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Use of Dienes' Stain in Diagnosis of Plant Mycoplasmal Diseases and Modification of Diagnostic Procedure

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Dienes 染色法을 利用한 마이코플라스마性 植物病의 診斷과 몇가지 染色方法의 改善

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ABSTRACT : Mulberry dwarf, paulownia witches' broom, jujube witches' broom, and sumach witches' broom are known to be associated with mycoplasmalike organisms(MLO) in Korea. Simple microscopic detection of MLO infection in these plants was attempted. Periwinkle plant was also tested. Application of 0.2% and 0.4% solution of Dienes' stain gave diagnostic value for MLO-induced diseases of periwinkle and mulberry. Among the various plant parts examined, young herbaceous stem just below the apical part gave the best result. Density of staining reaction was proportional to disease severity. Longitudial sections were superior to transverse sections in confirming MLO infection by staining. Light source without blue filter was useful for increasing the color contrast between sieve tube and xylem vessel and for eliminating misinterpretation. Paulownia, jujube, and sumach samples gave no clear difference in staining reaction between healthy and diseased sections even when various modifications of Dienes' staining procedure were tried.

INTRODUCTION

The first report on the association of mycoplasmalike organisms(MLO) with the plant diseases was made with employing electron microscopy[3]. Since then electron microscopic observation has contributed to reveal the association of MLOs with more than 200 diseased plant species[6]. Though electron microscopy is still the surest method for demonstrating MLO infection of suspected host plants, it offers some disadvantages in the sense that; (a) only a very small fragment of the material can be observed, (b) laborious and skilled techniques are required for the preparation of test material, and (c) expensive facilities and reagents are indispensable[1,2,5,8].

To overcome the above limitations in electron microscopy and to make rapid and simple detection of mycoplasmal etiology in suspected host plants, many attempts have been made with employing light and fluorescent microscopy[4,6,7,8,9].

The latest trial of such attempts employing bright field microscopy is the use of Dienes' stain which was originally used for colony staining in animal mycoplasma culture[2]. The purposes of the present study

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are to investigate the applicability of Dienes' staining method for the diagnosis of several MLO-incited plant diseases in Korea and to improve the technical procedures for better results.

MATERIALS AND METHODS

Plants Tested

Four species of woody plants naturally infected with MLOs were used for this study. They were mulberry (*Morus alba* L.), jujube (*Zizyphus jujuba* Mill.), paulownia (*Paulownia tomentosa* (Thunb.) Steud.), and sumach (*Rhus chinensis* Mill.). Diseased samples of natural hosts were taken from the field for each immediate use. Periwinkles (*Catharanthus roseus*) were artificially inoculated with mulberry dwarf mycoplasma using *Hishimonus sellatus* Uhler collected from the severely infected mulberry field, and also used for this study.

Plants showing mild to severe symptoms were selected and compared. Also, from a single plant, various stage of symptom development were examined and compared. Healthy samples of sumach were collected from mountains near Suweon. Healthy plants of the rest were pot-grown from seeds in insect-proof greenhouse.

Staining Method

Dienes' stain was prepared by dissolving 2.5g of methylene blue, 1.25g of azure II, 10.0g of maltose, and 0.25g of sodium carbonate in 100 ml distilled water. The stain was filtered through Whatman No. 1 filter paper and a series of dilutions (0.1-0.5 %, v/v) was made in distilled water. Preparation of sections and staining procedure suggested by Deeley et al.[2] were employed.

Modification of Dienes' Staining Procedure

To improve the Dienes' staining procedure used by Deeley et al., various modifications were attempted. Transverse and longitudinal sections with varying thickness (ca. 30-200 μm) were prepared. The staining and washing time were respectively applied from 5 to 30 min with various concentration of stain. Every microscopic observation was performed with or without light filter.

Two centimeter-long petioles and young herbaceous stems of infected and healthy mulberry were maintained respectively in 0.2% glutaraldehyde solution and the following 10 kinds of coagulant fixatives to examine how the fixation effects. They were ethanol (70 %), FAA, Craf III, Elaydes', Bouin's, Carnoy's, Farmer's, modified Keefe's, McWhorter & Weier's, and Zirkle's fluid. The fixed materials were sectioned and compared with fresh materials everyday from the next day after fixation.

Length Measurement of Sieve Element

Both infected and healthy samples of mulberry and periwinkle were free-hand sectioned in longitudinal direction and the length of sieve elements was measured.

RESULTS

General Observation of Staining Effect

Of the five MLO-infected plant species treated with Dienes' stain, sections of periwinkle and mulberry showed distinct difference in staining reaction between healthy and diseased samples, which led to useful diagnostic value. Jujube, paulownia, and sumach, however, showed little reliable difference for diagnosis, and some modified methods were also in vain to give distinct

difference between healthy and diseased sections.

In all sections of periwinkle and mulberry treated with 0.1-0.5% dilutions of Dienes' stain, the xylem vessel with secondary wall was colored turquoise blue. Cells in the cortex were stained pale blue or nearly discolorized and collenchyma cells usually remained pale purplish blue. The sieve tubes from healthy sections remained unstained (Fig. 1), while those of infected sections contained many regularly distributed areas that stained distinct dark blue (Fig. 2). These areas could be resolved under high magnification as groups of sieve elements with blue-stained contents.

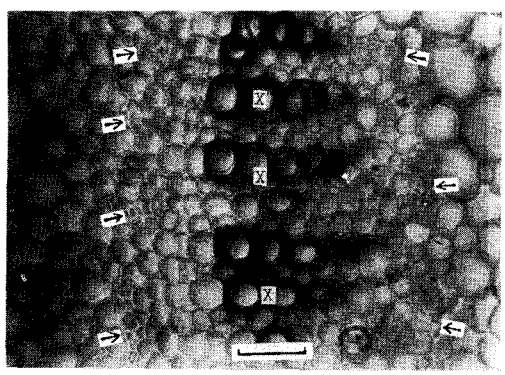

Fig. 1. The sieve tubes(arrows) of healthy periwinkle plant remain unstained, while xylem(x) vessel are colored turquoise blue. (bar = 50 μm)

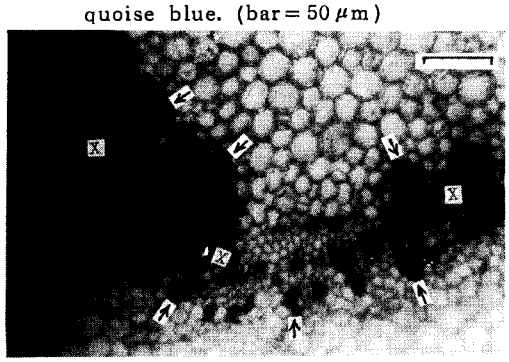

Fig. 2. Both internal and external sieve tubes(arrows) of infected periwinkle plant are stained distinct dark blue. Xylem vessels(X) are colored turquoise blue.(bar = 100 μ m).

Sieve tubes of both healthy and infected paulownia sections remained unstained when various dilutions of Dienes' stain were applied. In case of jujube, sieve tubes from severely infected sample were stained dark blue on rare occasions, and those of healthy sample sections remained unstained. In both jujube and paulownia, the cells other than sieve tubes stained as those of periwinkle and mulberry did. In case of sumach, however, the staining reaction of both infected and healthy samples was unsteady in all parts of cells including xylem vessel, and no color differentiation was observed among tissues subjected to various dilutions of Dienes' stain.

Detailed Observation in Periwinkle and Mulberry

Among various parts of diseased plant, the best staining effect to detect MLO infection was obtained with young herbaceous stem just below the apical part. Comparable result was attained from petiole and midvein of the infected leaf. Density of staining reaction was proportional to disease severity. In periwinkle, half part of a leaf usually showed more severe symptom than the other half part. [Sections of petiole from such a leaf were. usually more densely stained in the half part of complete ring of phloem.] In some cases, sections of periwinkle and mulberry at the early stage of symptom development remained unstained in sieve tubes.

Appropriate Combination of Procedure

Application of 0.2 and 0.4% solutions of Dienes' stain in periwinkle and mulberry respectively was most useful and made it possible to distinguish between infected and healthy tissue. Thick sections of infected sample readily prevented confirming strict sieve tube-stained reaction. Moreover, thick

sections of healthy sample usually induced misinterpretation. Thin sections less than 100 μm were suitable for clear observation and photographing. Though appropriate staining and washing time were variable to the thickness of section and concentration of stain, satisfactory results were obtained with staining for 5-10 min and washing for 10-20 min.

Sectioning Direction and Staining Reaction

Longitudinal sections were superior to transverse sections in staining reaction. In longitudinal sections of diseased periwinkle treated with 0.2% Dienes' stain, distinct blue-stained internal and external sieve tubes were apparent at each side of bright turquoise blue-stained xylem vessels(Fig. 3). The apparent difference in staining pattern between infected and healthy periwinkle was enough to be seen with hand-lens. In longitudinal sections of infected and healthy mulberry, comparable result was attained.

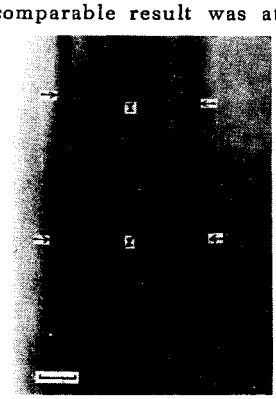

Fig. 3. In longitudinal section of mulberry dwarf mycoplasma infected periwinkle plant, the distinct blue-stained internal and external sieve tubes(arrows) are apparent at each side of bright turquoise blue-stained xylem vessels(X) (bar = 50 μm)

Light Effect

In both transverse and longitudinal sections of infected periwinkle and mulberry sample, better color contrast was obtained when light source without blue filter was used. In particular, longitudinal sections were useful in detecting the MLO infection with mild symptoms which are not easily detectable in transverse sections. Infected sieve tubes were colored purple, while healthy sieve tubes showed pale redish yellow. Cortex cells of both infected and healthy sections showed pale redish yellow. Xylem vessels of both infected and healthy sections were distinctly blue.

Effect of Fixatives

Stem sections of mulberry immersed respectively in 10 kinds of coagulant fixatives showed no difference between infected and healthy samples in staining reaction. Fixation in glutaraldehyde at room temperature decreased the density of staining reaction and prolonged fixation made cutting and staining difficult by making the tissue too soft. Materials fixed in glutaraldyhyde and stocked in a refrigerator, however, maintained the stainability of infected sections even after several monthes, though the staining reaction was not so clear as that of fresh materials.

Length of Sieve Element

In longitudinal free-hand sections, the sieve elements were readily observed under high magnification. The average length of sieve elements in periwinkle and mulberry was about 150 and 200 μm respectively. There was no distinct difference in the length of sieve elements between infected and healthy samples.

DISCUSSION

Deeley et al.(2) first applied Dienes' stain as a vital stain to detect MLO — incited

plant diseases, including spiroplasma etiology, with success. She and her colleagues confirmed the diagnostic value of Dienes' stain in all of the nine different plants species infected with MLOs or spiroplasmas. Among five plant species examined in this experiment, however, periwinkle and mulberry were found suitable for diagnosis of MLO etiology by Dienes' staining method. The negative results in the rest three plant species was possibly due to some substances, such as tainin, in the plant tissue, which make staining difficult.

Deeley et al. reported transverse sections were more useful than longitudinal ones (2). In this experiment, however, better staining reaction was obtained with employing longitudinal instead of transverse section. For sieve elements are positioned in longitudinal direction with average length of 150 μm in periwinkle and 200 μm in mulberry, routine transverse section would injure most of sieve elements, and MLOs within sieve element may be subject to flow out during staining and washing of sections, resulting in unstained sieve elements. Moreover, the high hydraulic pressure within the sieve tube reaching up some 20 atm (10). would asselerate leakage of MLOs because injury would cause a drop of turgor in the sieve tube and thus entry of water, which is also thought to be responsible for unstained sieve elements in transverse sections. On the other hand, in longitudinal sections, sieve elements are less injured and MLOs are well contained in sieve tubes.

Fixation was generally undesirable for Dienes' staining. And prolonged soaking of materials in water usually induced nonspecific staining in phloem. For practical use, polyvinyl packing of the materials with wet paper towel or glutaraldehyde fixation at 4 C seems to be feasible when immediate test is not available.

摘　　要

Dienes染色法을 國內에 發生하는 마이코플라스마性 植物病의 診斷에 適用해 보면서 本 染色法의 보다 効果的인 利用을 爲한 實驗에서 얻어진 結果를 要約하면 다음과 같다.

1. Dienes染色法을 通한 植物 마이코플라스마病의 診斷方法은 日日草와 뽕나무에서만 肯定的이었고, 대추나무, 오동나무 및 붉나무에서는 否定的이었다.

2. Dienes染色液의 0.2%와 0.4% 水溶液이 日日草와 뽕나무에 各各 適合했으며, 染色時間은 5～10分 그리고 脫色時間은 10～20分이 좋았다.

3. 正確한 顯微鏡的 觀察이나 鮮明한 寫眞을 위해서 切片의 두께는 50～100μm가 適當하였다.

4. 供試植物의 各 部位中에서 줄기 頂部 바로 밑의 部位가 좋은 結果를 나타냈으며, 葉柄이나 中肋도 거의 同等한 結果를 나타냈다.

5. 罹病植物에서 染色反應의 程度는 病勢와 比例했으며, 病徵發見 初期의 試料는 健全試料와 染色反應에 있어서 쉽게 區別되지 않았다.

6. 試料를 縱切할 경우 대부분의 管要素들이 傷處를 받지 않을뿐만 아니라 正確한 觀察이 容易하므로 試料를 橫切할 경우보다 效果的이었다.

7. 染色된 試料를 顯微鏡 觀察할 경우 filter를 使用하지 않고 텅스텐 赤色光을 그대로 使用하면 篩管要素와 導管要素 間의 색갈 對比를 增加시키며 正確한 解釋에 도움을 주었다.

8. 試料를 固定液에 固定시키면 染色反應이 방해되었으며, 試料는 新鮮한 것일수록 染色反應이 뚜렷하였다.

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