2. ARTIFICIAL CULTURE OF PLANT PATHOGENIC MYCOPLASMA-LIKE ORGANISM (MLO)

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Background information of the researches on plant pathogenic mycoplasma-like organism (MLO)

A number of species of plants in all parts of the world are found to be affected by MLO since the first discovery by Doi et al[6] in 1967. Serious loss is caused by MLO on more than three hundred crops or plants of economic importance, for example, rice in Asia, mulberry in Japan, and citrus in Africa and the United States.

Mycoplasma, also known as PPLO (Pleuropneumonia-like organism), has been recognized as a member of the microbial flora of animals since 1898 when Etinne Nocard and Emile Roux[21] succeeded to culture *Mycoplasma mycoides*, the agent of contagious bovine pleuropneumonia. Many other mycoplasmas have been discovered in animals and some of them were pathogenic. From the physiological and biochemical viewpoints, these mycoplasmas are similar to bacteria. The one important exception is that they lack a cell-wall and the cytoplasmas are bounded only by the delicate plasma membrane.

Characteristics of plant pathogenic mycoplasma-like organism

First, mycoplasmas can survive and grow only in an osmotically protected environment. Second, being free of the restrictions imposed by a rigid cell-wall, they can be deformed and can squeeze through narrow pores smaller than the diameter of the cell. This property may be important in connection with their spread through plant. Third, they are completely resistant to penicillin and other substances which disrupt the synthesis of bacterial cell-wall. Fourth, almost all of MLO are transmitted by insect vector, mainly leafhopper.

Research on MLO

Since the discovery of MLO in 1967, many reports from all over the world demonstrated the association of MLO with plant diseases by electron microscopy. However, according to the postulates of Robert Koch, proof that these organisms are pathogenic agents would require not only evidence of invariable association with disease but also a demonstration that the purified organism, preferably cultured in artificial media, can induce the disease when introduced in healthy plants.

The next step, therefore, was to attempt culture the presumed mycoplasmas in the laboratory, free of plants. Numerous attempts have been made to culture the causal agents if these disease agents can be grown in cell-free medium.

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Culture of spiroplasma

The stubborn disease agent of citrus, *Spiroplasma citri*, has recently been cultured *in vitro* by Saglio et al.[22,23] in France and Abd El-Shafy et al.[8,1] in the United States in 1971. This disease has since been induced in healthy clover and citrus plants by transmission of cultured S. *citri* through non-vector leafhopper, *E. plebejus*, by Markham et al.[5,16] in 1973 & 1974.

In 1970 Chen and Granados[2] achieved a long-term maintenance of the infectivity of corn stunt in a liquid medium. Although no colony was obtained on agar plates, evidence from negative staining and ultrathinsection electron microscopy of MLO maintained *in vitro* as well as infectivity tests suggested that limited growth took place in the primary cultures and that the organism in culture was the corn stunt agent. Recently a spiroplasma isolated from corn infected with corn stunt disease has been successfully cultured *in vitro* by Chen and Liao[3] in 1975.

Culture of other mycoplasma-like organism of plants

Successful results in the artificial culture of plant pathogenic mycoplasma-like organisms have been reported for the agents associated with sugarcane white leaf[14], clover dwarf[15], clover phyllody[7,10], citrus greening[9], lavender dépérissement[11], paulownia witches' broom[26], petunia aster yellows[26], gentian witches' broom[26], and Japanese honewort witches' broom[26]. Giannotti and Vago[10], working in France also have obtained mycoplasma cultures from a number of yellow diseased plants, including clover phyllody. When Giannotti's cultures were compared with known species, they were found to belong to *Acholeplasma laidlawii* species. Hamptom et al.[12] in 1969, succeeded in culturing *Mycoplasma galisepticum* from pea plant which is pathogenic to chickens. Several researchers have cultured *A. laidlawii* from plants but none of them was pathogenic.

According to Hayflick and Arai[13] in 1973, it should be noted that there are three ways of contamination by several mycoplasma species during the time of isolation and culture tests. At first mycoplasma contamination from medium components. For example, many researchers had an experience that horse serum used in mycoplasma-medium formulation was contaminated with *Acholeplasma laidlawii* or other species of mycoplasmas. Second passenger mycoplasma. In view of the fact that *A. laidlawii* can be found in soil and sewage, the isolation of this species from plant materials should be carefully examined. Third, mycoplasmas and obligate intracellular parasites. Due to the fact that some MLO can be found in healthy leafhopper vectors during our experiments the isolation of non-pathogenic MLO from leafhopper materials should also be scrutinized.

Culture of Western X mycoplasma-like organism (WXM)

In 1970, western X-disease of peach was recognized as a member of "yellows disease" group by Nasu et al[17]. Western X-disease agents showed some evidence of multiplication and pathogenicity in AcTc medium (Chiu anl Black, 1967[4]) for two weeks by Nasu et al[18] in 1974. Further culture experiments were repeated by us in Japan. Western X-disease on celery and leafhopper vectors, *Colladonus montanus*, were used which were received from University of California at Barkeley, California, through the courtesy of Prof. E. S. Sylvester.

Salivary glands containing WXM were removed from viruliferous insects and kept in various culture media, such as culture medium for animal mycoplasma, plant mycoplasma or some tissue culture media. Ultrastructural changes of WXM in salivary glands were observed and the effects of media were investigated. This method was very sensitive to detect the morphological changes of cultured WXM in salivary glands.

Breaking down of cytoplasma and unit membrane of WXM was observed when they

are cultured on Saglio's medium for *S. citri*. Similar results were also observed on some tissue culture media. In AcTc medium, however, fairly good growth of cultured WXM was observed in the early period of culture as shown by Nasu et al[18] in 1974, and then, a breaking down in shape of cultured WXM was observed when they were maintained in the same medium for a long time[20,24]. According to the results obtained from these culture experiments, it is concluded that these culture media could not be used to culture WXM.

Several attempts were made to determine better culture medium of WXM. At first, the favorable buffer solution for the maintenance of WXM was tested and it was found that glycine buffer of 0.1 to 0.01 M was effective to maintain a good shape of WXM[24]. As the next step, attempts were made on the effects of osmotic pressure to the shape of WXM in 0.1 M glycine buffer containing sucrose, sorbitol, or mannitol, respectively. From the results of experiments, it was concluded that sucrose was shown to be more effective for adjusting osmotic pressure than sorbitol and mannitol. It was also recognized that 0.9 M sucrose in 0.1 M glycine buffer was effective to maintain a good shape of WMX, which shown higher transmission percentage than other concentration of sucrose[24]. At the third step, the composition of artificial cell-free medium for WXM was tested. Many kinds of chemical elements were added one by one to 0.1 M glycine buffer containing 0.9 M sucrose and tested. More than two hundred media were checked. Best results[24] were obtained in a new liquid medium called No. 57-3 which consists of inorganic salts (10 kinds), amino acids (9 kinds), vitamins (5 kinds), bacto-yeastolate, carbohydrate (2 kinds), lactalbumin hydrolysate, adjusted to pH 6.5, and finally dissolved to 100 ml of 0.1 M glycine buffer containing 0.9 M sucrose. It was filtrated through 0.8 and 0.45 u millipore filter, divided into 10 ml stock glass tubes, and stored at 5°C.

Effects of pH of this medium on the growth of WXM were tested by adjusting the initial pH of liquid media to 5.5, 5.8, 6.0 and 7.0.

Relative morphological changes of cultured WXM were observed on 3, 7, 14, and 21 days after the beginning of culture. From the results of these experiments, it is clarified that WXM suffers great morphological damage when incubated 3 days at 28°C as shown in Fig. 2. However, it recovers from its morphological damage in 14 days after the start of culture as shown in Figs. 3 and 4. It is also concluded that the range of pH from 6.0 to 6.5 is the approximate optimal pH values for the growth of WXM. It is suggested that WXM in dead salivary glands in this medium can be maintained or may grow and increase in number similar to those in living infected salivary glands. The growth is retarded or stopped, however, when they were cultured for a long time in the same medium as shown in Fig. 5.

In infectivity tests[20], 20 pairs of salivary glands containing cultured WXM were removed, macerated with a small glass mortar (0.25 ml), and a small amount of them was injected into leafhopper nymphs by a fine glass needle. After injection, the leafhoppers were fed in group on healthy celery plants for 25 days. Those leafhoppers were transferred to celery test plants for inoculation for two weeks and then the survived insects were killed by insecticides. All exposed celery plants were held in the growth cabinet at 25°C for at least two months after inoculation. Several celery plants inoculated by injected leafhoppers, developed disease symptoms as follows: leafhoppers injected by WXM of 14 to 15 days culture 5.9% (1/17), and 22.0% (13/59); 14 to 17 days culture 2.4% (2/83); 15 days culture 1.0% (1/98); 21 to 22 days culture 5.7% (4/70); and 28 days culture 1.6% (1/16), respectively, whereas no cultured WXM, 69% (9/13). In this case, one of the reasons for the low transmission percentage seems to be related to the unstableness of culture medium itself, or to the lowered pH of medium due to metabolic substances. Otherwise, produced metabolic substances may inhibit the infection when cultured WXM are introduced into insect vectors.

Discussion and conclusion

Before the success of primary culture of WXM in dead salivary gland cells, many negative trials were repeated for several years. While the growth of WXM was tested in insect materials, attempts were also made to culture WXM from plant materials. However all attempts were not promising when checked by electron microscopy. It was suspected, therefore, that a liquid medium may not permeate into the intact phloem tissues of diseased plants, or the crude extract inoculum prepared from diseased tissues may contain some factors which are inhibitory[19].

Results of experiments showed that the WXM can be maintained at least for 28 days, multiplied in dead salivary glands in liquid medium, and then became plant pathogenic. Unfortunately, the test could not be made on the recovery of WXM from the inoculated plants because WXM was imported from U.S.A. under the permission of quarantine law, so that a runaway of any insect vectors must be carefully controlled. Inoculated plants, therefore, were kept in another growth cabinet after insecticide treatments. All insect vectors were killed by sucking insecticides through inoculated celery plants, when they fed on those plants for acquisition of WXM. So WXM recovery could not be tested from the inoculated celery plants. It means that not all of Koch's requirements were fulfilled. However, MLO were observed in phloem cells of inoculated celery plants.

Since WXM can not grow on cell-free medium for *S. citri* or corn stunt spiroplasma, it may be suggested that MLO varies very much among species in biological characteristics especially in the requirement of nutritional elements. Requirement of sterols for the growth may be regarded as one of the most important criteria to distinguish the difference between spiroplasma and other plant pathogenic MLO. *S. citri* or corn stunt spiroplasma grew very vigorously in serum media (containing 10 to 20% horse serum). On the contrary WXM can grow in serum-free media. Perhaps the most distinctive characteristics in the nutritional requirements of the WXM are for lipids and lipid precursors needed for membrane synthesis. The major difficulty is to provide the lipid materials in a liquid medium in an assimilable, nontoxic form resembling to that found in serum of a spiroplasma's medium. In the present medium, lactalbumin hydrolysate or some sterols from bacto-yeastolate have been supplied. It is much more difficult to furnish an adequate supply of the sterols to WXM.

Further difference in the nutritional requirement between spiroplasma and WXM for growth may be the protein compounds utilized by these organisms. *S. citri* and corn stunt spiroplasma grew very vigorously in a protein rich medium (PPLO-broth, serum or peptone) against which, WXM could not[25].

From these data, it may be concluded that WXM belongs to the different species or group of mycoplasma-like organism from plant pathogenic spiroplasma.

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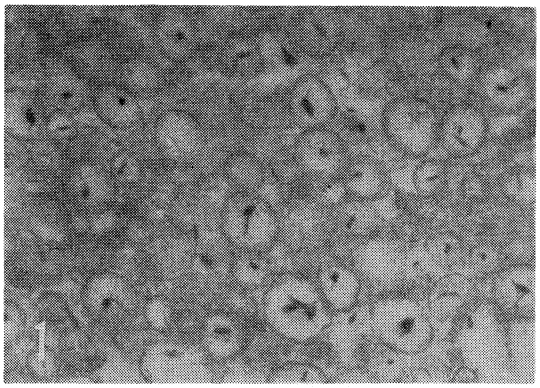

Fig. 1. Mycoplasmalike bodies in the salivary gland of *Colladonus montanus* leafhopper vectors infected with western X-disease.

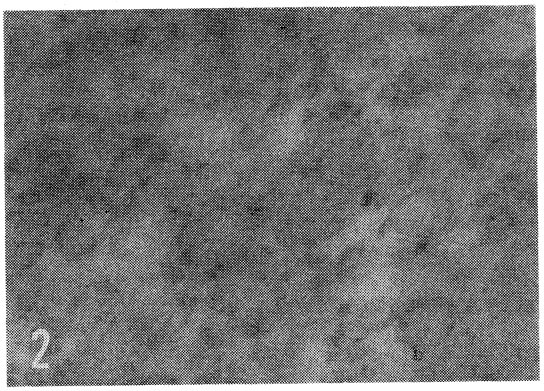

Fig. 2. Mycoplasmalike bodies after 3 days culture in a new liquid medium.

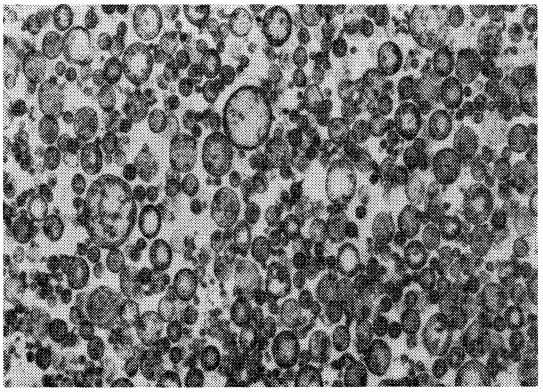

Fig. 3. Mycoplasmalike bodies after 14 days culture in a new liquid medium.

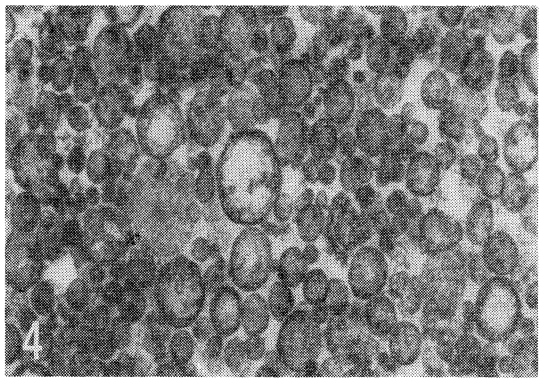

Fig. 4. **Mycoplasmalike bodies after 14 days culture in a new liquid medium.**

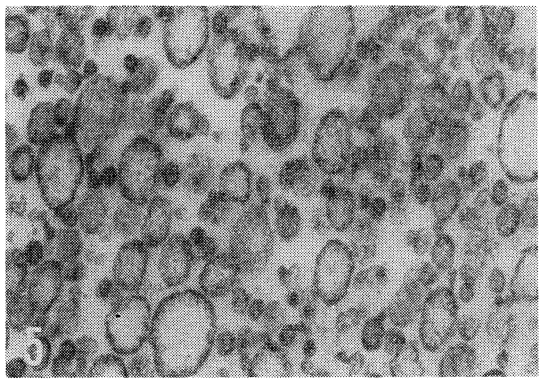

Fig. 5. **Mycoplasmalike bodies after 35 days culture in a new liquid medium.**

Discussion

D. A. Benigno, Philippines: If one interested to try to culture mycoplasma-like organisms how can we get hold of the medium you developed.

Answer: This medium is just developed at the beginning of this year. So, the composition of this medium is not published yet. If you like to try to culture some MLO in this medium, please refer to our paper after it is published. Now, we only say that the most important problem in this experiment is how to culture serially from cultured WXM in dead salivary glands. So, we are very sorry, we can not give you our medium now.

E. W. Kitajima, Brazil: Is it possible to cultivate salivary gland cells from non-viruliferous leafhopper and then infect them with western-X MLO?

Answer: I have no idea about your questions. But I guess, it may be possible to maintain salivary glands themselves for a few days, in different kind of medium. However, it may be impossible that WXM can infect cultured healthy salivary gland cells, because the osmotic pressure of the medium for salivary gland cell culture is so much different from that for WXM culture in dead salivary glands.