Witches' Broom Disease of Lime Trees in Oman: Transmission of a Mycoplasma-like Organism (MLO) to Periwinkle and Citrus and the Production of Monoclonal Antibodies Against the MLO

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ABSTRACT. The mycoplasma-like organism (MLO) associated with Witches' broom disease of lime (WBDL-MLO) in the Sultanate of Oman was transmitted from graft-inoculated lime seedlings to periwinkle by dodder. Transmission from infected periwinkles to lime seedlings was also achieved. Graft transmission from lime to several other citrus species is in progress. So far, Troyer citrange has developed symptoms and the MLO was detected in phloem. Monoclonal antibodies (MA) against the MLO were produced and used in immunofluorescence for the detection of the MLO in periwinkles and citrus. A DNA preparation enriched in WBDL-MLO DNA was also obtained.

Witches' broom disease of lime (WBDL) in the Sultanate of Oman is characterized by the presence of mycoplasma-like organisms in the phloem of affected, small-fruited acid lime trees (2). The disease is presently restricted to the northern coast of the Sultanate of Oman but it is spreading towards the southern part of the northern coastal plain. The most typical symptom of the disease is the appearance of witches' brooms (very small leaves on proliferating shoots) on the canopy of infected lime trees; their number increases with time, leading to tree death within 5 to 6 yr after first symptoms appearance (1, 2). Only small-fruited acid lime trees are grown in the affected area and, consequently, nothing is known about the susceptibility to WBDL of other citrus species. Also, the rapid spread of the disease and its MLO etiology suggest that it may have an insect vector. In this paper we present results on experimental transmissions of the MLO to various citrus species as well as to periwinkle plants. The infected periwinkles have also been used for the production of WBDL-MLO specific monoclonal antibodies and for WBDL-MLO DNA purification.

MATERALS AND METHODS

Graft-transmission of WBDL-MLO. Witches' brooms were collected from affected lime trees in the Sultanate of Oman. and taken to Bordeaux, France. Shoots from these witches' brooms were grafted on lime seedlings under quarantine in the greenhouse. Both shoots grafted on the lime seedlings and new shoots of the lime seedlings developed witches' broom symptoms. Symptomatic shoots from the lime seedlings were side grafted onto three seedlings of each of the following citrus species: Madame Vinous sweet orange, Troyer citrange and sour orange.

Transmission of WBDL-MLO to periwinkle plants by dodder. The transmission was performed as described for transmission of the greening bacterium with the use of dodder (3).

Production of monoclonal antibodies. Monoclonal antibodies (MA) specific for the WBDL-MLO were obtained by immunizing Balb/c mice with homogenates of WBDL-infected periwinkle midribs in the presence of a Balb/c mouse polyclonal serum prepared against homogenates of healthy periwinkle midribs. This procedure was described for the production of MA against phloem-restricted prokaryotes (8). WBDL-MLO specific hybridomas were selected by differential immunofluorescence on sections of healthy and infected periwinkle midribs.

Immunofluorescence. Immunofluorescence (IF) was carried out on

cryo sections (30 μm thick) of periwinkle or citrus midribs. The sections were incubated with MAs (hybridoma supernatant) for 30 min at room temperature (RT), washed 3 times with phosphate buffered saline, pH 7.4, (PBS) containing 0.05% Tween 20 (PBS-Tween) and then incubated with a 100-fold dilution in PBS of goat anti-mouse immunoglobulins labeled with fluorescein isothiocyanate (FITC) (Diagnostic Pasteur) for 30 min at RT. After washing with PBS-Tween, the sections were mounted in 50% glycerol in PBS, pH 7.4, and observed in an epifluorescent microscope Zeiss III RS with the filter combination BP 455-490/FT 510/LP 420.

Nature of the antigens recognized by MAs. The chemical nature of the MLO antigens with epitopes recognized by the MAs was determined by IF as described above except that the sections were submitted to one of the following treatments before addition of the MAs:
1. heating at 100C for 5 min; 2. digestion with proteinase K (0.5 mg/ml) for 90 min at 37C; 3. treatment with 50 mM periodate in PBS for 1 hr at RT in the dark; 4. treatment with 1 M NaCl for 30 min at RT; 5. treatment with β-mercaptoethanol for 1 hr.

Each treatment was followed by extensive washing with PBS.

Purification of WBDL-MLO DNA. DNA was extracted by the cetyltrimethylammonium (CTAB) procedure (9) from healthy or WBDL-infected periwinkle previously lyophilized and ground to a fine powder. Separating the MLO-DNA from plant DNA was performed by the procedure of Kollar *et al.* (5) after addition of a 0.5 mg/ml solution of bisbenzimide (Hoescht) to the DNA solution. The DNA solution was then mixed with cesium chloride and centrifuged for 84 hr at 32,000 rpm in a Beckman Ti60 fixed angle rotor. The DNA bands were visualized under UV light and the upper band containing MLO-enriched DNA was colleted.

DNA analysis. DNA extracted from healthy periwinkles and the CsCl purified MLO-DNA were digested by *Hind*III or *Eco*RI restriction enzymes (Bethesda Research Laboratories) at 37C during 3 hr in the buffer mixtures indicated by the manufacturer. Digested DNA was electrophoresed on 0.7% agarose gels at 50 V for 6 hr in 0.04 M Tris acetate buffer pH 8.0 containing 0.01 M EDTA (7). After Southern blotting of the DNA fragments on nylon membranes (Hybond) (10), they were hybridized for 24 hr at 42C in 5X SSC, 2X Denhardt's solution containing 50% formamide, with a ^{32}P-labeled probe containing the 16S ribosomal DNA gene of *Spiroplasma citri* (1.8 Kpb) (6). The specific activity of the probe was 7 x 10^6 cpm/μg.

RESULTS

Transmission of WBDL-MLO. WBDL-MLO was transmitted from lime to lime by grafting. The graft-inoculated lime seedlings developed symptoms 6 to 12 months after grafting when kept at 32C during the day and 27C at night. Among the three citrus species that were grafted, only one Troyer citrange developed symptoms after 6 months. The tree had very small leaves on shoots with short internodes.

Transmission of the WBDL-MLO to periwinkle plants by dodder was successfully achieved. Infected periwinkles developed very small leaves on highly proliferating shoots which is different from any symptoms previously reported for periwinkle affected by known MLO diseases. MLOs in the symptomatic Troyer citrange and periwinkle plants was confirmed by electron microscopy. As shown on Fig. 1, the phloem of graft inoculated periwinkle plants is packed with MLOs. This was also observed in some sieve tubes of naturally infected lime trees. Transmission of the MLO from periwinkle to lime was also accomplished with dodder. The MLO symptoms on the dodder-inoculated lime seedling were identical to those

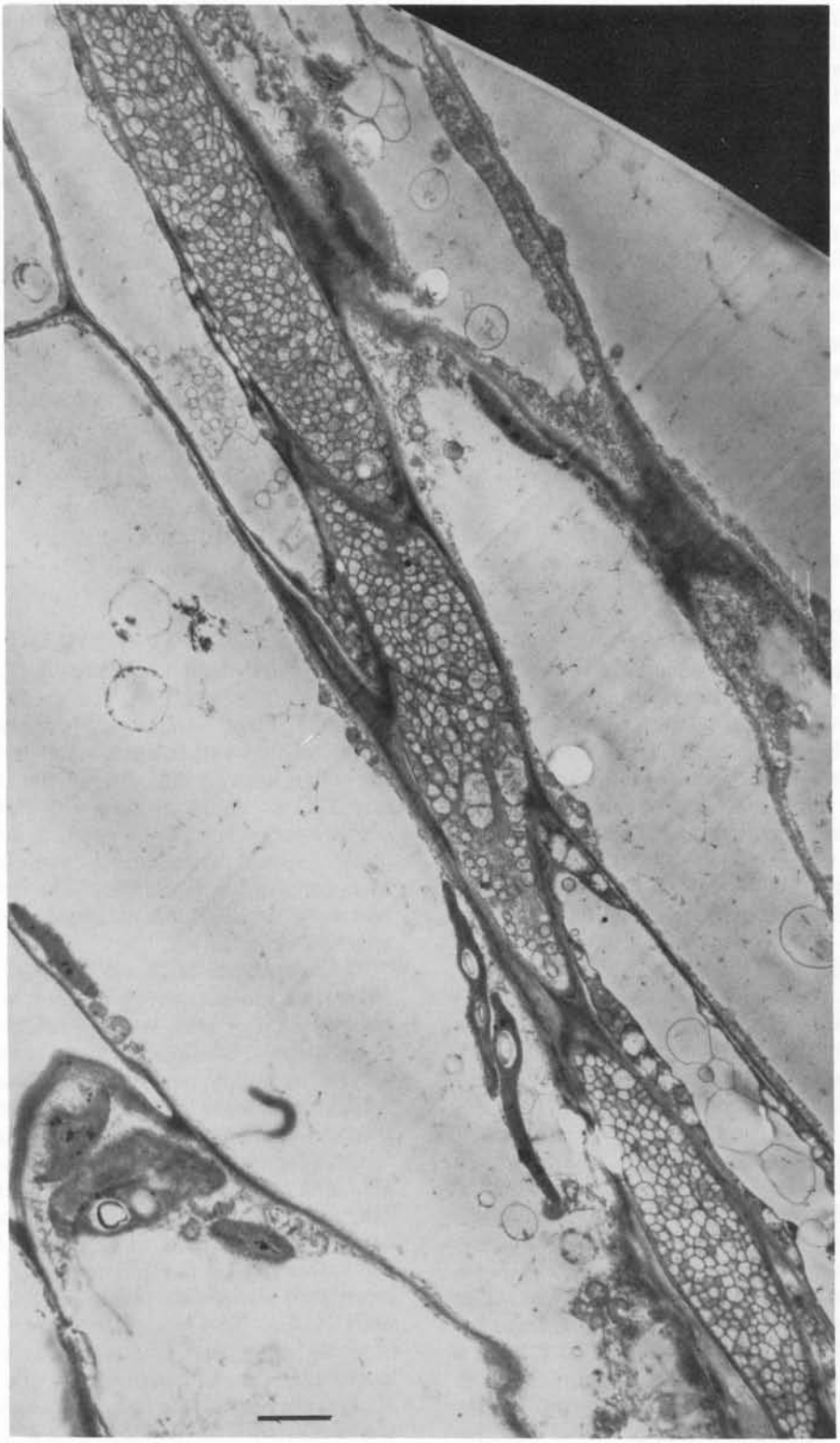

Fig. 1. Electron micrograph of a longitudinal section through a WBDL-MLO infected periwinkle leaf midrib showing phloem tissue packed with MLOs. Bar = 2 μm.

obtained after graft-transmission of infected lime tissue from lime to lime. A detailed description of the symptoms on periwinkle plants, lime seedlings and Troyer citrange is presented elsewhere (4).

Production of WBDL-MLO specific monoclonal antibodies. Two hybridomas, 7D5 and 1D11, secreting monoclonal antibodies specific from WBDL-MLO were selected by differential IF among 856 hybridomas obtained in one fusion experiment. They gave a strong positive green fluorescence in the phloem of infected periwinkles but no fluorescence in that of healthy ones. MA from hybridoma 7D5 was an IgG3 while that of hybridoma 1D11 was an IgM as demonstrated by the use of antibodies to mouse immunoglobulin classes and isotypes.

The specificity of the two MAs for WBDL-MLO was determined by IF on midrib sections of periwinkle plants infected with various phloem-restricted prokaryotes (MLOs, *Spiroplasma kunkelii*, *S. citri*). Table 1 shows that the two MAs reacted only with WBDL-MLO-infected periwinkles. No reactions were obtained with any other MLO-infected periwinkles or with periwinkles infected with *S. citri* or *S. kunkelii*. The chemical nature of the MLO epitopes recognized by the two MAs was investigated by IF after the midrib sections had been submitted to various chemical treatments as shown on Table 2. The two epitopes were proteinaceous as they did not react after a proteinase K treatment. They were also sensitive to high (100C) temperature. However the epitope recognized by 7D5 was sensitive to periodate and 1 M NaCl while that recognized by 1D11 was not. The action of β-mercaptoethanol is also different for each antibody. The epitope of 7D5 was dependent on disulfur bonds while that of 1D11 was not.

Detection of WBDL-MLO with MAs in infected lime and Troyer citrange seedlings. Immunofluorescence with 7D5 and 1D11 MAs on symptomatic lime and Troyer citrange seedlings that were graft-inoculated with WBDL-infected lime

TABLE 1
DETECTION OF THE WITCHES' BROOM DISEASE OF LIME—MYCOPLASMA-LIKE ORGANISM WITH MONOCLONAL ANTIBODIES BY IMMUNOFLUORESCENCE ON MIDRIB SECTIONS

Plant tested	Immunofluorescence on sections with monoclonal antibodies	
	7D5	1D11
Healthy periwinkle plant	–[z]	–
Periwinkle plant infected with the MLO of:		
• WBDL	+[y]	+
• Tomato stolbur	–	–
• Apple proliferation	–	–
• Aster yellows	–	–
• Cabbage chloranty	–	–
• Clover phyllody	–	–
• Hydrangea phyllody	–	–
• Gladolius phyllody	–	–
• Apricot chlorotic leaf roll	–	–
Periwinkle plant infected with		
• *Spiroplasma citri*	–	–
• *Spiroplasma kunkelii*	–	–
Healthy lime seedling	–	–
WBDL-infected lime seedling	+	+
Healthy troyer citrange seedling	–	–
WBDL-MLO-infected troyer citrange seedling	+	+

[z] – = No fluorescence in the phloem
[y] + = Green fluorescence in the phloem

TABLE 2
IMMUNOFLUORESCENCE ON SECTIONS OF THE WITCHES' BROOM DISEASE OF LIME—MYCOPLASMA-LIKE ORGANISM-INFECTED PERIWINKLE PLANTS WITH 7D5 AND 1D11 MONOCLONAL ANTIBODIES AFTER VARIOUS TREATMENTS

Treatments applied to the sections	Immunofluorescence with monoclonal antibodies	
	7D5	1D11
No treatment	–[z]	–
Heating at 100C for 5 min	–	–
Digestion with 0.5 mg/ml proteinase K for 90 min	–	–
Incubation with 50 mM periodate for 60 min	–	+
Incubation with 1 M NaCl for 30 min	–	+
Incubation with β-mercaptoethanol for 60 min	–	+

[z] + = positive immunofluorescence in the phloem; – = no immunofluoresence in the phloem.

tissue and in which MLOs were detected by electron microscopy resulted in a positive reaction in the phloem of the two citrus cultivars with each antibody. No reaction was observed with healthy lime or Troyer citrange seedlings (Table 1). The lime seedling inoculated by dodder with the WBDL-MLO from periwinkle plants also gave a positive IF reaction in the phloem.

Purification and analysis of DNA from healthy and WBDL-infected periwinkle plants. Hybridization patterns of *Eco*RI (lanes 3, 4) and *Hind*III (lanes 1, 2) digests of DNA purified from healthy (lanes 1, 3) and infected (lanes 2, 4) periwinkles with a probe containing the 16S ribosomal gene of *S. citri* (6) are shown in Fig. 2.

Only one broad DNA-band hybridized with the probe in the case of DNA extracted from healthy periwinkles (Fig. 2, lanes 1 and 3) and corresponds probably to chloroplastic rDNA. This indicated that the DNA from the upper band of the CsCl gradient was contaminated by plant DNA as shown by the presence of a band at the same level than that observed in tracks 1 and 3 (DNA of healthy plants). In lanes 2 and 4, corresponding to DNA purified from infected periwinkles, one additional band (Fig. 2, arrow head) was present. It corresponds very likely to MLO-DNA.

Fig. 2. Southern blot of Hind III (1,2) or EcoR I (3,4) cleaved DNA from healthy (1,3) and infected (2,4) periwinkle plants. Hybridization was done with the 16S ribosomal DNA gene of *Spiroplasma citri*.

DISCUSSION

We transmitted the MLO associated with WBDL to lime and Troyer citrange seedlings by grafting. This is the first report that citrus other than lime can be infected by the MLO. However, no conclusions can be drawn concerning the host range of the MLO in nature as only lime trees are grown in the affected area. WBDL-MLO was transmitted to periwinkle plants by dodder in which it multiplies to high titers and allowed us to obtain WBDL-MLO specific monoclonal antibodies and a DNA preparation enriched in MLO-DNA. The study of the chemical nature of the epitopes showed that carbohydrates were associated with the epitope of 7D5 and that the protein carrying the epitope was easily detached from the membrane by high ionic strength and hence was not transmembraneous. On the contrary, the protein recognized by 1D11 was likely to be a transmembraneous protein and no carbohydrates seemed to be involved in the antibody-binding site. These results indicate that the two MAs recognized two different epitopes on the WBDL-MLO. The MAs will help investigate whether insect species such as leafhoppers or psyllids, which are known to transmit MLOs, are natural vectors of the WBDL-MLO. These reagents will also be useful to compare the witches' broom MLO in Oman with the MLO associated with rubbery wood of citrus in India.

Finally, the DNA preparation enriched in MLO-DNA will be used for cloning; recombinant plasmids containing MLO-DNA will be selected and used to produce probes for a highly sensitive detection of the MLO.

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