In Vitro Studies of Mycoplasma-Like Organisms

M. SUGIURA, Ph.D. Agri., T. SHIOMI, B. Agri., and S. NASU, Ph.D. Agri.

Institute for Plant Virus Research, Tsukuba Science City, Ibaraki, Japan; National Institute of Agricultural Sciences, Tsukuba Science City, Ibaraki, Japan

Received January 4, 1983

We determined whether the western X mycoplasma (WXM) isolated from Colladonus montanus could be maintained in vitro by ultrathin sections or by assay of infectivity. Large spherical or small electron-dense bodies like those found in intact infected cells were observed in some media. Infectivity of WXM can be maintained for 28 days in cultured salivary glands in a newly developed medium, and for 281 days (seven passages) in modified AcTc, for 231 days (eight passages) in modified PC, 107 days (one passage) in spiroplasma medium, and 52 days (one passage) in modified GITC medium extracts. However, there is no evidence that WXM multiplied in any medium.

INTRODUCTION

Since the discovery of mycoplasma-like organisms (MLO) by Doi et al. [1] many scientists throughout the world have reported the association of MLO with yellows-type disease of plants. In only two mycoplasmas, Spiroplasma citri [2,3] and corn stunt spiroplasma [4,5] have Koch's postulates for proof of pathogenicity been fulfilled. To date, many attempts have been made to culture MLO in a cell-free medium, or to maintain MLO in vitro in organ culture [6]. Successful results have been reported in a cell-free medium with some plant pathogenic MLO such as sugar-cane white leaf in Taiwan [7], clover phyllody [8,9] and lavender withering in France [10], citrus greening [11] and sandal spike [12] in India, phyllody of Vinca rosea in Italy [13], and aster yellows in Canada [14]. However, the pathogenicity of these cultured or maintained mycoplasmas has not been clearly established in every case. Recently, spiroplasmas were isolated from plants infected with aster yellows [15–17], pear decline [17], and western X disease of peach. In 1970, western X disease of peach was recognized as a member of the MLO disease group [18]. In the study conducted by Nasu et al. in 1974, western X disease agents retained their pathogenicity in an AcTc medium for two weeks. Further experiments were continued in Japan from 1974 to 1981 [19–24]. The objective of this study was to use an infectivity assay to determine how to maintain western X mycoplasma (WXM) and mulberry dwarf mycoplasma (MDM) from leafhoppers in vitro.

MATERIALS AND METHODS

Diseased Plants

Western X diseased celery plants and mulberry dwarf diseased mulberry seedlings were obtained by transmitting the disease using leafhopper vectors. Celery plants...
infected with peach WXM and infected leafhoppers were received twice, in 1974 and 1981, from the Division of Entomology, University of California, through the kindness of Professor E.S. Sylvester. Diseased mulberry plants were also received from the National Institute of Sericulture, Tokyo, Japan.

Leafhoppers

The leafhopper vectors, *Colladonus montanus* and *Hishimonoides sellatiformis*, were allowed to feed on diseased plants in a greenhouse for several days during the nymph stages, while healthy vectors were fed on healthy celery and mulberry plants, respectively.

Extraction and Culture

Extraction and culture techniques for the organisms used in this investigation have been described previously [19,25,26].

Media

We sought a medium favorable for maintaining the infectivity of WXM or MDM, using the following: (1) modified tissue culture medium for *Agallia constricta* [27], (2) modified Grace's insect tissue culture medium (Gibco), (3) modified plant suspension culture medium (Gibco), (4) spiroplasma medium [28], and (5) a newly developed liquid medium (SM-1 medium). These media were filtered through a millipore filter (0.8 μ and then 0.45 μ, successively) and stored at 5°C. Each medium was used within a month after preparation.

Electron Microscopy

Ultrastructural changes of MLO in cultured salivary glands or in extracts were observed with a Hitachi HU-12 electron microscope.

Infectivity

To test for infectivity, a small amount of incubated extract or ten pairs of cultured salivary glands were removed from culture at the desired periods and injected into healthy nymphs of vectors at the third to fifth instar stage with a fine capillary glass needle under observation with a dissecting microscope. After injection, the groups of leafhoppers were allowed to feed on healthy plants for 25 days. They were then transferred to single test plants for inoculation for two weeks. All inoculated test plants were held in a growth cabinet or in a greenhouse under observation for at least two months after inoculation.

**TISSUE CULTURE MEDIUM FOR AGALLIA CONstricta**

(AcTc MEDIUM)

EM Observation

In the case of WXM, large spherical bodies like those found in an intact cell were observed when cultured in the AcTc medium. Three days after the start of culture, filamentous structures sometimes appeared between the large spherical bodies, and electron-dense masses assembled in peripheral areas in the cytoplasm inside the unit membrane of the large spherical bodies. Large spherical bodies, filamentous bodies, and cells budding from large bodies were observed. Small bodies were produced on the proximal tips of the filamentous bodies 22 days after the start of the culture, but these bodies were very different from those appearing in intact cells. However,
almost all of the WXM bodies showed a tendency to break down at 35 days of culture. When 0.9 M sucrose was added to AcTc medium, medium-sized spherical bodies developed, and a number of small electron-dense bodies were observed after eight days of culture. WXM bodies began to break down when culture was continued for more than five weeks. Breakdown of the cytoplasm and the unit membrane of the bodies was observed in AcTc medium with sucrose containing 10 or 20 percent horse serum, fetal bovine serum, and 1 percent cholesterol.

**Infectivity**

Infectivity of extracts from ten pairs of salivary glands infected with WXM in AcTc medium containing 0.9 M sucrose was 37.5 percent the day of extraction, 2 percent at one and three days of culture, 0 percent at seven days, and 12.5 percent at 14 days. The infectivity of leafhopper extracts in the same medium was 70 percent at the time of extraction, 4 percent one day after extraction, 0 percent at three days, 8.3 percent at seven days, and 0 percent at 14 days. In MDM, extract from ten whole leafhoppers in AcTc medium showed infectivity in 13.3 percent of tests immediately after extraction, and no infectivity in extracts studied one, three, seven, and 14 days after extraction. It is clear that the infectivity of WXM can be maintained in AcTc medium with sucrose for at least 14 days. We therefore tried to subculture extracted WXM in AcTc medium with sucrose (about 1,390 mOsm/kg). In this case, we used 20 leafhoppers in 5 ml AcTc medium. The results are shown in Fig. 1A. Sixteen days after extraction infectivity was 22 percent. Thereafter, diluted extract (1/50) in fresh medium at 19 days after the start of the first subculture showed 24 percent infectivity. This subculture was continued for 66 days. At 30 days of subculture, this culture showed 17.3 percent infectivity. An extract of the seventh subculture showed 20 percent infectivity. We tried to observe WXM particles in the pellet of the second subculture. A small amount of WXM material was observed in the pellet, but almost all of the WXM bodies had disappeared.

![Diagram](image.png)

**FIG. 1.** Infectivity of extract and subculture of WXM in the “plasmolysing” AcTc and PC medium.
EM Observation

Disintegration of cytoplasm and the unit membrane of WXM was observed when the organisms in salivary glands were cultured on GITC medium for 10 and 14 days; however, medium spherical bodies or small electron-dense bodies were observed at 14 days of culture in GITC containing 0.9 M sucrose and 0.5 percent fetal bovine serum.

Infectivity

In the case of WXM, extracts from ten leafhoppers in GITC medium with sucrose (1,680 mOsm/kg) showed 5 percent infectivity at the time of extraction, 0 percent at seven and 20 days, and 9 percent at 43 days after extraction. A 1/10 dilution of the extract in fresh medium 27 days after the start of the first subculture showed 13 percent infectivity. No infectivity was obtained at 40 or more days after checking. In the case of MDM, extracts of ten whole leafhoppers in GITC medium showed 59.6 percent and 37.9 percent infectivity at the time of extraction and one day later, respectively, but no infectivity at three, seven, and 14 days after extraction. Extracts with GITC medium with sucrose were infective (44 percent) at the time of extraction and one day after (17.2 percent) but not infective on longer culture.

PLANT SUSPENSION CULTURE MEDIUM (PC MEDIUM)

EM Observation

Polymorphic WXM bodies were observed at the early stages of culture in PC medium. At about 25 days after the beginning of culture, the cytoplasm and unit membrane of the bodies began to disintegrate. When culture was continued for more than five weeks, complete disintegration of large bodies occurred. In PC medium with 0.9 M sucrose and 1 percent bovine albumin, medium spherical bodies and small electron-dense bodies were observed at seven days, but they began to break down after about 45 days of culture. WXM bodies began to disintegrate within 14 days when they were cultured in PC medium with 10 or 20 percent fetal bovine serum, 1 percent casein, or polypeptone (Difco).

Infectivity

The infectivity of extract from 20 leafhoppers in PC medium with sucrose is shown in Fig. 1B. At 16 and 113 days after extraction, infectivity was 25.6 percent and 11 percent, respectively. Extract diluted 1/50 in fresh medium at 92 days after the start of the first subculture showed 18 percent infectivity. The second, third, fourth, and eighth subcultures showed 27 percent, 2 percent, 16 percent, and 4 percent infectivity, respectively. In the case of MDM, extracts in PC medium with sucrose were infective (50 percent) at the time of extraction but not at three, seven, and 14 days.

SPIROPLASMA MEDIUM [28]

EM Observation

WXM bodies had disintegrated completely within three days after the start of the culture.
Infectivity

WXM was infectious (4 percent) immediately after extraction but no infectivity was obtained at seven or 14 days after extraction, but when the extract was continued for 31 and 107 days, infectivity was 11 percent and 6 percent, respectively. The first subculture showed 6 percent infectivity 69 days after dilution but no infectivity was observed at 30 or 140 days after the start of subculture. No change of color occurred in the medium. In the case of MDM, infectivity was 40.6 percent at the time of extraction and not at other times.

A NEWLY DEVELOPED LIQUID MEDIUM (SM-1 MEDIUM)

Several systematic investigations were conducted, following ultrastructural changes of WXM in cultured salivary glands, to develop a favorable culture medium for WXM. The best results were obtained by using a newly developed liquid medium tentatively called SM-1 medium, which contains MgCl₂, 609 mg; MgSO₄, 36 mg; Ca(NO₃)₂, 20 mg; KCl, 80 mg; KNO₃, 8 mg; NaCl, 50 mg; NaH₂PO₄, 27 mg; Fe₂(SO₄)₃, 2.5 mg; MnSO₄, 1.5 mg; H₂BO₃, 1.5 mg; L-alanine, 22.5 mg; L-asparagine, 35 mg; L-cysteine, 2.2 mg; L-glutamine, 60 mg; L-glycine, 750 mg; L-leucine, 62.5 mg; L-lysine, 62.5 mg; L-methionine, 5 mg; DL-serine, 110 mg; L-tryptophan, 10 mg; ascorbic acid, 10 mg; biotin, 1 mg; folic acid, 1 mg; pyridoxine-HCl, 1 mg; thiamine-HCl, 1 mg; inositol, 5 mg; D-glucose, 400 mg; sucrose, 30,800 mg; lactalbumin hydrolysate, 1,000 mg; and yeastolate (Difco) 5,000 mg in distilled water, 100 ml, pH 6.50, 2,100 mOsm/kg. The solution was filtered successively through 0.8 and 0.45 µ millipore filters.

EM Observation

Morphological changes were observed in cultured salivary glands infected with WXM at 3, 7, 14, and 21 days. Great structural damage of large and medium spherical bodies was observed three days after the start of culture. Fibrous structures appeared between small electron-dense bodies. Budding cells were also abundant, and sometimes electron-dense cytoplasmic masses accumulated in the peripheral areas inside the unit membrane of the large spherical bodies. These polymorphic bodies were observed in cultured salivary glands in SM-1 medium at 7, 14, and 21 days after the start of the culture. The range of pH from 5.5 to 6.5 is approximately optimal for maintenance of morphology of the WXM bodies in this medium.

Infectivity

Leafhoppers injected with WXM after 14 or 15 days of culture produced disease symptoms in 5.9 and 22 percent of celery plants on which they fed. After 15 days of culture, they produced 1 percent infection; at 21-22 days, 5.7 percent; at 28 days, 1.6 percent. WXM which had not been cultured produced infection in 69 percent. An extract from ten whole leafhoppers showed 10 percent infectivity immediately but none after culture for 7 and 37 days after extraction. The same results were obtained in diluted extracts and in subculture. In the case of MDM, the extract was immediately infective in 75 percent, but no infectivity was detected after 3, 7, or 14 days.

DISCUSSION AND CONCLUSIONS

Before the success in maintaining infectivity of WXM in cultured salivary gland cells, many negative trials had been carried out over several years. While
maintenance of WXM was tested in leafhopper materials, attempts were also made to maintain WXM in plant materials, but none of these attempts were promising when checked by electron microscopy.

In these experiments five types of semi-artificial media were tested in an attempt to maintain WXM and MDM in vitro. There are two factors important for maintenance of MLO. One of these is adjustment of osmolality of the medium [29]. In the case of AcTc medium without sucrose, large spherical bodies of WXM were maintained only during the early stages of culture. The WXM bodies disintegrated when culture was continued for more than 35 days. But large or medium spherical bodies were observed at 8 and 15 days after the start of culture in AcTc medium with sucrose, and these polymorphic bodies showed a tendency to break down when culture was continued for more than 42 days in the same medium. A small amount of medium spherical bodies or small electron-dense bodies were observed in the pellet of the second subculture of an extract in AcTc medium with sucrose. No infectivity was detected in extracts at 3, 7, and 14 days after extraction in AcTc medium without sucrose but infectivity could be maintained for a long time (281 days in seven times subcultures) in AcTc medium with 0.9 M sucrose, as shown in Fig. 1A. In PC medium, the results were similar to those in AcTc medium with sucrose. In a spiroplasma medium, WXM bodies disintegrated completely at three days after the start of the culture, but extracts of the culture were infective 31 and 107 days after culture, and also 99 days after the start of the first subculture. In GITC medium with 0.9 M sucrose, almost all of the large spherical bodies disappeared, but small electron-dense bodies were observed at 14 days after the start of the culture. The extract was infective and continued to be active 43 days after extraction and in the first subculture. In SM-1 medium, polymorphic bodies of WXM like those seen in intact cells were observed at 14 and 42 days after the start of the culture. The infectivity of WXM can be maintained for at least 28 days in cultured salivary gland cells; however, extracts of salivary glands were infective only immediately after extraction. From the results of these experiments, it appears that both the infectivity and the morphology of WXM is fairly well preserved for a long time in the presence of high osmolality in media such as AcTc and PC with sucrose, but infectivity was not maintained in extremely high levels of osmotic pressure such as those in SM-1 medium. On the other hand, the infectivity of WXM was demonstrated in extracts with spiroplasma and GITC media. It may be suggested that there is no relationship between large or medium spherical bodies in cultured WXM, and the infectivity of WXM therefore is dependent on the number of small electron-dense bodies in the extract, but not on the large or medium spherical bodies of WXM.

The second important factor is the pH of the medium. According to Smith et al. [29], aster yellows were maintained for 24 hours at pH 6.0–6.25, but maintenance increased to 48 hours at pH between 6.5 and 7.5. The range of pH from 5.5 to 6.5 is approximately optimal in SM-1 medium, as judged by the morphology of WXM. It appears that the optimal range of pH for maintenance of MLO in vitro is very narrow.

Recently, spiroplasma was isolated from peach, periwinkle, and celery plants infected with two strains of western X disease of peach in California [15,17]. We therefore made attempts to isolate the spiroplasma from celery, Vinca rosea, and leafhopper vectors infected with WXM in 1974, 1976, and 1981. No spiroplasma was isolated from the diseased plants and leafhoppers. It is clear that the agent of western X disease of peach in our material is a true MLO, but not a spiroplasma. Consequently, it is concluded that multiplication of the organisms probably did not
occur in the extract and subculture, but the infectivity of WXM can be maintained in vitro for a long time. The cultivation of the agent of western X disease of peach is still an open question.

REFERENCES

1. Doi Y, et al: Mycoplasma- or PTL group-like microorganisms found in the phloem elements of plants with mulberry dwarf, potato witches’ broom, aster yellows or paulownia witches’ broom. Ann Phytopath Soc Japan 33:259–266, 1967
2. Abd El-Shafy Fudl-Allah, et al: Culture of a mycoplasma-like organism associated with stubborn disease of citrus. Phytopathology 62:729–731, 1972
3. Saglio P, et al: Isolation, culture and observation au microscope électronique des structures de type mycoplasme associées à la maladie du stubborn des agrumes et leur comparison avec les structures observées dans le cas de la maladie du Greening des agrumes. Physiol Vég 9:569–582, 1971
4. Chen TA, Liao CH: Corn stunt spiroplasma: Isolation, cultivation, and proof of pathogenicity. Science 188:1015–1017, 1975
5. Williamson DL, Whitcomb RF: Plant mycoplasmas: A cultivable spiroplasma causes corn stunt disease. Science 188:1018–1020, 1975
6. Jacoli GG: Sequential degeneration of mycoplasma-like bodies in plant tissue cultures infected with aster yellows. Can J Bot 56:133–140, 1978
7. Lin SH, et al: Isolation and cultivation of, and inoculation with, a mycoplasma causing white leaf disease of sugarcane. Phytopathology 60:795–797, 1970
8. Faire-Amiot A, et al: Essai de mise en culture de l’agent de la phyllodie du trèfle. Ann Phytopath 2: 251–258, 1970
9. Giannotti J, Vago C: Role des mycoplasme dans l’étiologie de la phyllodie du trèfle: culture et transmission expérimentale de la maladie. Physiol Vég 9:541–553, 1971
10. Giannotti J, et al: Obtention de culture de mycoplasmes à partir de Lavendula hybrida Reverchon atteinte de “dépérissement” et de Hyalestes absolutes Sigh., un vecteur probable de la maladie. C R Acad Paris 247:394–397, 1972
11. Ghosh SK, et al: Isolation and culture of mycoplasma associated with citrus greening disease. Curr Sci 40:299–300, 1971
12. Nayar RM, Ananthapadmanabha HS: Isolation and pathogenicity trials with mycoplasma-like bodies associated with sandal spike disease. J Indian Acad Wood Sci 1:59–61, 1970
13. Lombardo G, Pignattelli P: Cultivation in a cell-free medium of mycoplasma-like organism from Vinca rosea with phylloyd symptoms of the flowers. Ann Microbiol 20:84–89, 1970
14. Jacoli GG: Attempts to culture in vivo mycoplasma-like organisms from plants: a retrospective view. Phytopath Z 102:148–152, 1981
15. Kloepper JW, Garrott DG: Relation of in vivo morphology to isolation of plant spiroplasmas. Curr Microbiol 4:365–370, 1980
16. Kondo F, et al: Aster yellows spiroplasma: Isolation and cultivation in vitro. Proc Am Phytopath Soc 4:190–191 (Abst), 1977
17. Raju RC, Nyland G: Effects of different media on the growth and morphology of three newly isolated plant spiroplasmas. Phytopath News 12:216 (Abst), 1978
18. Nasu S, et al: Electron microscopy of mycoplasma-like bodies associated with insect and plant hosts of peach western X disease. Virology 41:583–595, 1970
19. Nasu S, et al: Primary culturing of the western X mycoplasma-like organism from Colladonus montanus leafhopper vectors. Appl Ent Zool 9:115–126, 1974
20. Nasu S, et al: Attempts at axenic subculture of western X disease agents in vitro. Ann Phytopath Soc Japan 44:102 (Abst), 1976 (in Japanese)
21. Nasu S, et al: Attempts at axenic culture of plant mycoplasmas (western X and mulberry dwarf MLOs). Memo Mycoplasma Soc Japan 5:9–13, 1978 (in Japanese)
22. Shiomie T, et al: Effects of plant cell suspension culture medium to morphology of western X mycoplasma-like organism in vitro. Ann Phytopath Soc 43:376–377 (Abst), 1977 (in Japanese)
23. Sugimura M, et al: Artificial culture of plant pathogenic mycoplasma-like organism (MLO). Tropical Agric Res Series 10:85–91, 1977
24. Sugimura M, et al: Attempts at axenic culture of some mycoplasma-like organisms. Food and Fertilizer Technology Center for the Asian and Pacific Region, Book Series 13:166–174, 1978
25. Nasu S, et al: Isolation of western X mycoplasma-like organism from infectious extracts of leafhopper and celery. Appl Ent Zool 9:199–203, 1974
26. Nasu S, et al: Extraction of western X mycoplasma-like organism from leafhoppers and celery infected with peach western X disease. Appl Ent Zool 9:53-57, 1974
27. Chiu RJ, Black LM: Monolayer cultures of insect cell lines and their inoculation with a plant virus. Nature 215:1076-1078, 1967
28. Davis RE: Spiroplasma associated with flowers of the tulip tree (*Liriodendron tulipifera* L.). Can J Microbiol 24:954-959, 1978
29. Smith AJ, et al: Maintenance in vitro of the aster yellows mycoplasma-like organism. Phytopathology 71:819-822, 1981