures of 6 to 8° C appeared the most suitable.

Another problem : the laid eggs soon enter in diapause and cannot hatch in the fields before the beginning of January. Three months in a humid cool room at 3-4° C was necessary to remove the diapause of the eggs in the wood.

Anticipate hatching in twenty days was then possible in a room with a hygrometry of 50 to 70% and a temperature between 20 and 25° C [12, 13].

Every three weeks, 20 new botanic species were sown, then transplanted into small cyclindric pots adjusted to small cages designed for leafhopper lodging (description in [13]).

The survival of *S. titanus* was tested on three plants of every species, each receiving 5 young leafhoppers.

We had to choose the most interesting botanic species. More than 300 were tested but a good survival of *S. titanus* was found on only 10 of them : some composacae, (*Cineraria, Bellis, Chrysanthemum*), some cruciferacae (*Nasturtium, Sinapis, Brassica*), polygonacae (*Rumex*), papillionacae (*Vicia sp.*). Among those species, broad bean, *Vicia faba* allowed only a medium length survival of the leafhopper, but it became the very suitable host plant for FD [13].

The next stage was to use *S. titanus* to inoculate FD to the species allowing sufficient survival of that leafhopper. But we needed first to know what stage of development of that insect was to be used.

We knew since KUNKEL and BLACK [1] that yellow's diseases are transmitted according to the *persistent mode*, which means that the pathogen agent taken by the stylets multiplies in the body of the vector, in the intestines, the salivary glands before making the insect infectious.

Its appeared that the larvae of *S. titanus* were able to acquire the FD pathogen agent but were poor at inoculating it. On the contrary, adults are very efficient for inoculation. We then tried to use for inoculations the whole adult life of the insect. In practice, we have to put in acquisition the larvae in their 4th stage of development in order to get them infectious at the time of their imaginal moulting.

Acquisition must be done by putting larvae on FD diseased grapevine. For a study throughout the year that is to be carried out in a greenhouse on cuttings showing FD symptoms. Such cuttings cannot be taken from diseased branches which cannot be propagated by cuttings. We have to take in winter symptomless vineshoots already inoculated during the former summer and being at the stage of *winter incubation*. The best is to take such branches from plants apparently healthy but situated around an area of active epidemy. We thus could hope to get about 20 % cuttings showing symptoms [4, 5].

We then put our 4th stage larvae on such cuttings showing symptoms. When the insects became adults they were transferred onto the botanic species chosen for a good survival of the insect. The inoculated plants were then placed in a greenhouse for incubation. We had to solve various problems : day length and lighting, particularly in winter. Very good symptoms were obtained on broadbean, *Vicia faba* and on several species of *Chrysanthemum* [13].

The final proof of the nature of the obtained symptoms was brought by carrying out the return of the FD pathogen agent from the herbaceous plants to grapevine with *S. titanus*. Nearly all the healthy grapevines which received the vector insects infected on *Vicia faba* reproduced FD symptoms. The returns were more difficult from diseased *Chrysanthemum*, *S. titanus* surviving badly on these plants once old and diseased [13].

Broadbean, *Vicia faba* was retained as the best host plant. It is easily obtained by sowing, grows rapidly and FD symptoms appear in less than a month. Besides, it has proved a good source of the FD pathogen agent [13, 14]. The varieties Seville, Strube and Arla were particularly suitable (Fig. 1).

Figure 1 - Healthy broadbean, *Vicia faba* (left) and broadbean showing symptoms of Flavescence dorée (right), inoculated by leafhopper.
(Photograph CAUDWELL, according to Ref. 12, 13)

2) Search for an experimental leafhopper vector usable throughout the year and introduction of a working cycle *V. faba-Euscelidius variegatus*

The natural vector of FD, *S. titanus* is not convenient for research. It has only one generation per year and cannot be obtained in continuous raising : we obtain few eggs and they soon enter in diapause [13]. Moreover that insect, very alive but fragile is difficult to handle. It was then necessary to search if other species of leafhopper could transmit FD from *V. faba* to *V. faba*.

Bois noir of grapevine, found in Burgundy since 1960 was at that time so far considered a non epidemic form of FD in a geographic area not colonised by *S. titanus* [3] (still called *S. littoralis*). By luck the leafhopper species handled in searching the vector of *Bois noir* lead to discover that it was possible to transmit FD from *V. faba* to *V. faba* by two species of Jassidae leafhoppers which survived badly on grapevine, *Euscelis plebejus* FALL and *Euscelidius variegatus* KBM. It was a burgundian strain of that last species which has given the best transmissions. It has the advantage to show no diapause of any kind and an indefinite number of generations per year [14].

In order to establish if the symptoms obtained on the broadbeans inoculated by *E. variegatus* were those of FD, we had to try the return of the disease to grapevine. The simplest way was to try that operation by *S. titanus* which lives poorly on broadbean and well on grapevine. That return was successful with 75 % transmission [14].

We had thus obtained a model cycle *V. faba – E. variegatus* for FD. That disease could henceforth benefit from the same conveniences of study as the yellow's diseases of herbaceous plants, *Aster yellow's*, *Stolbur* and others.

It remained to organise the raising of *E. variegatus* in order to dispose of the insect in every stage throughout the year.

3) Organization of the raising of *E. variegatus* for use throughout the year

The discovery of the wallless prokaryotes (MLO) in the cells of plants affected by yellow's diseases did not bring out any additional technique for the study of those diseases, except their observation *in situ* [27].

But the model cycle *V. faba – E. variegatus* lead us to develop an infectivity test by injection of healthy leafhoppers and then to use it to improve the media of extraction, of survival and possibly of culture of the pathogen agent. We then decided to turn the activity of our laboratory in that new direction.

If we intend to launch 6 to 9 infectivity test every week, each of them involving inoculation of 40 young healthy leafhopper it required the weekly production of : 240 to 360 leafhoppers healthy young adults and 360 to 540 infectious leafhoppers [11].

That objective had to be reached in a continuous manner without any slacktime. We were then obliged to start a new raising cage every two weeks, and in that way, each cage was replaced by the 5th following one. A rotation of six cages was then necessary [11]. (Fig. 2)

In order to dispose at any time of young adults to be injected, we had to start each cage with larvae in their 5th stage whose age was perceptible with an approximation of $\pm$ 3 days : the leafhoppers were thus arranged into groups of nearly the same age in successive cages.

We also had to avoid consanguinity and apparition of genetic isolats. Therefore we started every cage with 5th stage larvae taken from three different cages.

In order to prevent the occurrence in the cages of parasites of the vegetal host, we were led to alternate the hosts. Maize was chosen for leafhopper raising and laying eggs. The FD diseased broadbeans became the alternative host when we had to make the whole cage infectious. In the usual practice it was sufficient to infect one out of two cages [11] (Fig. 2).

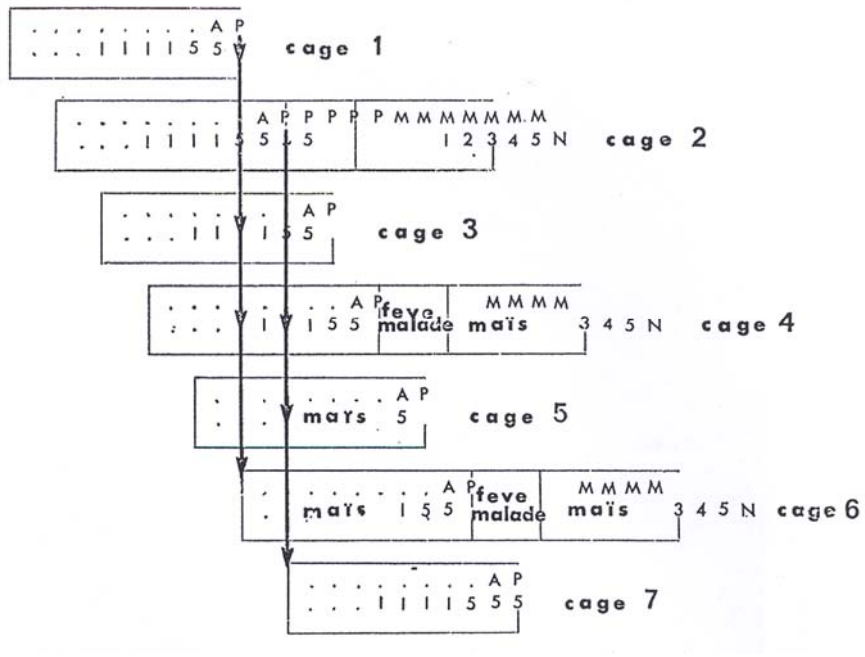

Figure 2 - Organization of the raising of the leafhopper *Euscelidius variegatus* for an use throughout the year (according to CAUDWELL and LARRUE, 1977, Ref. 11).
A new cage is thrown again every two weeks on maize (*Zea Maïs*).
One out of two cages is infected from the 10th to the 13th week by FD infected broadbean (without maize) for infected leafhopper production. Thus, the infectious adults could be used during seven weeks without any risk of mixing with the next non infected generation.
(5 = 5th stage larvae ; A= Adults ; P = Egglaying ; M = Infectious)

That organization of the raising of the leafhopper *E. variegatus* for a regular use of the infectivity test was not a simple one. It required an important supply of greenhouse, an insectarium, the permanent mobilization of several persons and a strict planing. We have to pay homage to Jean LARRUE who was in charge with his fellow workers over numerous years.

4) Developing an infectivity test by vector injection

An infectivity test by injection of infectious extracts to the vector insect was devised in 1920 by WEIGL for human rickettsioses [34]. It was applied by BLACK to plant pathogen agents transmitted by leafhopper according to the persistent mode when he tried to test if that pathogens multiplied in the body of its vector [1].

The model cycle *V. faba – E. variegatus* lead to attempting such test for FD. It is a complex one as it involves the multiplication of the pathogen agent successively in the vector body then in the plant, according to the natural cycle of the disease : we counted a latency period of three weeks for the vector to become infectious, then four additional weeks to get the symptoms on broadbean.

But that test appeared as the only means to establish the occurrence of the infectivity and to measure it in the extracts without making any hypothesis about the nature of the pathogen agent.

In practice we inoculated for each test 40 leafhoppers “young adults” soon after their imaginal moulting. We utilized for the injection PASTEUR pipettes twice drawn while the leafhoppers were kept motionless, ventral side up, by a piston working in a cylinder closed on the top by a stretched parafilm [20].

The inoculated leafhoppers were first kept on two healthy broadbeans for the three weeks incubation in the insect body.

They were then encaged three by three on young healthy broadbeans for one week to make almost sure that the infectious leafhoppers had time to transmit the disease. The plants, once rid of the leafhoppers, were put in a greenhouse, in conditions favourable to symptoms expression. The results were thus expressed according to the ratio of broadbean becoming diseased [7].

5) Statistical study of that infectivity test in order to express the results in numbers of infective units (IU) per millilitre

The results of the infectivity tests expressed in proportions of broadbeans becoming diseased gave only relative indications: good, bad, better etc… It was then necessary to express these results in numbers of Infectious Units (IU) per millilitre, according to the practice in bacteriology and virology. We were able to solve the problem by statistical methods [7].

Let us suppose at the outset that *one infectious leafhopper is necessary and sufficient to make a plant to develop the disease.* If **n** leafhoppers are caged on one plant, the number of **non** infectious leafhoppers follows a binomial rule with the parameters **n** and **q** (**q** being the probability for one leafhopper to stay healthy).

The probability P for one plant to remain healthy, in other words, the probability that all the leafhoppers put on it were non infected is then :

A) $P = q^n$ that is $q = \sqrt[n]{P}$

That relation A enables us to go back from the proportion of plants becoming diseased to the proportion of leafhoppers being infectious, each plant having received **n** leafhoppers.

That relation A and the calculation of its security intervals show that most of the sensitivity of the test is lost when we put more than three inoculated leafhoppers per plant. However we had to proceed to a certain bulking to limit the number of plants to be kept simultaneously in the greenhouse. We adopted the practice of caging three inoculated leafhoppers per plant.

We have now to examine if the percentage of injected leafhoppers that became infectious allows to go back to the number of infectious units (IU) per millilitre of the injected solution.

Let us now suppose that *only one Infectious Unit is necessary and sufficient to make a given leafhopper infectious.*

The Infectious Units being distributed at random into the injected drops, we could get 0, 1, 2…K Infectious Units per drop. The Poisson distribution could then fit, provided we use properly diluted solutions.

That Poisson distribution will have as average value the number M of infectious units per drop :

$$q_k = e^{-M} \cdot \frac{M^k}{K\,!}$$

Which gives for K= 0 (No IU per drop) :

B) $q = e^{-M}$ that is $\mathrm{Log}_e\, q = -M$

Then, according to that second hypothesis, *the average number M of injected Infectious Units per leafhopper can be estimated by the neperian logarithm of the probability q that a leafhopper would not be infected.*

Both relations A and B give a value of q, (the probability for one leafhopper to be **non** infected). It is then possible to reduce them to the following single relation C :

C) $P = e^{-nM}$ that is $\text{Log}_e P = -nM$

The average number M of Infectious Units per injected drop can be calculated from the probability that one plant would not be infected.

The volume of the injected drops being estimated at 0,0005 ml per insect, the concentration (number of Infectious Units per ml) become :

$$\frac{M \times 1 \text{ ml}}{0{,}0005 \text{ ml}} = 2000\ M$$

The results of relation C have been summarized by the Abacus of Fig. 3 which appeared in our publication [7]. It became the tool of an every day use.

The results expressed in number of Infectious Units per ml are minima…Indeed, it could occur that only one infectious leafhopper was not sufficient to make a plant diseased or that only one IU was not sufficient to make a leafhopper infectious.

In that respect our assistant Catherine KUSZALA who was in charge of the use and improvement of the infectivity test has shown that male leafhoppers are significantly more sensitive to inoculum than females [29]. We then recommend to inject males only.

On the other hand we have suggested broadbean, *Vicia faba* as the best host plant for infectivity tests of yellow's diseases. Easy to grow, it rapidly reacts giving specific symptoms. It would then be a good substitute for Periwinkle *Catharantus roseus* in the study of those diseases [7].

We will now show that it was indeed the infectivity test which enabled to solve the problems set by the phytoplasm-yellow's diseases. That was in conformity with the expectations and choices of our working team.

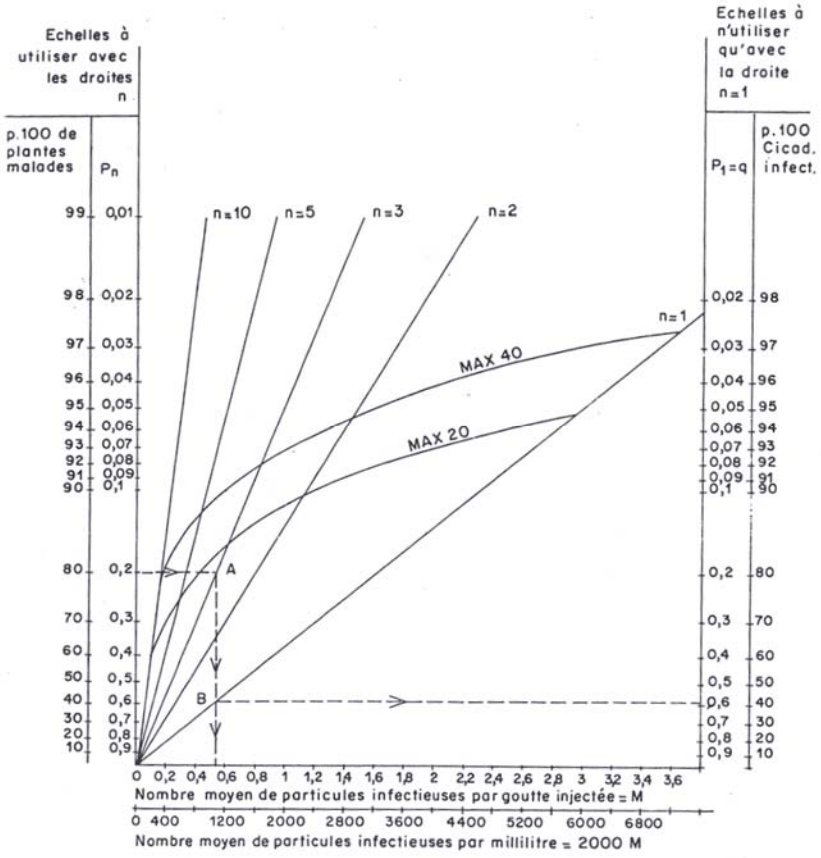

Figure 3 - Relation between the observed percent of diseased plants in the infectivity test and the mean number of infectious units (IU) per ml in the injected solution (taking into account the number n of leafhoppers per plant).
(according to CAUDWELL, 1977, Ref. 7).
The way of reading is given by the arrows on the dotted lines :
the given example is that of a result of 80 p 100 diseased plants,
each of them having received three injected leafhoppers (n = 3).
We obtain on the X-axis 0,55 IU per injected drop, thence 1000 IU per ml.

In case of need, the Y-axis in the right gives the percent of injected leafhoppers that became infectious, by using the line n = 1.

6) Use of the infectivity test to increase concentration in Infectious Units of the extracts

Let us keep in mind that the aim was to obtain the pathogen agent purified and concentrated in order to have access to its characterization and to diagnosis methods.

The questions were numerous:

– We had to look for the best sources of IU: the best test plant, which part of that plant, at what stage of infection.

– Which insect vector species, sex, stage of development.

– We had to improve the media to prevent aggregation of IU among them or with the host tissues.

– We had to search the best survival conditions of the IU, their resistance to freezing (composition, pH, osmotic pressure) etc…

The research lead to use as a source of infectivity the broadbean apex showing young symptoms, and as insect vector the male leafhoppers *E. variegatus* in infectious stages.

A substantial improvement of the extraction and survival was obtained with 747 medium whose composition for 100 ml was as follow [19] :

35 ml TC 199 Morgan medium.

35 ml stock solution close to BM 22 Vago medium.

20 ml horse serum.

10 ml yeast extract prepared in the laboratory with a pH of 6,5 and an osmotic pressure of 300 millimoles.

Further improvements led to retain media 1379 and 1464. The former rich in Sera and biologic complexes must be used for survival and long run operations. The second, deprived of biological compounds but permitting a good phytoplasm (MLO) extraction was used for sera preparation with serological tests in view [9].

Both 1379 and 1464 media allowed to dilute the extracts 100 times before the infectivity tests. That brought the possibility to compare numerous media or purification methods simultaneously [9].

In medium 1379 the infectious extracts could be kept frozen at – 80° C for several month without any loss of infectivity.

A purification method has been recommended : two successive clarifications, double prefiltration, one filtration (450 nm) and sedimentation [9].

7) Valorization of those results by serological crossing of both hosts, the test plant and the vector insect

The successive approaches, according to the results of the infectivity tests brought to extracts partially purified containing 10^5 to 10^6 IU, respectively for broadbean or leafhopper extracts.

Those concentrations did not allow to detect the pathogen agent *in vitro* by electron-microscopy at the magnification of 10.000 x that would be necessary to recognize them, according to their image in ultra thin sections. The same problem occurred with serological tests. But any additional concentration would also multiply impurities.

The complex cycle of the phytoplasms on two successive hosts very different, the plant and the leafhopper allowed to overcome those difficulties.

To make clear, let us take the case of visualization. We express the concentration in number of particles per millilitre. In microscopy that ml spreads in a thin layer surface k which is considered identical either in optic (between both plates) or in electron microscopy (the dried drop on the grid).

The greater the magnification the smaller the field surface in the microscope. We have the following relation :

$$\text{Concentration} = \frac{\text{1 particle x k}}{\text{field surface}}$$

That relation becomes linear in logarithmic coordinates (Fig. 4).

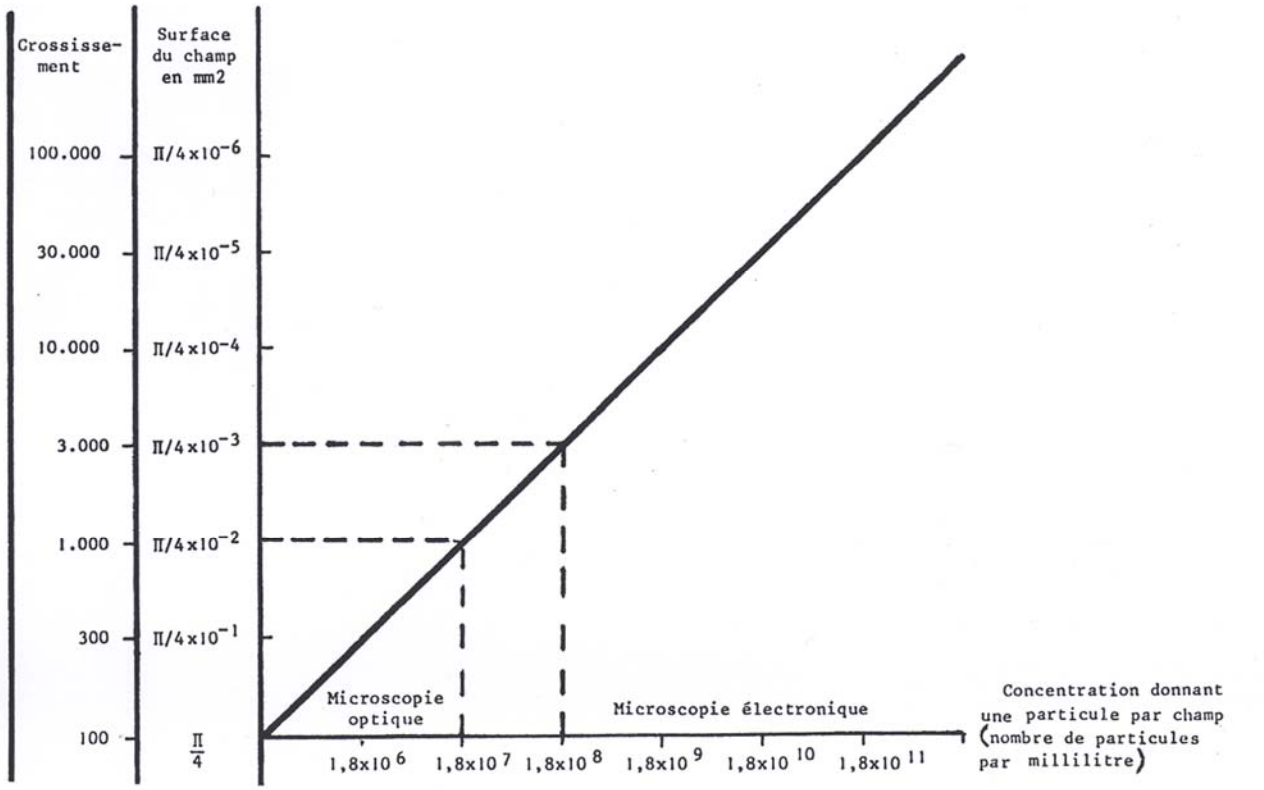

Figure 4 - Correlation between the magnification of an optic or electronic microscope and the solution concentration giving one particle per field.
(according to CAUDWELL, Ref. 8 and 9).
On the Y-axis, the magnification of the microscope and in parallel the solution surface wich can be observed in the field.
On the X-axis, the solution concentration giving one particle per field.

NB : That figure, correctly published [8] was unfortunately given with a misprint in the equation formula. It has been corrected in the next issue of the same journal (Febr. 1978) and later in another publication [9].

That Fig. 4, valid for any pathogen or microscopic object, shows that we need a concentration of 1,8 x 10^9 particles per ml to see one particle in the microscope field at the magnification of 10.000 x.

A factor 1000 was then lacking in our extract concentrations to see the pathogen agents in the liquid media. It probably was the same problem for the serological tests.

That factor 1000 could be gained through the serological methods, new at that time, using immunoadsorbents. Those methods were indeed 100 to 1000 times more sensitive than the former methods of serology or of visualization.

However those methods on immunoadsorbents require that at least one of both reagents, the antibody or the antigen should be perfectly specific : that was not the case.

We then thought that it was possible in the case of FD and of its modelised cycle to take advantage of the availability of two very different hosts, the plant and the leafhopper vector.

We decided to use *an anti infectious leafhopper serum against diseased broadbean extracts* [21, 22, 23]… and reciprocally *an anti diseased broadbean serum against infectious leafhopper extracts* [24, 25].

The normal proteins of the plant and of the leafhopper being very different our poorly specific sera, thanks to that crossing, became *specific by difference* and perfectly useful as shown in the next chapter.

8) The world's first serological results for phytoplasma diseases and first visualization of their pathogen agent in a liquid medium

In order to test our sera, *specific by difference* we first chose the technic of immunosorbent-electronmicroscopy (ISEM) which is a combination of immunoadsorbant serology and visualization. That choice was induced by the fact that the visualization of phytoplasms (then called MLO) had been very often hoped for over many decades but never obtained.

Several rabbits were immunized either with concentrated extracts of FD broadbeans, or with infectious leafhopper extracts [21, 22, 23, 24, 25].

The technique for ISEM was mainly that described by LESEMANN and PAUL for plant viruses [30].

The freshly carbon-coated grids of the electron microscope are incubated for 5 min on a buffered solution of *Staphylococcus aureus* protein A which allows to use undiluted antiserum. The grids are rinsed with buffer, then incubated for 5 minutes on the undiluted FD broadbean antiserum and rinsed again. In order to prevent non specific reactions, a FD leafhopper antigen is used to react for 15 minutes with the FD broadbean antiserum. Tween 20 at the final concentration of 0, 05 % has been added to the antigen to hinder direct adsorptions on the grid. After rinsing, the preparations are fixed with glutaraldehyde 1 %, rinsed again, and negatively stained with an ammonium molybdate solution 2 or 6 % for electron microscopic examination.

The protocol revealed many globular, yeast shaped vesicles showing occasional budding. Some of them were closely linked together like dumb bells or by thin peduncles. The average diameter was about 300 nanometers. They take the dye but keep a white, well delimited edge (Fig. 5).

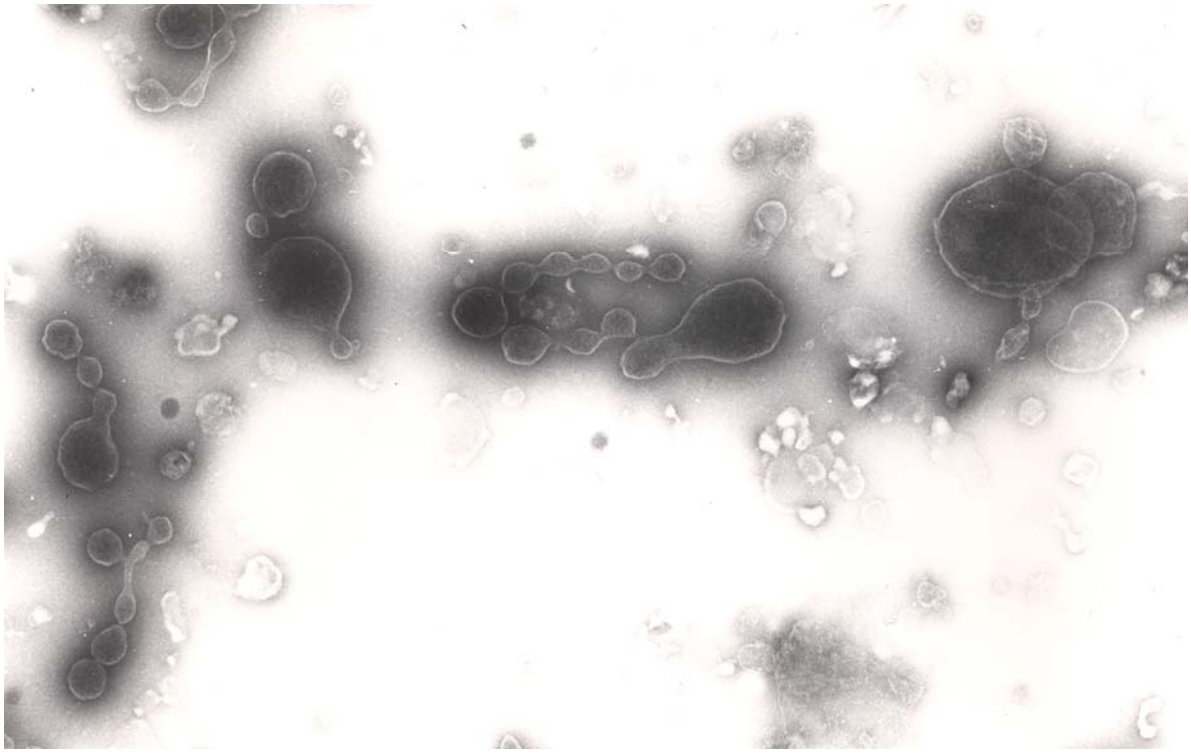

Figure 5 - Observation of the pathogen agent (phytoplasma) of grapevine Flavescence dorée in liquid medium by immunosorbent electronmicroscopy (ISEM). Photograph by R. MEIGNOZ.
(According to CAUDWELL et Al, 1981, 1982, 1983, Ref. 21, 22, 23, 24, 25).
That result was the world's first visualization of the phytoplasma pathogen agent of the yellow's diseases and the first result obtained by polyclonal antibodies for that kind of pathogen.

None of the various controls, including in the same protocol either a 0 Serum of the same rabbit prior to the first injection, or a healthy broadbean antiserum, has ever shown such vesicles [21, 22, 23].

Identical results were reciprocally obtained using FD broadbean extracts with FD leafhopper antisera [24, 25].

The simultaneous testing of the FD pathogen and of other yellow's diseases as the PHY disease, the *Stolbur* and the *Clover phyllody* could show that the ISEM proved a specific method, able to distinguish several yellow's diseases [28].

It appears that these results first published in 1981 were the world's first report of polyclonal antisera for the yellow's diseases of the plants and the world's first visualization of their phytoplasm pathogen agent in a liquid medium.

They are the conclusions of the ten years research conducted in our laboratory in order to find diagnosis methods for that kind of diseases. Unfortunately, these ten years, most important for their results has been completely ignored in the handbooks and synthesis mentioned in our introduction and the latters are now used to sum up history for new generations of student and searchers.

EPILOGUE

I would like to mention the further developments which soon followed that important ten years research.

In 1981, our laboratory received a group of three colleagues, Elisabeth BOUDON-PADIEU, Raymonde MEIGNOZ and Charles SCHNEIDER, coming from a neighbouring laboratory. They enabled us to make better use of the electron microscope, owing particularly the technicity of R. MEIGNOZ. That group could then soon be associated to the research around ISEM [21, 22, 23, 24, 25].

On the other hand, the electron microscopy, reorganized in its turn as a "Service commun de microscopie electronique", has been joined to our laboratory by recruiting a graduate engineer Jeannine LHERMINIER.

Both events made it possible to share the tasks in one time when new ways were opened by the need to test other immunoadsorbents :

1) The plastic supports for the immunoenzymatic methods (ELISA, DOT BLOT). That part has been devolved to E. BOUDON-PADIEU (publications from 1986 onwards). In 1992 those methods could be applied to the direct diagnosis of grapevine tissues owing to adjunction of high amounts of detergents (Triton x 100, 3 to 4 % or chaps 5 %) in extraction solutions [10].
2) The Ultrathin sections of infectious leafhoppers or of FD plants, according to the immunocytological methods (colloïdal gold or others). Here too appeared necessary to use FD broadbean antisera for sections of leafhoppers and reciprocally. (Publications of J. LHERMINIER from 1987 onwards and thesis by that author in 1993).
3) Purification of Phytoplasms (MLO) of FD on hydrazid affinity columns which allowed elution and regeneration of the column (Publications of A. SEDDAS and R. MEIGNOZ from 1993 onwards and thesis by A. SEDDAS in June 1994).

The model cycle of FD on broadbean and *E. variegatus* and the extraction media 1376 and 1464 were also at that time the basis of two other important results:

- Obtaining of monoclonal antibodies for grapevine Flavescence dorée (Publication of Y. SCHWARTZ from 1988 onwards and thesis by that author in 1989). Easy and infinite production of pure and specific antibodies for that diseases was then made possible, suppressing the need of serological crossing of both hosts the plant and the vector.
- The cloning of DNA Flavescence dorée phytoplasm and its application to the detection of the grapevine phytoplasmas (Publication of Xavier DAIRE from 1990 onwards and thesis by that author in June 1994).

That time of the thesis of X. DAIRE was for me the age of the retirement from INRA and from all the responsabilities in the laboratory of mycoplasmas and arboviruses.

CONCLUSION

It is to be hoped that the present compilation shows to students, young searchers and professionals of grapevine that no difficult problem can be spontaneously solved.

The ten years study which was the aim of that compilation was specifically focused on the various handicaps that the phytoplasms pathogens of the plant yellow's diseases set to the pathologist. In fine that work brought about the world's first polyclonal sera for that kind of pathogens and their world's first visualization in a liquid medium. It was then surprising to see them completely ignored in historical specific publications.

The further results of our laboratory, given as epilogue were also the fruit of the same original ten years study.

It is interesting to note that grapevine which was often at the origin of discoveries in plant pathology has once more brought its contribution to one of the most difficult problems encountered in that discipline in the last decades, as it depended on none of the fields already explored.

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