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Application of Immunofluorescent Staining and Tissue-blotting Techniques for the Detection of a Mycoplasmalike Organism Associated with Sweetpotato Witches' Broom

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ABSTRACT

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Immunofluorescent staining and tissue blotting techniques were applied in the detection and differentiation of a mycoplasmalike organism (MLO) associated with sweetpotato witches' broom (SPWB) with monoclonal antibodies. SPWB-MLO was differentiated from MLOs associated with paulownia, loofah, and *Ipomoea obscura* witches' brooms, aster and ash yellows, and rice yellow dwarf in both serological tests. SPWB-MLO was also proved to be serologically related to peanut and asparagus bean witches' broom MLOs, and MLOs associated with witches' broomed weeds, *Rhynchosia minima* and *Alysicarpus vaginalis.* Using both techniques, SPWB-MLO was readily detected in tissue sections of roots, stems, and leaf midribs prepared from diseased sweetpotato and periwinkle, and also in sections of the fleshy receptacle and the basal part of the ovary prepared from diseased periwinkle.

Key words: monoclonal antibody, immunodiagnostics, tissue-blotting techniques.

INTRODUCTION

In Taiwan, sweetpotato witches' broom (SPWB), a disease associated with a nonculturable mycoplasmalike organism, was first discovered in a geographically isolated area, the Penghu Islands, in 1969 (16). The disease occurs annually because of climate, cultivation practices, and rather simplicity of domestic plants on the Islands. SPWB disease has now been recorded in many areas and countries besides Taiwan (14).

Like other phytopathogenic MLOs, SPWB-MLO remains unculturable. Detection of SPWB-MLO is always tedious and difficult. Host contaminants present in the MLO immunogens and DNA from host organelles coexists with MLO DNA take the biggest responsibility for the frustrations in the generation of specific antibodies and DNA probes.

Recently, the hybridoma techniques and molecular cloning techniques have been applied in our lab to generate specific monoclonal antibodies (14) and cloned DNA probes (9) for SPWB-MLO. Monoclonal antibodies and cloned DNA probes are now concurrently applied in the detection of SPWB-MLO for the ecological studies routinely and will be applied in the screening of SPWB-MLO-free propagating tissues (9).

In this paper, we describe the application of monoclonal antibodies for SPWB-MLO in the detection of SPWB-MLO resided in various parts of tissues with immunofluorescent staining and tissue blotting techniques. Both techniques were proved to be very effective and convenient approaches for the detection and differentiation of SPWB-MLO.

MATERIALS AND METHODS

Diseased plants

Diseased plants of sweetpotato, *Ipomoea obscura* (L.) Lam., loofah, paulownia, peanut, and asparagus bean witches' brooms, aster (New Jersey strain) and elm yellows, rice yellow dwarf, and witches' broom diseases of *Rhynchosia minima* (L.) DC, and *Alysicarpus vaginalis* (L.) DC tested in this study were originally obtained as previously described (14). Among

these, tissue sections from diseased plants of rice yellow dwarf and peanut, asparagus bean, *R. minima*, and *A. vaginalis* witches' brooms were prepared from original hosts; all the others were prepared from infected periwinkle. Freehand sections of leaf midribs, stems, roots, fleshy receptacles or ovaries were prepared for the immunofluorescent staining and tissue blotting from healthy and MLO-infected plants mentioned above.

Monoclonal antibodies

Five stable hybridoma clones, Mas 6, 16, 21, 35, and 40 produced in the previous study (14) secreting specific monoclonal antibodies against MLO associated with sweetpotato witches' broom were used in this study. Hybridoma cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco Lab., Grand Island, NY). Monoclonal antibodies were harvested from culture supernatants when cell titers reached 1×10^7 cells per milliliter, and used for immunofluorescent staining and tissue blotting.

Immunofluorescent staining

Cross sections of various tissues were prepared from healthy and MLO infected plants described in diseased plants by freehand sectioning. Indirect immunofluorescent staining was carried out by following the procedure described previously (14). In brief, sections were fixed in cold acetone, incubated in undiluted hybridoma culture supernatants, and then incubated in fluorescein isothiocyanate (FITC)-conjugated antimouse IgG + IgA + IgM (Caltag Laboratories, San Francisco, CA). Stained samples were mounted on microslide with glycerol-phosphate-buffered saline (glycerol-PBS, 9:1, V/V) and examined with an Olympus epifluorescence microscope as described before (14). Photomicrographs were made with a 35-mm Olympus automatic photomicrographic system (PM-10ADS) with ASA400 film (Kodak Tri-X Pan, or Ektachrome).

Immunological detection by tissue blotting method

Cross or longitudinal sections of different tissues were prepared from healthy and MLO-infected plants described above by freehand sectioning as those for immunofluorescent staining. Tissue imprints were obtained by pressing the fresh cut surface onto the nitrocellulose membranes (0.45 μm pore size, Schleicher and Schuell, Dassel, Germany) for 3 sec. The blots were then treated with the similar immunological method described before (13,15) for the detection of SPWB-MLO antigens. Membranes were first immersed in PBS containing 2% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were then incubated with undiluted hybridoma culture supernatant at room temperature for 2 h. After being washed with three changes of PBS containing 0.05% (V/V) Tween 20 at 15-min intervals, membranes were incubated with alkaline phosphatase-conjugated goat antimouse IgG + IgM (Jackson Immuno Research Laboratories, Inc., West Grove, PA) diluted 10,000-fold in PBS for 2 h at room temperature. Membranes were then washed three times as before and visualized by incubating the membranes in the dark in a solution containing colorimetric substrate, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Upon development, blots were examined directly for imprints of root sections of sweetpotato or using a stereo microscope for those of other sections.

RESULTS

In the immunofluorescent staining and tissue-blotting analyses, SPWB-MLO-specific monoclonal antibodies only recognized tissue sections prepared from plants infected with MLOs that caused witches' broom diseases of sweetpotato, peanut, asparagus bean, *R. minima*, and *A. vaginalis* collected from the Penghu Islands. The antibodies did not react with sections prepared from plants infected with MLOs that caused *I. obscura*, loofah, and paulownia witches' broom, aster and elm yellows, and rice yellow dwarf, same as those results obtained from indirect enzyme-linked immunosorbent assay (ELISA) (14).

In the immunofluorescent staining, all five monoclonal antibodies reacted positively to the sections of midribs, stems and roots of infected periwinkle, sweetpotato, and all the other host plants that cross reacted to SPWB-MLO monoclonal antibodies mentioned above and showed FITC-specific fluorescence in phloem elements. Representative micrographs showing the results of immunofluorescence tests on the cross sections of roots of SPWB-MLO-affected sweetpotato, periwinkle and of peanut witches' broom (PNWB) MLO-infected peanut were shown in Figure 1. Micrographs revealed the FITC-stained cross sections of fleshy receptacle and basal part of the ovary of SPWB-MLO-affected periwinkle were shown in Figure 2. Micrograph showing the positive FITC-staining reaction on the cross section of stem from witches' broomed *A. vaginalis* was shown in Figure 3.

In tissue-blotting tests, all five monoclonal antibody reacted positively to the sections prepared from the same tissues as described above that reacted positively in immunofluorescence tests. Representative photo and dissecting micrograph showing the bluish or blackish purple color reaction on the blots were shown in Figure 4. There was no color development in tissue blots printed by uninfected healthy plants and plants affected with MLOs that did not react with the monoclonal antibodies described above.

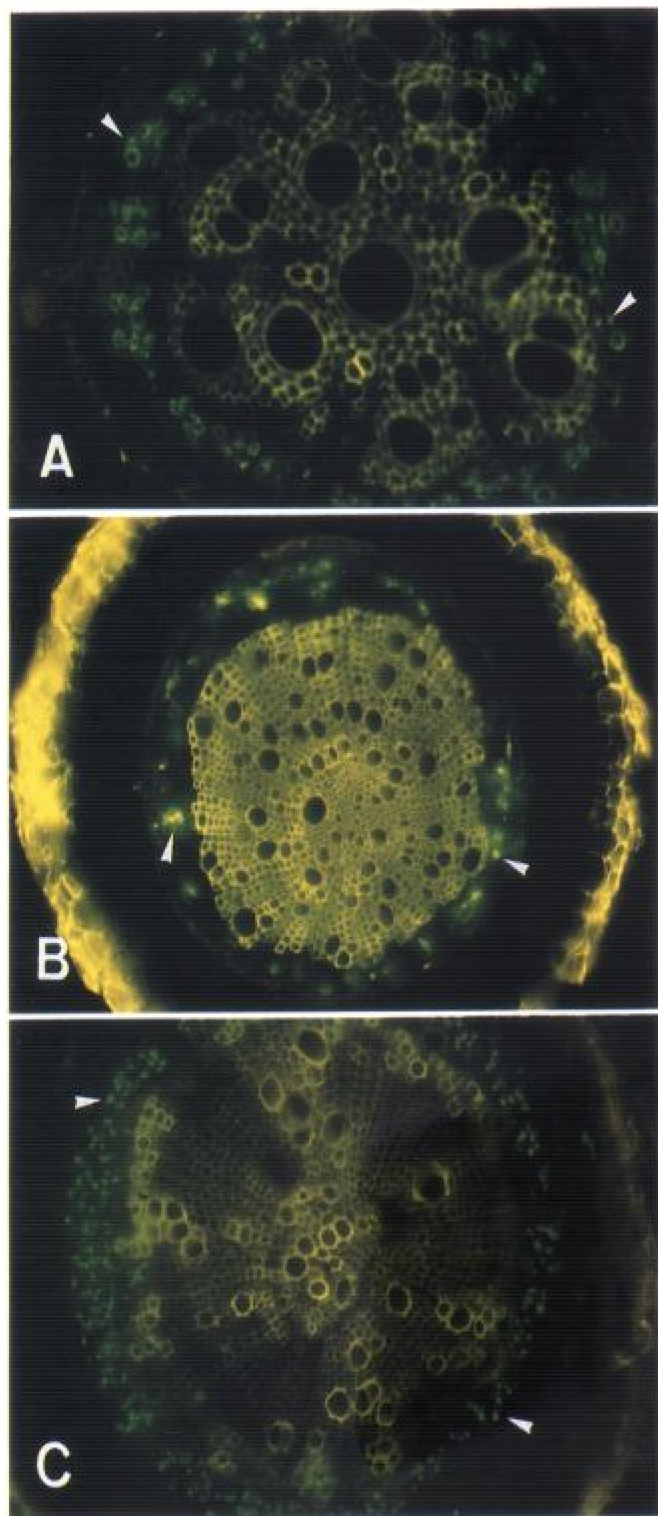

Fig. 1. Immunofluorescent staining on cross sections of roots from sweetpotato witches' broom mycoplasmalike organism (SPWB-MLO)-infected sweetpotato (A), SPWB-MLO-infected periwinkle (B), and peanut witches' broom mycoplasmalike organism (PNWB-MLO) -infected peanut (C) reacted with monoclonal antibody for SPWB-MLO from undiluted culture supernatant of hybridoma MA40. Fluorescent micrographs show the greenish yellow autofluorescence in cuticle and xylem areas, and the fluorescein isothiocyanate-specific apple-green fluorescence (arrows) in phloem tissues. (× 100).

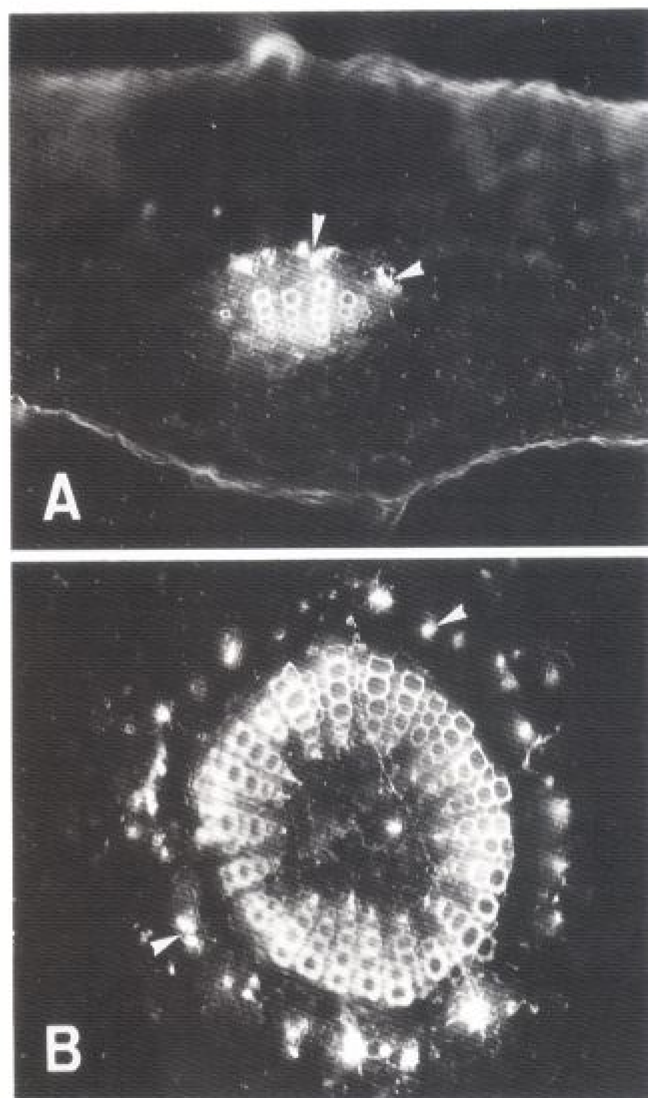

Fig. 2. Immunofluorescent staining on cross sections of the fleshy receptacle from sweetpotato witches' broom mycoplasmalike (SPWB-MLO)-infected periwinkle (A), and the basal part of the ovary from SPWB-MLO-infected periwinkle (B) reacted with monoclonal antibody for SPWB-MLO from undiluted culture supernatant of hybridoma MA21. Fluorescent micrographs show the greenish yellow autofluorescence in cuticle and xylem areas, and the fluorescein isothiocyanate-specific apple-green fluorescence (arrows) in phloem tissues. (× 100).

DISCUSSION

For the diagnosis of plant diseases caused by MLOs, a sensitive, rapid and specific method for the pathogen detection is essential. Monoclonal antibodies and cloned DNA probes are now proved to be the most effective reagents available for the detection of phytopathogenic MLOs (1-11,14). Approaches including indirect ELISA, immunofluorescent staining and tissue-blotting had been proved to give most promising results

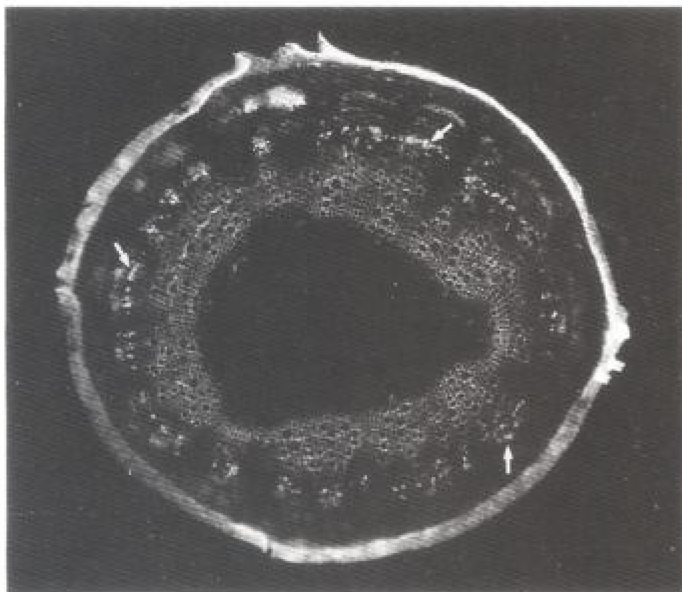

Fig. 3. Immunofluorescent staining on cross section of stem from witches' broomed *Alysicarpus vaginalis* (L.) DC reacted with monoclonal antibody for SPWB-MLO from undiluted culture supernatant of hybridoma MA35. The fluorescent micrograph shows the greenish yellow autofluorescence in cuticle and xylem areas, and the fluorescein isothiocyanate-specific apple-green fluorescence (arrows) in phloem tissues. (× 40).

when monoclonal antibodies were used for the disease detection (3,4,7,11-14). Among these approaches, tissue-blotting techniques used in this study and previous report (9) could be the most efficient and applicable method for the detection of SPWB-MLO in epidemiological studies either with monoclonal antibodies or DNA probes. It allows a large number of sample to be screened simultaneously in a very short period of time and is now adopted for the routine screening of SPWB-MLO-free propagating tissues and for the ecological studies. Recently, we also demonstrated that tissue-blotting techniques incorporated with monoclonal antibodies could be the only effective serological approach for the detection of *Xylella fastidiosa*, the causal agent of marginal scorch of pear in Taiwan, that presented sparsely in the xylem tissues of pear (*unpublished data*).

Polymerase chain reaction (PCR) is another effective and applicable method that can detect the SPWB-MLO at very low cell density when effective oligonucleotide primers are available. Specific oligonucleotide primer pairs for PCR were recently designed for the amplification of specific SPWB-MLO DNA fragment in our lab (9). Both of the serological and genetic approaches, tissue blotting and PCR assays, are now adopted in our lab and applied in the routine screening of the presence of SPWB-MLO either in plants or insect vectors with similar sensitivity.

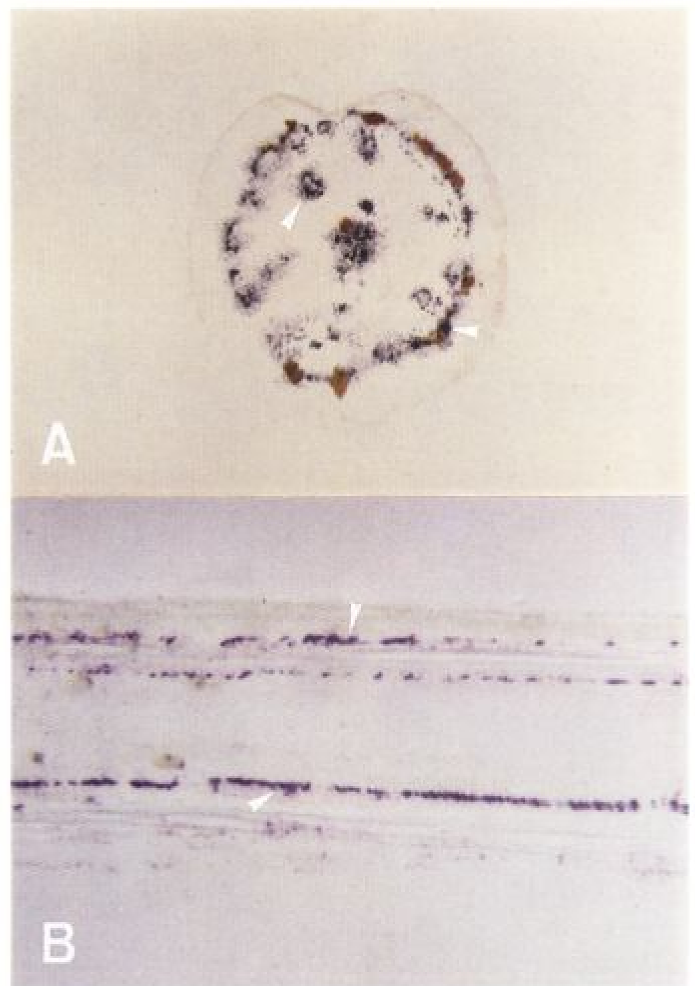

Fig. 4. Immunological detection of sweetpotato witches' broom mycoplasmalike organism (SPWB-MLO), and peanut witches' broom mycoplasmalike organism (PNWB-MLO) in tissue blots of infected root and stem on nitrocellulose membrane. The blots were reacted with monoclonal antibodies for SPWB-MLO from undiluted culture supernatant of hybridoma MA35 showing the bluish or blackish purple color reaction on the blots (arrows). A, cross section of root from SPWB-MLO affected sweetpotato (× 3.4); B, micrograph of longitudinal section of stem from PNWB-MLO affected peanut (× 13.5).

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摘　　要

沈偉强、林長平．1994．利用免疫螢光染色及組織轉印技術偵測甘藷簇葉病病原似菌質體．植病會刊 3:79-83．（台北市　國立臺灣大學植物病蟲害學系）

利用單元抗體將免疫螢光染色及組織轉印技術應用於甘藷簇葉病 (SPWB) 病原似菌質體 (MLO) 之偵測及鑑別上。利用此二種血清技術可將 SPWB-MLO 與引起泡桐、絲瓜、*Ipomoea obscura* 等簇葉病，翠菊、白楊黃化病及水稻黃萎病等病原似菌質體予以鑑別區分。SPWB-MLO 亦被證實與花生、菜豆、小葉括根及山地豆等簇葉病之病原似菌質體有血清相關性。利用此二種血清技術可精確的偵測出在 SPWB 罹病甘藷及日日春之根、莖、及葉部中肋等組織切片中之 SPWB-MLO，此外在罹病日日春之花托及子房基部亦可偵測到 SPWB-MLO。

關鍵詞：單元抗體，血清偵測，組織轉印技術。