Witches' Broom Disease of Lime (WBDL) in Iran

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ABSTRACT. Witches' broom disease of lime (WBDL) is a caused by "*Candidatus* Phytoplasma aurantifolia". The disease was first observed in the Sultanate of Oman in the 1980s and found to be present in the United Arab Emirates (UAE) in 1989. A putative leafhopper vector, *Hishimonus phycitis*, reproducing actively on lime trees, was identified in 1991 in Oman and found to be also present in the UAE in 1993. In July 1997, symptoms of the disease were observed in the southeastern region of Iran near Nikshar (Dapas Kur) and Qasr-e-Qand. Joint serological and molecular characterizations confirmed that the symptoms observed in Iran are those of WBDL and that, on the basis of ELISA and PCR, the WBDL phytoplasma in Iran is indistinguishable from that in Oman and the UAE. *H. phycitis* was easily recovered by D-Vac® aspiration not only from lime trees in the affected region but also from lime trees in regions free of the disease. From the extent and severity of the symptoms observed and the presence of the vector, some trees must have become infected 10 yr ago. Eradication of the 500 or so affected trees is underway. A few individuals of *Diaphorina citri*, the psyllid vector of Huanglongbing, were also collected in the survey. This is the first report of *D. citri* and of *H. phycitis* in Iran. The origin of WBDL is discussed.

Witches' broom disease of lime (WBDL) is a caused by "*Candidatus* Phytoplasma aurantifolia" (10). The disease was first observed in the Sultanate of Oman in the 1980s (1) and found to be present in the United Arab Emirates (UAE) in 1989 (7). A putative leafhopper vector, *Hishimonus phycitis*, multiplying actively on lime trees, was identified in 1991 in Oman and found to be also present in the UAE in 1993 (4). As early as 1985, the southern regions of Iran were identified as possible sites for WBDL occurrence (2) and this concern was reiterated in 1995 (5). Eventually, in July 1997, symptoms of the disease were observed by members of the Iranian Plant Pests and Diseases Research Institute in the southeastern region of Iran near Nikshar (Dapas Kur) and Qasr-e-Qand, i.e. approximately 100 km north of the coastal town of Chah Bahar and 100 km west of the Pakistani border. A joint Irano-French mission surveyed the affected regions, from November 25 to December 5, 1997, and collected plant material and leafhoppers for serological and molecular characterization of the involved pathogen. We show here that the disease in Iran is identical to that in Oman and the UAE.

MATERIALS AND METHODS

Leaves were collected on symptomatic and asymptomatic lime trees in the affected areas and their midribs were used for ELISA with WBDL-specific monoclonal antibodies (6), and PCR amplification of "*Candidatus* Phytoplasma aurantifolia" 16S rDNA with universal primer P1 (forward) and specific primer WB3 (reverse) (10). Lime leaves from nonaffected areas were also analyzed.

One of us (M.S.) provided various phytoplasma-infected plants for *Rsa*I treatment and RFLP characterization of the PCR amplified 16S rDNA of the phytoplasmas with universal phytoplasma primers P1 (forward) and P7 (reverse) (9). Leafhoppers and other insects were collected with a D-Vac aspirator.

RESULTS

The symptoms of WBDL are very characteristic. They were first described in Oman (1,3), later in the UAE (7). The symptoms seen in the two affected areas in Iran (Fig. 1, Table 1) are identical to those present

in Oman and the UAE. Only lime trees were affected in Iran. From the extent and severity of the symptoms observed in both areas, some trees must have become infected at least ten years ago, in the mid or late 1980s. In Oman, the disease was first encountered in the early 1980s but probably occurred there earlier.

All lime leaves with WBDL symptoms gave strong positive ELISA and PCR reactions (Table 1). The reagents used in these assays, monoclonal antibodies for ELISA (6) and primers for PCR (10), were obtained with the Omani strain of "*Candidatus* Phytoplasma aurantifolia". As shown in Table 1 and Fig. 2, when these reagents were used to analyze symptomatic lime leaves from Iran (Table 1 and Fig. 2, lanes 1, 3, 4, 5, 6, 9), reactions were as strong as those with Omani WBDL leaves (Table 1 and Fig. 2, lane D). Therefore, on the basis of ELISA and PCR, the phytoplasma associated with WBDL in Iran is indistinguishable from that in Oman and the UAE. Lime leaves of sample 7 were from a symptomless shoot growing from a stump of a cut-down WBDL tree. A faint amplified DNA band was obtained.

The leafhopper *H. phycitis*, suspected as the vector of the WBDL phytoplasma in Oman and the UAE (4), was easily recovered by D-Vac® aspiration not only from lime trees in the affected regions but also from lime trees in regions free of the disease such as Chah Bahar, Jiroft, and especially Minab and Duran where many large lime orchards occur (Table 2). Interestingly, in the affected Loriyani (Qasr-e-Qand) area, 2 of 11 *H. phycitis* leafhoppers captured gave a positive PCR result. In Hadji Abad (Dapas Kur), the other affected region, only three leafhoppers were captured, because time was short, and this is probably

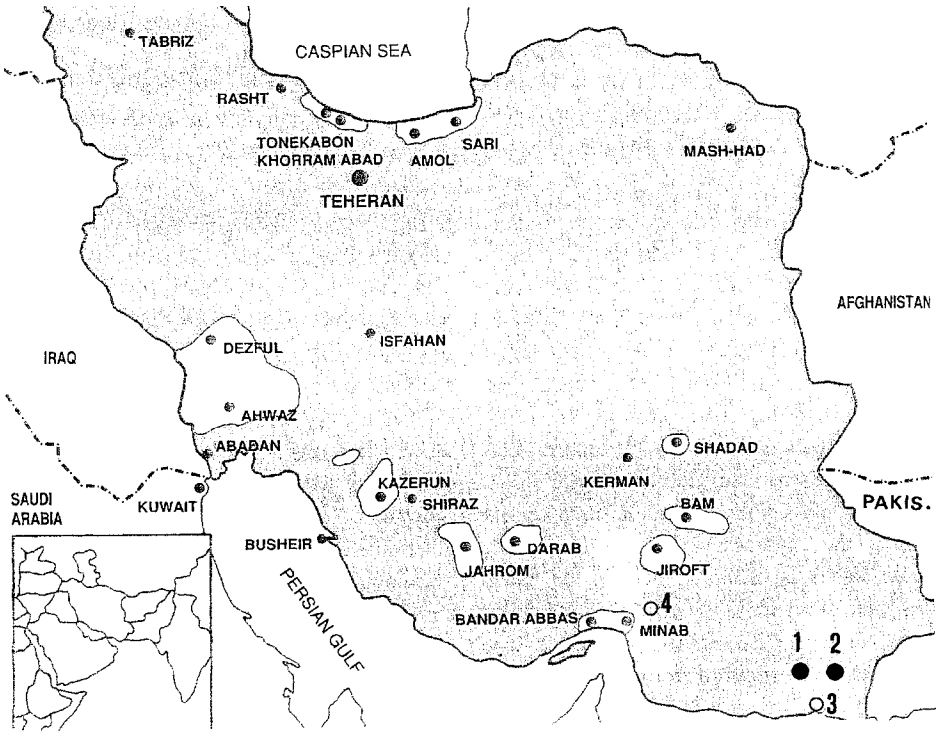

Fig. 1. Map of the Islamic Republic of Iran showing the major citrus-growing regions (white areas) and the two WBDL-affected zones: Dapas-kur (Nikshar) (1); and Qasr-e-Qand (2). Locations of Chah-Bahar (3) and Rodan (4) are also indicated.

TABLE 1
ELISA AND PCR DETECTION OF *CANDIDATUS* PHYTOPLASMA AURANTIFOLIA IN IRAN

Area	Sample no.	Cultivar	Symptoms	ELISA (OD_{405})	PCR
Dapas kur,					
Hadji Abad village	1	Lime	WBDL	2.140	3+
"	2	Sweet orange	Rosette	0.032	-
Qasr-e-qand,					
Loriyani Village	3	Lime	WBDL	2.150	3+
"	4	Lime	WBDL	1.280	3+
"	5	Lime	WBDL	0.910	3+
Toukal Village	6	Lime	WBDL	0.460	3+
"	7	Lime	Symptomless from WBDL stump	0.130	+
"	8	Lime	Symptomless	0.025	-
"	9	Lime	WBDL	1.150	3+
Rodan					
Moez Abad village	10	Lime	Symptomless	0.054	-
Bordeaux glasshouse	A	Lime	Healthy	0.045	-
	C	Periwinkle	Healthy	0.052	-
	B	Lime	WBDL	2.315	3+
	D	Periwinkle	WBDL	2.390	3+

too low a number for positive insects to be detected. In Jiroft, a large citrus-growing area free of WBDL (Fig. 1), *H. phycitis* was captured on lime trees but not on neighboring Valencia and Washington navel sweet orange trees nor on lemon trees.

Table 3 lists various phytoplasma diseases observed in Iran and the hosts to which these phytoplasmas were transmitted either naturally, or by dodder or *Circulifer haematoceps*, a leafhopper vector of the citrus stubborn pathogen, *Spiroplasma citri*. The 16S rDNAs of the phytoplasmas were amplified by PCR and the amplified DNAs were restricted by endonuclease *Rsa*1 (8). Figure 3 shows the restriction profiles. Interestingly, phytoplasmas from samples 23, 25, 29 and 31 gave the same profiles as the WBDL phytoplasma.

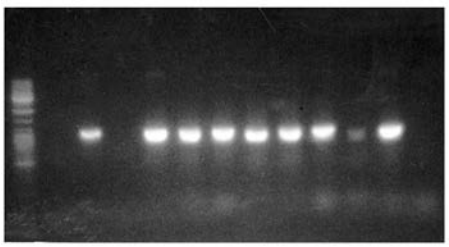

Fig. 2. PCR detection of *"Candidatus* Phytoplasma aurantifolia" 16 S rDNA in Iran. DNA extracted from healthy (A, C) and Omani WBDL-infected (B, D) lime (A, B) and periwinkle (C, D) leaves from Bordeaux glasshouse. DNA extracted from symptomless (7, 8) and symptomatic (1, 3, 4, 5, 6, 9) lime leaves from Iran (see Table 1). Notice that leaves of sample 7 were from a symptomless branch of a WBDL-affected stump. M: Molecular weight marker (1 kbp ladder). The PCR amplified DNA band has a size of 1 kbp.

Finally, among the insects captured in Kahir and Loriyani (Table 2), the psyllid *Diaphorina citri* (Kuwayawa) was present. This insect is the Asian vector of *"Candidatus* Liberibacter asiaticum", the bacterium associated with Huanglongbing (greening) in Asia. This is the first report of *D. citri* west of Afghanistan and Pakistan.

DISCUSSION

On the basis of symptomatology and results of ELISA and PCR obtained with reagents specific of *"Candidatus* Phytoplasma aurantifolia" from Oman, it is clear that the phytoplasma inducing WBDL in Iran cannot be distinguished from that present in Oman and the UAE.

TABLE 2
PCR DETECTION OF "*CANDIDATUS* PHYTOPLASMA AURANTIFOLIA" IN *HISHIMONUS PHYCITIS* LEAFHOPPERS FROM LIME TREES IN IRAN

Area of capture	Number of *H. phycitis* captured	Presence of WBDL in Area	PCR[z]
Chah Bahar (Kahir[y])	11	No	0/11
Dapas Kur (Hadji Abad)	3	Yes	0/3
Qasr-e-Qand (Loriyani[y])	11	Yes	2/11
Jiroft	17	No	0/12
Rodan (Badd-E-Mola)	9	No	0/9
Rodan (Moez Abad)	12	No	0/12
Minab (Cheragh Abad)	9	No	0/9

[z]Number of PCR-positive leafhoppers over number of leafhoppers analyzed.
[y]Area where *Diaphorina citri* (Kuwayawa) was also captured.

In Iran, the putative leafhopper vector, *H. phycitis*, is present on lime trees in the WBDL-affected areas, as well as in disease-free regions such as Chah Bahar, Jiroft, Minab and Rodan. This observation is relevant as far as the origin of WBDL is concerned. Indeed, *H. phycitis* was discovered in 1991 on lime trees in Oman, and in 1993 in the UAE (4). This was the first time *H. phycitis* was reported in the Arabian Peninsula, even though the insect is well known in India. Therefore, it was thought that the leafhopper had been introduced into Oman recently (1960s to 1970s), had picked up the WBDL phytoplasma on some wild or cultivated plant, and had transmitted it to lime. Prior to the work reported here, *H. phycitis* was thought to be absent from Iran. It is likely that the use of the D-Vac® aspirator made it possible to easily capture and detect the insect. In the UAE also, the leafhopper could only be seen once the D-Vac® was used. For these reasons, it might well be that *H. phycitis* has been present in Oman and the UAE, as well as in Iran, for much longer times than initially thought. If so, the origin of WBDL would not be related to the recent introduction of the putative vector.

H. phycitis is the main leafhopper captured on lime trees in Oman, the UAE and Iran. It is also the only leafhopper found to give positive crush-blot hybridizations with WBDL phytoplasma specific probes in Oman (4)

TABLE 3
PHYTOPLASMA DISEASES AND HOST IN IRAN

Sample no.	Disease	Transmission	Host	Origin
20	Almond brooming	Dodder	Periwinkle	Shiraz
21	Almond brooming	Natural	Almond	"
22	Phyllody	"	Periwinkle	Jiroft
23[z]	Virescence	"	Periwinkle	Chah-Bahar
24	Eggplant big bud	Dodder	Periwinkle	Shiraz
25[z]	Alfalfa witches' broom	Dodder	Periwinkle	"
26	Alhaghi proliferation	Dodder	Periwinkle	"
28	Stubborn	*C. haematoceps*	Periwinkle	"
29[z]	Broadbean phyllody	Natural	Broadbean	"
30	Wild lettuce witches' broom	"	Wild lettuce	"
31[z]	*Ocimum basilicum* w. broom	"	*O. basilicum*	Chah-Bahar
T[x]	WBDL (Bordeaux)	Dodder	Periwinkle	Oman

[z]These samples gave the same RFLP profiles after *Rsa*I-treatment of phytoplasma 16S rDNA amplicons (see Fig. 3).

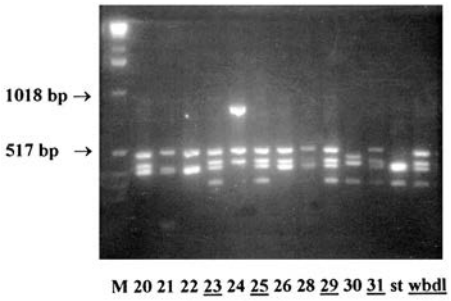

Fig. 3. Agarose gel electrophoresis of *Rsa*I-treatment PCR amplified 16S rDNA from various phytoplasmas in Iran. Size of untreated 16S rDNA amplicons is 1.7 kbp. M: molecular weight marker (1 kbp ladder); 20 to 26, 28 to 31: see Table 3 for sample description; st: stolbur-phytoplasma-infected periwinkle leaves; WBDL: sample T of Table 3 (periwinkle infected with "*Candidatus* Phytoplasma aurantifolia" from Oman). Underlined samples have the same *Rsa*1 profiles.

or positive PCR reactions as shown here (Table 2) with Iranian *H. phycitis*. Yet, it could not be shown experimentally so far that *H. phycitis* transmits the WBDL phytoplasmas in the glasshouse. However, lack of transmission does not necessarily mean that *H. phycitis* is not the vector. For instance, *Circulifer haematoceps*, the well-known leafhopper vector of *Spiroplasma citri*, fails to transmit certain strains of the spiroplasma. *Diaphorina citri*, the psyllid vector of the Huanglongbing liberibacter gives only very low percentages of transmission in the glasshouse.

WBDL was first observed in Oman in the 1980s and in Iran about 10 yrs later. The region in Iran where WBDL occurs is very remote. It is far from the coast (Fig. 1). Had the disease appeared at Chah Bahar on the coast, where lime trees grow, one could have hypothesized that the disease was introduced with infected plant material from Oman or the UAE. This does not seem to be the case. Therefore, the origin of WBDL in Iran is as puzzling as it is in Oman.

It has just been reported that a phytoplasma-associated witches' broom disease of lime trees occurs in India (8). This disease and WBDL seem to have similar symptoms. *H. phycitis* was captured on the lime trees in India also, but failed to transmit the phytoplasma in controlled conditions. No serological or molecular assays with WBDL-specific reagents have been carried out yet. Until such work is completed, the relationship between Indian witches' broom and WBDL as known in Oman, the UAE and Iran remains to be established.* However, assuming that WBDL is present in India, and probably also in Pakistan, the origin of disease might be in the Indian subcontinent from where it could have been moved by contaminated vectors, possibly *H. phycitis*, into Oman and Iran.

WBDL is a lethal disease. Trees die within 5 to 10 yr after the first witches' brooms have appeared. Therefore it is not easy to determine, years later, when the disease first occurred in a given region. As WBDL seemed to be restricted to Oman and the UAE for about 10 yr before it was reported in Iran, the assumption was that the disease occurred first in Oman and only later in Iran. Perhaps this is wrong. The disease might have entered both regions at about the same time, but independently of each other. It remains to be seen if the Indian subcontinent is the origin of WBLD in both Oman and Iran.

On the other hand, it was shown previously that the WBDL phytoplasma and the phytoplasmas of alfalfa, sesame and sunhemp phyllodies were closely related (10). Interestingly, four phytoplasmas from Iran (Table 3) as well as the WBDL phytoplasma, gave the same RFLP profiles after *Rsa*I treatment of their 16S rDNA amplicons (Fig. 3). Even though more such comparative work must be done, it might well be that the phytoplasma responsible for WBDL has been present in the affected regions in various plant spe-

cies long before it appeared in lime. It was first noticed in lime probably because the disease is economically important and particularly conspicuous and severe in lime. If alternative hosts of the WBDL phytoplasma exist, re-infection of lime will occur even if affected lime trees are eradicated, an operation now taking place. Therefore, identification of such putative hosts is of paramount importance for the control of WBDL.

****Note added in proof.*** On the basis of photos made available by D. K. Ghosh in December 2000, it appears that the multiple sprout formations (MSFs) with broom-like appearance seen on lime and mandarin trees in Nagpur (India) are clearly different from those of WBDL as seen on lime trees in Oman, the UAE and Iran. A few MSFs, similar to those observed in the Nagpur region, occurred also in Oman, even though seen very seldomly. In 1986, one lime tree in Oman showed both a MSF and a WBDL witches' broom. MSF in Nagpur was seen on both lime and mandarin trees; WBDL has never been observed on mandarin trees, and the WBDL phytoplasma could not be graft-transmitted to mandarin. WBDL in Oman, the UAE and Iran spreads rapidly and is extremely destructive; this has not been reported as such from India. From these results if seems that WBDL and MSF are different, and that MSF is not caused by "*Candidatus* Phytoplasma *aurantifolia*". This conclusion should be confirmed by serological and molecular tests. We thank D. K. Ghosh for having made photos of MSF on lime and on mandarin available, and C. N. Roistacher for fruitful discussions. For information on the Nagpur affection, see Ghosh et al. 1999, Plant Disease 83(3): 302, and Current Science 77(1): 174-177.

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