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Transmission and Histochemical Detection of Mulberry Dwarf Mycoplasma in Several Herbaceous Plants

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뽕나무 오갈병 마이코플라스마의 몇가지 草本植物에의 傳染과 組織化學的 檢定

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ABSTRACT

Transmission of mulberry dwarf mycoplasma (MDM) from diseased mulberry to 5 herbaceous plants (periwinkle, white clover, Ladino clover, red clover, and Chinese milk vetch) through insect vector, *Hishimonus sellatus*, was confirmed by symptom expression and microscopic evidences. The earliest symptom appearance was noticed on periwinkle in which incubation period was 25-30 days, while it ranged 35-40 days in the other plant species. The common symptoms of MDM infected plants were characterized by poor plant growth with accompanying discolorations of leaves (chlorosis with vein clearing in periwinkle, reddish in red clover, brownish in white and Ladino clovers, and yellowish in Chinese milk vetch). Mycoplasmal infections were diagnosed light microscopically by Dienes' and toluidine blue staining of hand-cut and Epon-embedded sections, respectively. In Dienes' stain, all the plants infected with MDM showed specific staining reaction in phloems. In toluidine blue stain, mycoplasmal existence was noted by granular appearance in sieve tubes which were confirmed to be mycoplasma-like organisms under an electron microscope.

Key words: mulberry dwarf mycoplasma, *Hishimonus sellatus*, insect transmission, histochemistry.

要 約

뽕나무 오갈병 마이코플라스마가 媒介昆虫인 마름무늬매미충에 의하여 5가지 草本植物(일일초, 화이트클로우버, 라디노클로우버, 레드클로우버, 자운영)에 傳染되었음이 病徵發現과 光學 및 電子顯微鏡的 方法에 의하여 確認되었다. 傳染된 植物에서는 마름무늬매미충이 적어도 25일 이상 생존하였고, 産卵이 確認되었다. 이 病의 潛伏期간은 일일초에서 25～30일, 클로우버類와 자운영에서는 35～40일로 나타났다. 感染된 植物의 공통된 病徵은 잎의 變色으로, 일일초는 葉脈透化 및 黃化, 화이트 및 라디노클로우버는 褐色, 레드클로우버는 赤色, 자운영의 잎은 黃色으로 各各 나타났다. 한편 클로우버類와 자운영에서는 上記한 잎의 變色과 함께 植物體에 萎縮症狀이 나타났다. Dienes 染色에 의한 光學顯微鏡的 診斷方法은, 모든 罹病植物의 줄기 篩部가 特異하게 染色되어 뽕나무 오갈병 마이코플라스마의 檢出에 신빙성이 있고, 사용하기에 간편한 것으로 나타났다. Toluidine

blue 染色에 의한 光學顯微鏡的 方法과 電子顯微鏡 관찰로, 罹病植物組織內에서 마이코플라스마의 存在가 確認됨으로써 이 病의 傳染이 立證되었다.

INTRODUCTION

Mulberry dwarf disease caused by mulberry dwarf mycoplasma (MDM), is the most serious disease of mulberry in Korea and Japan (1, 10). In nature, the etiological agent is transmitted by the rhombic marked leafhopper (*Hishimonus sellatus*). Both the disease and its vector have been extensively studied in both Japan and Korea, but, unfortunately, there is still only limited information available concerning host range of MDM. Additionally, there is a need for more rapid and less expensive detection techniques for the organism within infected plant tissue.

This study was, therefore, initiated to investigate the transmission of MDM to selected herbaceous plants by the leafhopper vector and histochemical and electron microscopic detection of the infectious agent within infected plants.

MATERIALS AND METHODS

Transmission of MDM. Adults of *H. sellatus* (rhombic marked leafhopper) were collected from the mulberry field of Sericultural Experiment Station, Office of Rural Development, Suweon, Korea. A group of about 2000 adults were reared in diseased mulberry plants for about 45 days to get 5th instar nymphs or adults of the next generation which were allowed to feed on 10 selected plants (*Catharanthus roseus, Trifolium repens* 'White', *T. repens* 'Ladino', *T. pratense, Astragalus sinicus, Apium graveolens, Trigonotis peduncularis, Solanum nigrum, Phryma leptostachya,* and *Portulaca grandiflora*). These plants had been cultured in plastic pots with steam sterilized soil in a greenhouse. Each plant was exposed to 20 insects with a nylon screen cage placed on each pot. The temperature in the greenhouse varied from 25±2°C in the daytime to 15±2°C at night during the experiment. The number of living leafhoppers and symptom appearance were investigated at intervals of 5 days.

Light and Electron Microscopic Detection of MDM. Dienes' stain(2) was applied to detect the MDM in infected plants. Cross sections (20-50 μm) of upper stem parts of plants were cut by hand with a razor blade. The sections were stained with 0.2% of the stock solution (0.25 g of sodium carbonate, 10 g of maltose, 2.5 g of methylene blue, 1.25 g of azure II, and 100 ml distilled water) for 10 min., decanted in distilled water for 10 min., and examined under a compound light microscope.

For electron microscopy, pieces of midribs (2-4 mm in length) of infected plants were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH. 7.0) and 0.2% osmium tetroxide, followed by dehydration in ethanol series, and embedded in Epon medium. Embedded samples were sectioned to about 80 nm in thickness with a ultramicrotome. The sections were stained with uranil acetate and lead citrate to observe under a JOEL 100X electron microscope. Thick sections of the Epon-embedded samples (0.5-2 μm in thickness) were also prepared to monitor infection sites by staining them with 1% toluidine blue and to test diagnostic efficacy.

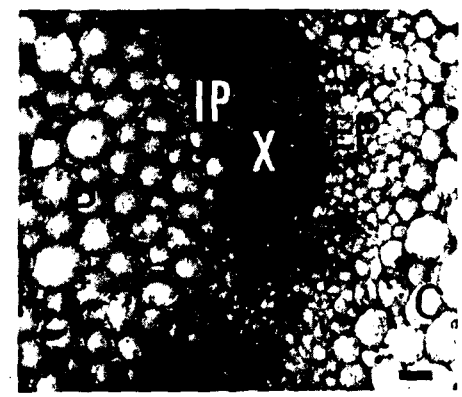
RESULTS

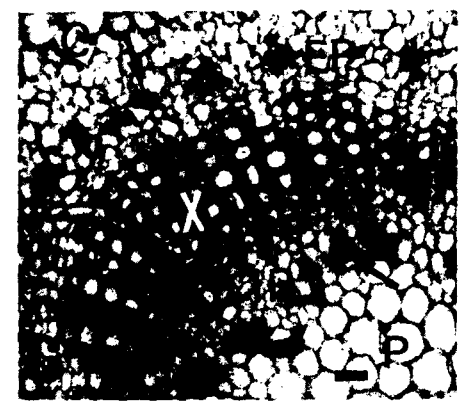
Transmission and Symptomatology. Symptoms appeared on *Catharanthus roseus* (periwinkle), *Trifolium* species (white, red, and Ladino clovers), and *Astragalus sinicus* (Chinese milk vetch) on which the

Table 1. Transmission of mulberry dwarf mycoplasma to herbaceous plants by viruliferous *Hishimonus sellatus*.

Plant species	No. plants inoculated	No. symptom-expressed plants	Incubation period (days)
Catharanthus roseus	5	5	25-30
Trifolium pratense	3	3	35-40
Trifolium repens 'Ladino'	3	3	35-40
Trifolium repens 'White'	3	2	35-40
Astragalus sinicus	3	3	35-40
Apium graveolens	3	0	–
Trigonotis peduncularis	3	0	–
Solanum nigrum	3	0	–
Phryma leptostachya	3	0	–
Portulaca grandiflora	3	0	–

Figs. 1-3. Symptoms on herbaceous plants infected with mulberry dwarf mycoplasma (MDM) : (1) Periwinkle with leaf chlorosis and vein clearing 25 days after inoculation. Arrow: necrosis of flowers. (2) Red clover with leaf discoloration and plant stunting (arrow).(3)Chinese milk vetch with proliferated and stunted shoots (arrow) 35 days after inoculation.





Figs. 4-6. Light micrographs of periwinkle: (4) Toluidine blue stain of an infected midrib section with granular appearances (arrow) in sieve tubes, indicating the existence of MDM bodies. Healthy (5) and diseased stem section (6) with Dienes' staining showing no staining reaction (5) and dark stained regions (arrows) in phloems (6), respectively. Note internal (IP) and external phloem (EP). C; cortex, P; pith, X; xylem. Bars; 25 μm.

leafhoppers survived at least 25 days after inoculation feeding and the 2nd generation nymphs were observed. All of the plants mentioned above showed characteristic symptoms (Tab. 1). The incubation periods were 25-30 days in periwinkle and 35-40 days in other hosts, respectively.

The common symptoms of supposedly infected

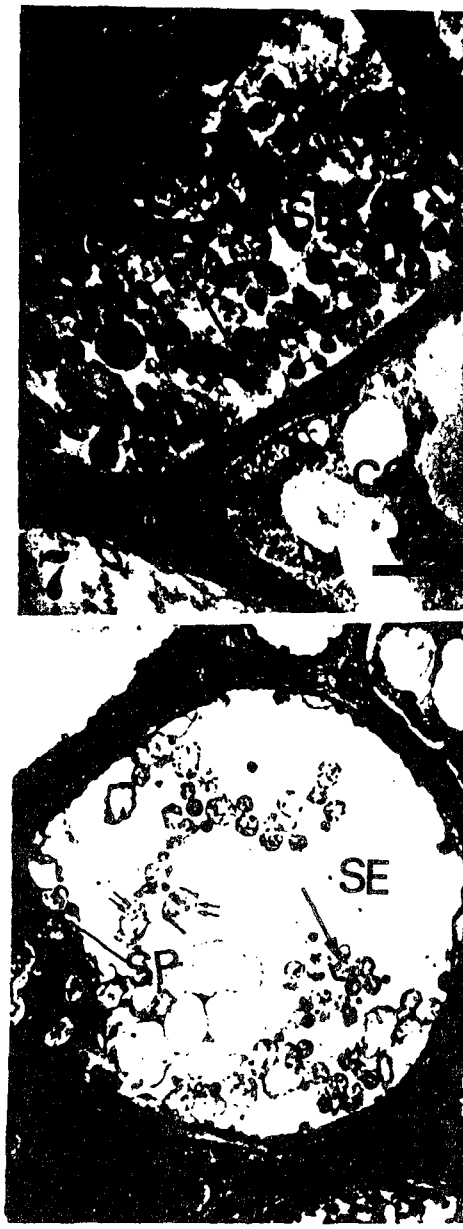
plants were characterized by poor plant growths, usually followed by severe stunting of upper plant parts (Figs 2-3). Discoloration occurred in most of the symptom-expressed plants with variable colors depending on plant species. On periwinkle, chlorosis with vein clearing symptoms appeared on leaves 25-30 days after exposure to viruliferous insects, followed by discoloration and necrosis of flowers (Fig. 1). The vein clearing symptoms firstly appeared from upper plant parts and advanced downward with time until wilt and death of a whole plant.

In clovers and Chinese milk vetch, poor growths were noticed, accompanying discloration of leaves (brownish in white and Ladino clovers, reddish in red clover, and yellowish in Chinese milk vetch) and plant stunting (Figs 2 & 3). Later, plants became withered, leaving clusters of stunted shoots.

Light and Electron Microscopic Detection of MDM. Stem sections of the plants with characteristic symptoms as well as diseased mulberry showed positively stained areas in phloems, while healthy plants had no particular staining reaction by Dienes' stain solution (Figs 5 & 6). Especially the diseased periwinkle, which have both internal and external phloems in the stem, had well defined stained regions inside and outside xylem vessels (Fig. 6). Lignified cell wall such as those of xylem vessels and phloem fibers also showed distinct staining reactions by Dienes' stain, but differed in color from infected phloem cells. Infected phloem cells stained true blue, whereas xylem vessels and phloem fibers colored turquoise and light blue, respectively.

The infected phloem areas were also differently stained with toluidine blue from other plant cells, when Epon-embedded sections were used for the light microscopy. Infected phloem cells stained dark blue, while primary and secondary cell walls appeared purplish blue tints. Some sieve tubes in these phloems showed an indication of mycoplasmal existence in the cells; i.e., granular appearances which were confirmed to be MLO (mycoplasma-like organism) particles under the electron microscope (Figs. 4 & 7).

Mycoplasma-like organisms were located in sieve tubes or differentiating sieve tubes of infected plants (Fig. 7). MLO particles had membranes with electron dense materials spread inside the cells (Fig. 7). Surrounding tissues appeared to be damaged by mycoplasmal infection and in some sieve tubes, degenerated MLO-looking particles were noted (Fig. 8).



Figs. 7-8. Electron micrographs of periwinkle (7) and red clover (8) showing micoplasma-like organisms (arrows) in sieve tubes (SE). Note degenerated MLO-looking particles (double arrow in (8)). CC; companion cell, SP; sieve plate. Bars; 0.5 μm (7) and 2 μm (8).

DISCUSSION

In this experiment, transmission occurred from

dwarf diseased mulberry to 5 herbaceous plant species through the insect vector, *H. sellatus*, as confirmed by macro- and microscopic evidences. Comparing the times of a noticeable symptom expression among plants, incubation period differed among the infected host species, shorter in periwinkle (25-30 days) than the others (35-40 days). Also specific symptoms were variable depending on host species, all of which appeared to be associated with poor plant growths. Symptoms on clovers and periwinkle infected with MDM were different from those infected with aster yellows and sandal spike disease, respectively (3,5). The symptoms of diseased periwinkle plants were similar to those described in jujube witches' broom which shares the same vector, *H. sellatus* (9). These suggest that the classification of MLO may be possible by symptomatology.

Dienes' stain seems to be a simple and quick method and has a diagnostic value for the light microscopic detection of MDM, because all the plants infected with MDM showed specific staining reaction in phloems as in other mycoplasma diseases reported by Deeley et al. (2). As revealed by toluidine blue stain of Epon-embedded materials, these stained areas were composed of MLO containing sieve tubes and parenchymal phoem cells affected by MDM infection. In a healthy periwinkle, some phloem cells colored light blue tints (Fig. 5), but not thickly stained as in the diseased plants. This suggests that the diseased cytoplasmic contents might have stronger stainability to Dienes' stain than healthy cytoplasm, improving specific staining reactions.

Symptoms were not noted on decline-infected pears of which mycoplasma bodies were found only sparsely in sieve tubes (6). Also in our experiment, it was not possible to obtain significant staining reactions in phloems before symptoms appeared on the inoculated herbaceous hosts. These suggest that the light microscopic detection of MLOs may not be possible until the disease has been advanced enough to disturb plant metabolisms or plug sieve tubes with mycoplasmas, so that apparent external symptoms can be expressed.

It was not always possible to observe MLO bodies in the infected plants under the electron microscope. The occasional failure in detecting MLOs in ultra-thin sections may be due to uneven distribution of mycoplasma bodies in the sieve tubes or degeneration of the organisms. In this respect, toluidine blue staining of thick sections of embedded materials are convenient to locate infection sites before electron microscopic observation.

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