

## Witches' Broom Disease of Lime

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### Abstract

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Witches' broom disease of lime (*Citrus aurantifolia*) (WBDL) is caused by Candidatus *Phytoplasma aurantifolia*. The WBDL phytoplasma is closely related to the phytoplasmas of alfalfa, sesame, and sunhemp phyllodies. WBDL was first described in the Sultanate of Oman where thousands of lime trees were killed since the 1980s. It was seen in the United Arab Emirates (UAE) in 1989, and by 1993 all regions were affected. As predicted, the disease was reported next in Southeast Iran in 1997, but must have been there, many years before. Finally, witches' broom was reported as a new disease of lime in the Nagpur region of India in 1999. Monoclonal antibodies and PCR primers for 16S rDNA amplification have been obtained for the Omani phytoplasma. These Omani-specific reagents detect equally well the WBDL-phytoplasma from the Emirates and Iran. Whether the Nagpur phytoplasma also reacts with these reagents could not yet be studied. Natural spread of WBDL is very fast. 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 and in Iran in 1997. The leafhopper is well known in India. Even though *H. phycitis* appears as the most likely vector of the WBDL agent, experimental transmissions have so far been unsuccessful. The most severely and widely affected species is small fruited acid lime (*C. aurantifolia*). However, in the UAE, sweet limes (*Citrus limetta*, *Citrus limettoides*) showed severe symptoms. In the field, sweet orange, mandarin and grapefruit trees have never shown symptoms and were free of the WBDL agent. In the greenhouse, these species did not become infected following graft inoculation with infected lime shoots. Control is by prevention. Affected trees are easily spotted on the basis of their characteristic witches' brooms. Specific reagents are available to confirm visual diagnosis. Symptomatic trees must be removed. Finally, the origin of WBDL, a relatively new disease of citrus, will be discussed.

### Introduction

Small-fruited acid limes (*Citrus aurantifolia* (L.) Swingle) have been produced in the Sultanate of Oman along the northern coastal plain, or Batinah, for many generations. In the late 1970s, lime growers witnessed a severe decline of lime trees. Affected trees were characterized by the presence of conspicuous witches' brooms, hence the name "witches' broom disease of lime" (WBDL) (2, 3, 5).

Witches' brooms are easily detected on the trees by their compactness and their small, pale-green leaves. The smaller the leaves, the more severe the disease. In the early stages of the disease, the trees show only one, then a few, witches' brooms, with the other parts of the trees remaining symptomless, except for absence of new shoots. These early witches' brooms are soon followed by many others in various parts of the trees. In the advanced stages of the disease, the leaves of the older witches' brooms become dry and die, but remain attached (9). Eventually, the dead leaves drop, leaving naked twigs and shoots as the only evidence of former witches' brooms. After the first witches' brooms have appeared, the trees decline rapidly and, within a few years, they die: WBDL is a lethal disease.

As will be summarized below, the disease is due to a plant mollicute (mycoplasma) and more precisely, a phytoplasma. When we first described the disease from 1986 on (2, 3, 5), the phytoplasmas were not yet recognized as genuine mollicutes, and they were given the name "Mycoplasma-Like Organisms" or MLOs. Here, we will use throughout the name phytoplasma which was officially adopted in 1992 (16).

In addition to the phytoplasmas, plants can be infected by members of a second group of mollicutes: the spiroplasmas. The spiroplasmas are helical and motile, and *Spiroplasma citri*, the causal agent of citrus stubborn disease, was cultured as early as 1971 (13). In comparison, the phytoplasmas have no characteristic morphology, they are not motile, and they have never been cultured so far.

The mollicutes, including the spiroplasmas and the phytoplasmas, are true bacteria, but they lack a cell-wall. They are phylogenetically related to Gram-positive bacteria with low guanine (G) and cytosine (C) in the DNA.

While *S. citri* was the first mollicute of plant origin to have been obtained in culture, the WBDL was the first recognized phytoplasma disease of citrus.

The phytoplasma etiology of WBDL is based on several lines of evidence and will be summarized hereafter. These studies were conducted with plant material collected in 1986 in the Sultanate of Oman or derived from this material in our glasshouses in Bordeaux.

### Electron microscopy

Electron microscopy observations showed that the sieve-tubes of leaf-midribs from all witches' brooms examined contained phytoplasmas (2, 3, 5). The phytoplasmas are often extremely numerous in the sieve-tubes of midribs from very small leaves; such sieve-tubes can be literally filled with organisms. In larger leaves, the phytoplasmas are less numerous. Hence, the severity of symptoms is positively related to the number of phytoplasmas in the sieve-tubes. The phytoplasmas are restricted to the sieve-tubes.

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## Graft- and dodder-transmission of the WBDL-phytoplasma

Lime seedlings graft-inoculated with pieces of shoots or twigs from witches' brooms developed typical witches' broom symptoms, 6 to 12 months later, and phytoplasmas were present in the symptomatic leaves. Graft-transmissions of the WBDL-phytoplasma to a variety of citrus species and cultivars will be described below.

Transmission of the WBDL-phytoplasma to periwinkle (*Catharanthus roseus*) seedlings by dodder (*Cuscuta campestris*) was successfully achieved (8). The plants developed very small leaves on highly proliferating shoots. Phytoplasmas were present in the symptomatic periwinkle leaves. Back transmission of the phytoplasma from symptomatic periwinkle plants to lime seedlings by dodder was also obtained, the seedlings showing characteristic symptoms of WBDL. Through graft-transmission of the WBDL-phytoplasma from periwinkle to periwinkle, a large supply of phytoplasma-infected plant material can be obtained, and has made it possible to develop WBDL-specific reagents: monoclonal antibodies, DNA probes and PCR primers.

## Monoclonal antibodies and the serological detection of the WBDL-phytoplasma

Monoclonal antibodies (MA) specific for the WBDL-phytoplasma were obtained by immunizing Balb/c mice with homogenates of WBDL-infected periwinkle midribs (8, 9). Specific hybridomas were selected by differential immunofluorescence (IF) on sections of healthy and infected periwinkle midribs. In one fusion, two such hybridomas, 7D5 (IgG3) and 1D11 (IgM), were obtained among 856 hybridomas tested (8). In another fusion, 11 MAs, including 2H3 (IgG1), could be selected (9).

The specificity of the MAs for the WBDL-phytoplasma was determined by IF on midrib sections of periwinkle leaves infected with various phloem-restricted mollicutes. All MAs gave positive reactions with WBDL-phytoplasma-infected midrib sections, but not with sections containing other phytoplasmas, *S. citri*, *Spiroplasma kunkelii*, or the liberibacter of huanglongbing (greening) (8, 9).

MA 2H3 was amplified and successfully developed into a reagent for the specific detection of the WBDL-phytoplasma by IF or ELISA (7). Strong positive reactions were obtained with witches' broom leaves from all areas tested in the Sultanate of Oman.

## DNA probes and the relation of the WBDL-phytoplasma with other phytoplasmas

Periwinkle plants infected with the WBDL-phytoplasma were used for partial purification of the phytoplasma DNA by the bisbenzimidazole technique (11). The phytoplasma DNA fraction was cloned in plasmid pUC18 and amplified in *Escherichia coli* (1). Several recombinant plasmids had inserts that hybridized with the DNA extracted from plants infected with the WBDL-phytoplasma, but not from healthy plants (8, 10). Inserts I1H (probe P1), I10H (probe P3), and I28H (probe P4) were studied in more detail. In dot-blot hybridizations, probes P3 and P4 reacted strongly with DNA from symptomatic WBDL-phytoplasma infected plants, while probe P1 reacted more faintly. The specificity of the three probes was determined. In addition to the WBDL agent, two other phytoplasmas gave strong positive dot-blot hybridizations: the phytoplasmas of sunhemp (*Crotalaria*

*juncea*) and sesame (*Sesamum indicum*) phyllodies. However, when probe P3 was used in Southern hybridizations on *Hind* III-restricted DNA extracted from phytoplasma-infected plants, the hybridization patterns were different for the WBDL-phytoplasma and the two phyllody phytoplasmas (7, 9, 17). The alfalfa phyllody phytoplasma gave also a positive reaction in Southern hybridization, but the reaction was faint. These results show that the WBDL-phytoplasma is closely related to, but different from, the sunhemp and the sesame phytoplasmas.

## Characterization of the WBDL-phytoplasma

Until recently, only bacteria available in culture lent themselves to characterization and could be given latin binomial species names. However, with the advent of molecular techniques, the situation has changed. In particular, the sequence of the 16S ribosomal RNA or, to be more precise, the sequence of the gene coding for the 16S ribosomal RNA (16S rDNA), can now be determined easily: the 16S rDNA is first amplified by PCR with universal 16S rDNA primers, and the amplified DNA is sequenced either directly or after cloning. In this way, the 16S rDNA sequence from a new bacterium can be compared with the many 16S rDNA sequences of known bacteria deposited in gene banks. Closely related bacteria have very similar 16S rDNA sequences, distantly related bacteria have much less 16S rDNA sequence similarities.

Even though the phytoplasmas are not available in culture, their 16S rDNA can be PCR-amplified, using as target DNA the total DNA of plants infected with the relevant phytoplasma. Prior to the PCR step, the interfering chloroplast 16S rDNA has to be cut by *Bcl*I to prevent its amplification. The amplified DNA is sequenced, and its sequence compared to the 16S rDNA sequences present in the gene banks. In this way, the name of the bacterial species with the most similar 16S rDNA sequence is obtained, and indicates to what bacterial group the phytoplasma belongs. This is how it was shown that the phytoplasmas are members of the division *Mollicutes*, and that the known phytoplasmas represent about 20 groups or phylogenetic clusters (potential species) (14).

The 16S rDNA sequence of the WBDL phytoplasma was determined and compared with the 16S rDNA sequences of the other phytoplasmas (17). A phylogenetic tree was constructed. The 16S rDNA sequence of the WBDL phytoplasma was determined and compared with the 16S rDNA sequences of the other phytoplasmas (17). A phylogenetic tree was constructed. The WBDL-phytoplasma was found to cluster within the faba bean phyllody phytoplasma group. Other members of this group are the phytoplasmas of sesame and sunhemp phyllodies, a result in agreement with those from DNA hybridizations (see above), sweet potatoe witches' broom, peanut phyllody, and tomato big bud.

From the 16S rDNA sequence of the WBDL phytoplasma, a specific primer, WB3, was designed and used with universal primer rp1 for PCR detection of the phytoplasma in plants (17).

In addition to the 16S rDNA sequence, other molecular data were obtained for the WBDL-phytoplasma. The 16S – 23S ribosomal spacer region was sequenced. The genome size of the WBDL agent was determined by pulsed-field gel electrophoresis, and found to be of 720 kbp. On the basis of these molecular data, including the Southern hybridization profiles obtained with the WBDL-phytoplasma DNA-probes,

and following the *Candidatus* proposal of Murray and Schleifer (12), the WBDL agent was given the name *Candidatus* Phytoplasma aurantifolia (17). It is the first phytoplasma to receive a *Candidatus* designation.

### Search for the putative insect vector of the WBDL-phytoplasma

In 1986, WBDL was restricted to the northern part of the Batinah and extended from Al Murayr, immediately south of the border with the United Arab Emirates (UAE), to Saham. Within these limits, the most severely affected areas were those of Liwa and Shinas, Al murayr being third. The orchards in the affected region totaled 29,232 trees of which 6,291 (21.5 %) showed WBDL. By 1987, the disease had spread not only to the southern Batinah, as far as Barka, but also to inland areas, such as Al Rustaq and Dan'k. In 1989, the first cases were seen in the UAE, and by 1993, most citrus growing regions were affected.

In affected orchards, the disease spreads rapidly. In 1990, in a given orchard, 7.6% of 251 trees were affected. One year later, the trees with witches' brooms amounted to 41%.

Because of the rapid spread of the disease, an insect vector was suspected. The phytoplasmal nature of the WBDL agent pointed towards leafhoppers and psyllids, as these insects are known to be vectors of phytoplasmas. Insects were captured in the Sultanate of Oman in May 1993 with a D-Vac aspirator. Leafhoppers were separated into species. Individual leafhoppers were crushed with a glass rod directly onto a nylon N+ membrane, and the "crush-blot", each with about one hundred insects, were used for hybridization with 32P-labeled probe P3.

Among the leafhoppers captured some species were new to the Arabian Peninsula and were reported for the first time (7). The major result of this work concerned one of the new leafhopper species discovered: *Hishimonus phycitis*. *H. phycitis* was the only species that tested positively in DNA hybridizations with the WBDL-phytoplasma probe P3. In addition, this leafhopper was the only one consistently and almost exclusively found on lime trees (7).

Interestingly, *H. phycitis* is well known in India. Acid lime is given as one of the host plants on which the leafhopper is able to live and reproduce. It is vector of a severe and widely distributed phytoplasma disease of eggplant: little leaf disease. It transmits also the phytoplasma of *Parthemium hysterophorus* phyllody.

In summary, *H. phycitis* could be the vector of the WBDL phytoplasma for the following reasons: the leafhopper is consistently found on lime trees, it is the only reacting positively with the DNA probe produced against the WBDL phytoplasma, and it is known to be a vector of phytoplasmas. In the orchard, WBDL seems to spread from tree to tree. This type of spread would be expected of a vector living and multiplying on the affected trees. However, experimental transmissions of the WBDL phytoplasma with *H. phycitis* have not yet been obtained, and hence proof that this leafhopper is indeed the vector of the WBDL agent is not yet on hand. *H. phycitis* remains however the most likely candidate for the role of vector, not only in the Sultanate of Oman, but also in the UAE and Iran (see below).

### Geographical distribution of WBDL and comparison of the phytoplasmas involved

WBDL was first reported in Oman in 1986, but as judged from the presence of very severely affected trees, the disease must have occurred much earlier, probably in the 1970s. By late 1992, the entire Batinah as well as inland regions were affected. In the UAE, the disease was first seen in 1989, and by early 1993, lime trees in most citrus growing regions showed symptoms. In addition to lime, citron, Indian Palestine sweet lime and sweet limetta were also affected (15).

In Iran, it was in July 1997 that members of the Iranian Plant Pests and Diseases Research Institute observed witches' brooms on lime trees in a remote area of the southeastern region of the country, 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. About 500 trees were found affected. When we visited the area in December 1997, we noticed that several trees were severely affected and others had already died, indicating that the disease must have been present since many years. We collected plant material and leafhoppers in the affected areas, as well as in regions where the disease was absent: Cha Bahar, Jiroft, Rodan and Minab (4).

In all three countries, the symptoms and the progress of the disease are identical. The availability of specific reagents, Mas, DNA probes, and PCR primers, has made it possible to compare the WBDL-phytoplasmas from the three countries. On the basis of symptomatology, ELISA with MA 2H3 and PCR amplifications with primers rp1 and WB3, the WBDL-phytoplasmas from Oman, the UAE, and Iran are the same: the causal agent of WBDL is *Candidatus* Phytoplasma aurantifolia.

*H. Phycitis*, first detected in Oman, was also found in the UAE and in Iran (4), once a D-Vac aspirator was used to collect the insects from the trees. In Iran, *H. phycitis* was present on lime trees not only in the WBDL-affected regions, but also in regions free of the disease (Chah-Bahar, Jiroft, Minab and Rodan). The WBDL-phytoplasma could be detected by PCR in individual *H. phycitis* leafhoppers from the affected area, but not in those from the WBDL-free regions (4).

It has been reported in 1999 (10) that a phytoplasma-associated witches' broom disease of lime trees occurs in the Nagpur region of India, a major Indian citrus region. No serological and/or molecular assays with *Candidatus* Phytoplasma aurantifolia-specific reagents could be carried out. Thus, the relationship between the Indian phytoplasma and the WBDL-phytoplasma remains unknown.

### Host range of WBDL

In nature, the most widely affected species is small-fruited acid lime. In the UAE, citron, Indian Palestine sweet lime and sweet limetta also show severe symptoms in the orchards, and they give positive IF reactions with WBDL-phytoplasma specific Mas, and positive DNA hybridization reactions with probe P3 (7).

Experimentally, the WBDL phytoplasma could be graft-transmitted to the following citrus species which showed symptoms of witches' broom disease within 24 months after graft-inoculation with symptomatic lime shoots: *C. aurantifolia*, *C. excelsa*, *C. hystrix*, *C. ichangensis*, *C. karna*, *C. macrophylla*, Etrog citron, Meyer lemon, Rangpur lime, rough lemon, and Troyer citrange (6).

Presence of the WBDL-phytoplasma in these symptomatic species was confirmed by ELISA with specific Mas. The following species did not become infected, despite the development of good witches' broom symptoms on the lime shoots grafted on these species as inoculum: *C. clementina*, *C. deliciosa*, *C. halimi*, *C. junos*, *C. latifolia*, grapefruit, Cleopatra mandarin, sweet orange, *Fortunella margarita*, *Microcitrus australis*, and *Severinia buxifolia*. It is worthwhile noting that sweet orange, clementine, and grapefruit are among the species that did not become infected. Either the phytoplasma cannot move through the graft union from the infected lime inoculum to the cultivar, or the above cultivars are resistant. In the orchard, these species have remained symptomless even when adjacent to severely affected lime trees, and none or only very few *H. phycitis* leafhoppers were captured on these species.

As already indicated, the WBDL-phytoplasma could be transmitted from lime seedlings to periwinkle plants, and back to lime, by dodder.

## Conclusion

A phytoplasma is always present in plant material (citrus or periwinkle) showing symptoms of WBDL, whether the infection occurs naturally in the orchard or has been obtained experimentally by graft inoculation or dodder transmission in the greenhouse. Presence of the phytoplasma is based on electron microscopy, serological reactions with

Mas, DNA probes, and PCR primers for DNA amplification of 16S rDNA. The same phytoplasma is involved in Oman, the UAE, and Iran, and has been characterized on the basis of molecular properties. These results fulfil the first of the four Koch's postulates. The three others cannot be carried out, as the phytoplasmas are not available in culture. There is, however today, general consensus to believe that, when a well-characterized phytoplasma is associated with a disease, this phytoplasma is the causal agent of the disease. Therefore, *Candidatus Phytoplasma aurantifolia* is the etiological agent of WBDL.

Control of WBDL is by prevention. Affected trees are easily spotted on the basis of their characteristic witches' brooms. Specific reagents are available to confirm visual diagnosis. Symptomatic trees must be removed. However, as indicated above, the WBDL-phytoplasma and the phytoplasmas of alfalfa, sesame and sunhemp are related. It might well be that the WBDL-phytoplasma has been present in the affected regions in various plant species long before it appeared in lime. It was first noticed in lime probably because in lime the disease is economically important, and particularly conspicuous and severe. If alternative hosts of the WBDL-phytoplasma exist, reinfection of lime will occur even if affected lime trees are eradicated. Therefore, identification of such putative hosts is essential for the control of WBDL.

## المخلص

بوفيه، أ. م. ومونيك جارنيه. 2000. مرض مكنسة الساحرة لأشجار اللايم. مجلة وقاية النبات العربية. 18: 148-152.

ينشأ مرض مكنسة الساحرة لأشجار اللايم (*Citrus aurantifolia*) عن *Phytoplasma aurantifolia*. ويرتبط الكائن المسبب للمرض بعلاقة وثيقة مع الفيتوبلاسما المسببة لتورق الأزهار في الفصاة/ الجت، السمسم والقتب. وقد وصف هذا المرض لأول مرة في سلطنة عمان، حيث تسبب في موت آلاف الأشجار منذ الثمانينات. وتمت رؤيته أيضاً عام 1989 في دولة الإمارات العربية المتحدة، وانتشر إلى كافة دول المنطقة في 1993. وكما كان متوقعاً، سجل المرض في جنوب شرق إيران عام 1997 ولو أنه يعتقد بوجوده هناك قبل ذلك بعدة سنوات. وأخيراً سجل المرض كمرض جديد على اللايم في مقاطعة ناجبور الهندية عام 1999. وتم الحصول على أجسام مضادة وحيدة الكلون واسمات PCR للفيتوبلاسما العمانيّة. واستطاعت هذه الأجسام، الخاصة بالعزلة العمانيّة، كشف العزلتين الإماراتية والإيرانية، ولا يعرف بعد فيما إذا كانت العزلة الهندية تتفاعل مع هذه الأجسام. ويحدث الإنتشار الطبيعي للمرض على نحو كبير. وقد تم تعريف نطاق ورق ناقل (*Hishimonus phycitis*)، يتكاثر بنشاط على أشجار اللايم في عمان عام 1991، وتبين وجوده أيضاً في دولة الإمارات عام 1993 وفي إيران عام 1997 وهو معروف أيضاً في الهند. على أن محاولة نقل الفيتوبلاسما على نحو تجريبي لم تكلل بالنجاح. ويصيب المرض بشدة وعلى نحو واسع اللايم صغير الثمرة (*C. aurantifolia*). وفي الإمارات العربية، أظهرت أشجار اللايم الحلو (*C. limetta* و *C. aurantifolia*) أعراضاً مرضية شديدة. ولم تظهر أشجار البرتقال الحلو، والماندرين والكريفون أعراض المرض تحت الظروف الحقلية وكانت خالية من العامل الممرض. كما أن هذه الأشجار لم تصب تحت ظروف الدفيئة عقب تطعيمها بفروع لايم مصابة. تعد إزالة الأشجار المصابة فضلياً وسائل المكافحة، حيث يمكن كشف الأشجار المصابة بسهولة من خلال الأعراض الظاهرية المميزة التي تبديها، كما تتوافر وسائل تشخيصية متخصصة لتأكيد الأعراض الظاهرية المميزة. وسناقش الباحثان أصل مكنسة الساحرة وهو المرض الحديث نسبياً على الليمون/ الحامض.

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