Witches' Broom Disease of Lime Trees: Monoclonal Antibody and DNA Probes for the Detection of the Associated MLO and the Identification of a Possible Vector

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ABSTRACT. A total of 18 monoclonal antibodies and 17 DNA probes specific for the witches' broom disease of lime (WBDL)-MLO have been produced. MA 1E2 and probes I1H, I10H and I28H were used for the detection of the MLO in plants and/or insects in the Sultanate of Oman and the United Arab Emirates. Among 30 different leafhopper species tested, only one species, *Hishimonus phycitis*, reacted positively both with the MA and the DNA probe (I10H) used. In addition, leafhoppers of this species were captured exclusively on lime trees. This leafhopper is known as the vector of the MLO of eggplant little leaf disease in India. *H. phycitis* is therefore a candidate insect-vector of WBDL-MLO, although, experimental transmission of the disease has not yet been obtained.

Witches' broom disease of lime (WBDL) appeared in the Northern coastal plain of the Sultanate of Oman, near the border with the United Arab Emirates (UAE) probably in the 1970s. Since then, the disease has spread within Oman and extends all along the coastal plain from the border with the UAE to Muscat. Since 1989 it has also appeared in the oases of the coastal mountain range, but it has not yet been observed in the southern part of the country near Salalah. The disease appeared in the UAE in 1988-1989 (10) (1993 data in Note Added in Proof).

In 1986, we showed that a mycoplasmalike organism (MLO) was associated with the disease and that the MLO could be transmitted to periwinkle plants and back to lime by dodder (2, 3). Experimental graft-transmissions of the MLO to lime and Troyer citrange were also successful but not to sweet or sour orange seedlings (11). Recently, transmissions of WBDL-MLO to lemon, rough lemon, and trifoliate orange seedlings have also been obtained (unpublished data).

WBDL-infected periwinkle extracts were used to produce monoclonal antibodies (MAs) specific for the WBDL-MLO. In two lymphocyte fusions, a total of 18 WBDL-MLO-specific MAs were obtained (10, 11). In addition, partial purification of WBDL-MLO-DNA resulted in the production of 17 recombinant plasmids containing an insert of WBDL-MLO-DNA (10). The nature of the causal agent and the rapid spread of the disease strongly suggest that the disease is actively transmitted by an insect vector. In this paper, we report the use of WBDL-specific MAs and DNA probes for the detection of the MLO in plant and insects in the Sultanate of Oman and in the UAE.

MATERIALS AND METHODS

Plant samples. The specificity of MAs and DNA probes used in this work was determined by testing them against a collection of MLOs maintained in periwinkle plants in a greenhouse in Bordeaux as well as against the MLOs of sesame phyllody and sunhemp phyllody (kindly provided by E. Seemuller, Biologische Bundensanstalt, Heidelberg, Germany). Possible cross reactions with three citrus pathogens, *Spiroplasma citri*, the greening BLO and tristeza virus, were also examined.

Plant samples from more than a hundred trees were collected in the Sultanate of Oman and tested by ELISA for the presence of WBDL-MLO as described below. Leaf samples from nine trees were also received from

the UAE and tested by ELISA and DNA-DNA hybridization.

Collection of leafhoppers. Leafhoppers were captured in the Sultanate of Oman during a mission from April 29 to May 13, 1991 with a D-Vac aspirator in lime orchards, fields cultivated with other crops and non-cultivated areas. The insects were separated into species and tested either individually by DNA-DNA hybridization or in batches of five to ten insects by double antibody sandwich ELISA (DAS-ELISA).

DNA-DNA hybridization. Individual leafhoppers were crushed with a glass rod directly onto a Nylon N+ membrane previously soaked into 5 SSC. The DNA was denatured by incubating the membrane for 30 min with 0.4N NaOH. The membrane was air dried, sealed in a plastic bag and carried back to Bordeaux, France for hybridization. Hybridization was performed as described by Garnier et al. (11) with probe I10H labeled with 32Pα dCTP. This probe was selected because it gave the strongest hybridization signals.

DAS-ELISA was done as previously described (12). Five to ten insects (according to their size and availability) were crushed with 300µl of PBS buffer in a glass homogenizer. The liquid phase was collected and added to the wells of an ELISA plate previously coated with MA 1E2 at 10µg/ml. The alkaline phosphatase conjugate of MA 1E2 was used at a 1/1000 fold dilution. Plant extracts were prepared by grinding leaf midribs in two volumes of PBS in a Polytron homogenizer. The homogenate was filtered through four layers of cheese cloth and deposited in the wells of the ELISA plate as before. The ELISA reactions had to be read visually, as no ELISA plate reader was available in Oman.

RESULTS

Specificity of the reagents used to detect the WBDL-MLO. Table 1 shows that MA 1E2 is specific for WBDL-MLO as no reactions were observed with a collection of ten different MLOs tested, or three citrus pathogens. However, dot-blot hybridization between any one of the three probes (I1H, I10H and I28H) tested and the DNAs from 12 different MLOs tested, showed that the MLOs of sunhemp and

TABLE 1
SPECIFICITY OF THE REAGENTS USED TO DETECT THE WBDL-MLO

Tested material	ELISA with MA IE2	DNA-DNA hybridization with probes I1H	I10H	I28H
Lime witches' broom MLO	+	+	+	+
Sunhemp phyllody MLO	–	+	+	+
Sesame phyllody MLO	–	+	+	+
Tomato stolbur MLO	–	–	–	–
Apple proliferation MLO	–	–	–	–
Aster yellows MLO	–	–	–	–
Cabbage chloranty MLO	–	–	–	–
Clover phyllody MLO	–	–	–	–
Gladiolus phyllody MLO	–	–	–	–
Elm proliferation MLO	ND[z]	–	–	–
Apricot chlorotic leafroll MLO	ND	–	–	–
Citrus rubbery wood MLO	–	–	–	–
Eggplant little leaf MLO	ND	–	–	–
Hydrangea phyllody MLO	–	ND	ND	ND
Spiroplasma citri (citrus stubborn)	–	–	–	–
Citrus greening BLO	–	–	–	–
Citrus tristeza virus	–	–	–	–

[z]ND = ND: not determined

sesame phyllody hybridized with the probes, indicating that these MLOs share sequence homologies with WBDL-MLO. However, the southern hybridization patterns were different for the WBDL-MLO and the phyllody MLOs as shown on Fig. 1 for probe I10H. This DNA polymorphism indicates that the WDBL-MLO and the phyllody MLOs are not the same.

Detection of the WBDL-MLO in plants. Strong positive ELISA reactions were obtained with witches' broom leaves of lime trees from all areas tested in the Sultanate: Sohar, Liwa, Al-Murayr and Nizwa, as well as with witches' broom leaves from all areas tested in the UAE (Hatta, Fujairah). Leaves of the following Omani plants, showing MLO-like symptoms were also tested: fasciated sapotilla with flat limbs, alfalfa with leaf yellowing, unidentified plant with witches' broom-like proliferation, and a symptomless sesame shoot. None of these samples gave positive reactions.

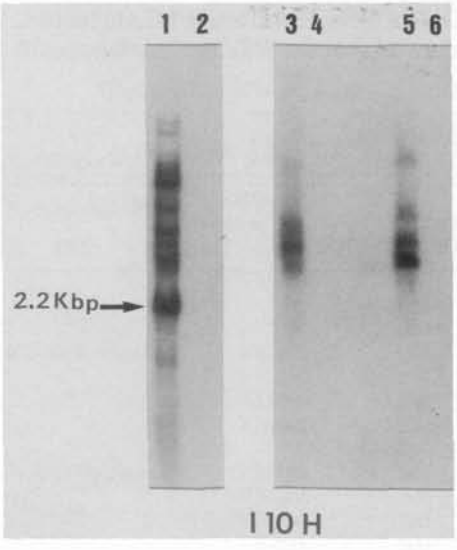

Fig. 1. Southern blot hybridization with probe I10H of *Hind*III-digested DNA extracted from WBDL-MLO-infected periwinkle midribs (track 1), healthy periwinkle midribs (track 2), phyllody-MLO-infected sesame midribs (track 3), healthy sesame midribs (track 4), phyllody-MLO-infected sunhemp midribs (track 5), healthy sunhemp midribs (track 6).

In the UAE, (but not in Oman in 1991), citrus other than lime is also grown to some extent and witches' broom symptoms have been observed on citron, Mediterranean sweet limetta and Indian Palestine sweet lime (9). All were found positive by ELISA and DNA hybridization.

Detection of WBDL-MLO in insects. Tables 2 and 3 list the leafhopper species captured on lime trees and on plants other than lime. One leafhopper species, *Hishimonus phycitis* (Distant 1908) (Fig. 2), was consistently found on lime trees. In a total of 34 hopper-captures on lime trees, more than 580 *H. phycitis* were collected, hence an average of about 20 leafhoppers per capture. One to three trees were sampled per capture. Subsequent captures done immediately on the witches' brooms of the same lime tree yielded appreciable numbers of *H. phycitis*. In addition, *H. phycitis* was more abundant on witches' brooms than on symptomless areas of infected trees or trees without symptoms. Hoppers of a few other species were occasionally captured on lime but only in very low numbers. Table 2 and 3 show that *H. phycitis* was the only hopper tested that gave positive ELISA reactions as well as positive DNA hybridizations (Fig. 3).

DISCUSSION

The use of MAs and DNA probes for detection of the WBDL-MLO has confirmed the widespread distribution of the WBDL-MLO in the Sultanate of Oman and the UAE. In addition to small-fruited acid lime, other natural hosts of the MLO have been identified: citron, sweet limetta and Indian Palestine sweet lime (9). Beside the natural hosts, experimental transmission to lemon, rough lemon, Troyer citrange and trifoliate orange has been achieved.

Because of the rapid spread of the disease, an insect vector was suspected. Among the hoppers captured some species are new to the Arabian Peninsula and are reported here for the

TABLE 2
ELISA REACTION AND DNA HYBRIDIZATION OF LEAFHOPPERS CAPTURED ON WBDL-INFECTED LIME TREES WITH WBDL-MLO SPECIFIC ELISA AND DNA PROBES

Leafhopper species	No. of captured	ELISA[z]	DNA-DNA hybridization[z]
Hishimonus phycitis	>580	30/66	36/180
Agallia sp.	5	0/1	
Austroagallia sinuata	3	0/1	
Bampurius sp. or *Savanicus* sp.	4	0/1	0/3
Batracomorphus chahbaharus	1		
Chloropelix canariensis	1		
Deltocephalinae	5	0/2	
Empoasca sp.	2	0/2	
Goniagnathus guttulinervis	1	0/1	
Laodelphax striatelus	1		
Ommatissus binotatus	3	0/1	
Recilia beieri	2	0/1	

[z]No. of insects reacting positively / no. tested

TABLE 3
ELISA REACTION AND DNA HYBRIDIZATION OF HOPPERS CAPTURED ON PLANTS OTHER THAN LIME

Leafhopper or planthopper species	No. of captured	ELISA[z]	DNA-DNA hybridization[z]
Acacimenus makranus	4	0/1	0/1
Aconurella prolixa	124	0/4	
Akotropis sp.	4	0/3	0/3
Anaceratagallia laevis	42	0/2	
Austroagallia sinuata	38	0/4	
Balclutha rufofasciata	16	0/1	
Bampurius sp. or *Savanicus* sp.	6	0/1	
Chiasmus conspurcatus	1	0/1	
Chloriona sp.	29	0/4	
Chloropelix canariensis	61	0/6	0/17
Cicadulina bipunctata	15	0/3	0/3
Circulifer dubiosus	>136	0/3	
Circulifer opacipennis	> 61	0/1	
Empoasca sp.	43	0/1	0/4
Falcotoya minuscula	6		0/5
Neoaliturus pulcher	1	0/1	
Nisia nervosa	11	0/1	
Opsius richteri	> 50	0/1	0/6
Philotheria group *striata*	2	0/1	
Recilia beieri	61	0/7	0/7
Reptalus lindbergi	11	0/2	0/4
Sempia capreola	30	0/11	

[z]No. of insects reacting positively / no. tested

first time; two are planthoppers: *Falcotoya minuscula, Nisia nervosa*; the others are leafhoppers: *Balclutha rufofasciata, Batracomorphus chahbaharus, Hishimonus phycitis, Opsius richteri, Recilia trifasciata, and Sempia capreola.*

The major result of the work in Oman concerns one of the leafhopper species new to the Arabian Peninsula: *H. phycitis*. *H. phycitis* was the only leafhopper that tested positively in ELISA and DNA-DNA hybridization. Batches of *H. phycitis* found positive

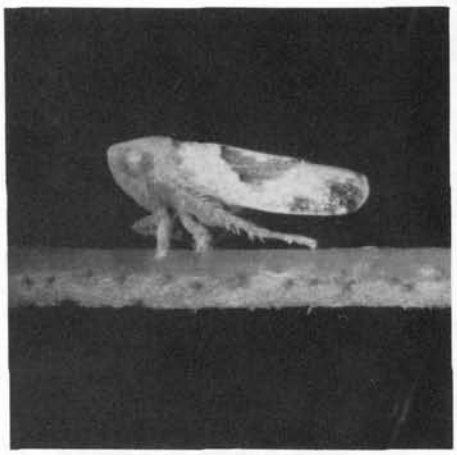

Fig. 2. *Hishimonus phycitis* χ40.

by one technique were also found positive with the second. This leafhopper was the only one consistently and almost exclusively associated with lime. A few *H. phycitis* were captured on sweet lime trees, and only one was found in a capture from two rare sweet orange trees growing near lime trees.

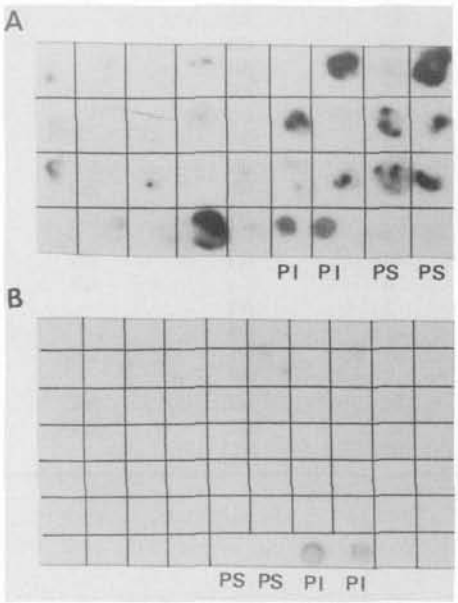

Fig. 3. Crush-blot hybridization of *Hishimonus phycitis* leafhoppers (panel A) or leafhoppers other than *H. phycitis* (panel B) with probe I10H. PS: DNA extracted from healthy periwinkle midribs; PI: DNA extracted from WBDL-MLO-infected periwinkle midribs.

Among the many non-citrus plants sampled, only watermelon and the surrounding weeds yielded two specimens.

The percentage of positive leafhoppers captured from WBDL-affected trees was on average 20%. It cannot be totally excluded that other hopper species might react positively with the WBDL-MLO reagents. In our studies, because of limited availability, only small numbers of hoppers other than *H. phycitis* were tested. Higher numbers of such hoppers must be used to assess their infection with WBDL-MLO.

Interestingly, *H. phycitis* has been long known in India. Acid lime is given as one of the host plants on which the leafhopper is able to live and reproduce (1). In India, it is vector of a severe and widely distributed MLO disease of eggplant: little leaf disease (1). Even though WBDL-MLO-specific MAs and DNA probes do not cross react with the eggplant MLO (Table 1), thus showing that two different MLOs are involved, the fact that *H. phycitis* transmits the eggplant MLO shows that it has MLO-vector capability. More recently, *H. phycitis* was shown to transmit phyllody disease of *Parthemium hysterophorus* (14). Other *Hishimonus* species are also vectors of MLO diseases. *H. sellatus* is the vector of mulberry dwarf (13), jujube witches' broom (15) and *Ligustrum ovalifolium* witches' broom (4), and *H. concavus* is the vector of *Luffa cylindrica* witches' broom (5).

In summary, the following results suggest that *H. phycitis* could be the vector of WBDL-MLO: the leafhopper is consistently found on lime trees, it is the only one reacting with MAs and DNA probes produced against the WBDL-MLO and it is known to be a vector of MLOs.

The way WBDL is spread in Oman and the UAE agrees well with the hypothesis of *H. phycitis* being the vector. Indeed, the spread of the disease within an orchard is very rapid; in one orchard containing 251 trees, there were 7.6% symptomatic trees in 1990 and

41.2% in 1991. From the time the first witches' broom appears in an orchard to the time the entire orchard is dead, only 5 to 6 yr elapse. This suggests tree to tree spread and explains why, in WBDL-affected zones, symptomless orchards could be found across the road from severely affected orchards. But once the disease has reached a hitherto unaffected orchard, spread is very fast. Tree to tree spread would be expected of a vector living and multiplying on the affected trees.

Proof that *H. phycitis* is the WBDL-MLO vector is, however, not yet on hand. So far experimental transmission of the WBDL-MLO with *H. phycitis* leafhoppers raised in the Bordeaux greenhouse and fed on WBDL-affected lime seedlings has not yet been obtained, but such transmissions are difficult (A. Varma, personal communication). Also, there is a remote possibility that the MLO detected by DNA hybridization in the *H. phycitis* leafhoppers captured on WBDL-affected lime trees is not the WBDL-MLO but perhaps the sesame or sunhemp phyllody MLO shown in this paper to cross react with the WBDL-MLO probe. However, as *H. phycitis* is not a vector of the phyllody MLOs in India, and no sesame or sunhemp phyllody has been observed in Oman this is considered unlikely. It must also be pointed out that the positive ELISA reaction given by *H. phycitis* leafhoppers were rather weak. In spite of these observations *H. phycitis* remains the best candidate as a vector for WBDL-MLO.

H. phycitis has not previously been reported from Oman and the Arabian Peninsula. It is not mentioned in the Fauna of Saudi Arabia (6, 7, 8). The leafhopper could have been introduced from India where it is widespread. Movement of citrus plants occurs from India to Oman. In 1987, one of us (J.M.B.) witnessed the introduction of hundreds of lime seedlings affected by citrus tristeza vein-clearing and canker lesions from India to Oman by air. The development of WBDL in recent years in Oman might be related to the introduction of *H. phycitis* into the Sultanate. If so, as no WBDL is known to occur in India, the leafhopper most probably arrived free of the WBDL-MLO and acquired it from some native plant.

ACKNOWLEDGMENT

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Note added in proof: Two of us (J.M.B. and M.G.) visited the UAE in February 1993 on invitation from the UAE authorities. As reported by the UAE workers (9) WBDL could be observed, not only on lime but also on citron, Indian Palestine sweet lime and sweet limetta. *H. phycitis* was captured with a D-Vac aspirator from each WBDL-affected tree sampled. From 1988-1989, when the disease first appeared in the UAE, to February 1993, progress of WBDL has been astonishingly fast. All citrus growing areas are now affected, and many lime orchards are completely destroyed. Thus the situation in the UAE is now similar to that in Oman.

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