Witches’-Broom, a Lethal Mycoplasmal Disease of Lime Trees in the Sultanate of Oman and the United Arab Emirates

Small-fruited acid limes (Citrus aurantifolia (L.) Swingle) have been grown in the Sultanate of Oman along the northern coastal plain, or Batinah, (Fig. 1) for many generations. The people of Oman say that Sinbad the Sailor came from the Batinah coast and that the presence of sun-dried lime fruits on his boat might have been one of the reasons for the success of his voyages. In many Arabic countries, limes are preferred to lemons. Limes as an export crop, after being second to dates for a long time, became by the early 1970s the most important export commodity apart from oil. In 1971, limes were worth 333,300 Omani rials (1 rial = $2.5 U.S. in 1990) while dates were worth only 118,000 rials.

In the late 1970s, lime growers witnessed a severe decline of lime trees. Because the presence of witches’-brooms on affected trees was the most characteristic symptom, the disease was named witches’-broom disease of lime (WBDL). From the time the first witches’-broom appears, only 4-5 years elapse before the tree dies. By 1986, several orchards were completely destroyed and farmers began to grow banana plants instead.

Two surveys by one of us (J.M.B.) in April 1986 and April 1987 showed WBDL to spread rapidly to unaffected areas in the Batinah. In 1989, the disease was seen for the first time in the neighboring United Arab Emirates (UAE).

This paper summarizes for the first time the work that has been done on the geographical distribution of WBDL, the association of mycoplasmalike organisms (MLOs) with the disease, transmission of the WBDL MLO to rutaceous and nonrutaceous hosts, back-transmission of the MLO from periwinkle (Catharanthus roseus (L.) G. Don) to lime, and production of monoclonal antibodies and DNA probes for detection of the WBDL MLO. These reagents will be most useful in the search for the putative insect vector of the disease agent. Short partial reports on WBDL have appeared (3,5).

Symptomatology and Etiology

In the Sultanate of Oman, the disease is characterized by the appearance and progressive development of witches’-brooms, first in one part of the tree, then throughout the canopy (Fig. 2A,B,C). The leaves on the witches’-brooms are small to very small (Table I) and pale green to yellow (Fig. 2D); after some time, they become dry but remain attached to shoots, then eventually fall (Fig. 2E). Very few flowers and fruits are present on the witches’-brooms.

Witches’-brooms are often seen on plants infected by mycoplasmalike organisms (MLOs). MLOs are associated with more than 300 diseases of plants (12). They are known today to be true mollicutes (10), but because attempts to obtain them in culture have failed, they are considered more and more as obligate parasites. Mollicutes are not primitive prokaryotic organisms; they have evolved from ancestors of gram-positive bacteria and have a low percentage of guanine and cytosine in the DNA, as do the clostridia (13).

MLOs are unambiguously associated
with the witches'-broom disease of lime trees (Table 1 and Fig. 3). MLO populations in the sieve tubes of leaf midribs collected on witches'-brooms from trees infected in the field were estimated by means of electron microscopy. The highest number of MLOs was found in the smallest leaves, while larger leaves contained fewer organisms (Table 1). Many sieve tubes of small leaves (0.5–1 cm long) were packed with MLOs.

**Distribution and Spread**

In the Sultanate of Oman, in April 1986, the disease was restricted to northern Batinah and extended from Al Murayr, immediately south of the border with the UAE, to near Saham (Fig. 1). Within these limits, the most severely affected areas were those of Liwa and Shinas, Al Murayr being third. According to the survey of 1986, the orchards in the affected area totaled 29,232 trees, of which 6,291 (21.5%) showed WBDL. The percentage of affected trees was similar in each of the three areas most affected. The Liwa area had the highest number of affected trees (4,274). This area had approximately 185 orchards with at least one infected tree per orchard; 1–10% of trees were affected in 95 (51.3%) of the 185 orchards, 10–20% in 31 (16.7%), and 90–100% in 15 (8.1%). In both Liwa and Shinas areas, 7% of the orchards had more than 90% affected trees.

No affected trees were noticed in the Sohar area in April 1986, but by April 1987, several orchards had trees showing symptoms of the disease. These observations indicated that the disease was spreading to hitherto unaffected zones. Up to 1986, the Sohar-Saham area was the southern limit of the affected region. By April 1987, the disease had progressed south of this border area, and witches'-brooms on lime trees were seen not only in the Barka area (southern Batinah), but also inland, in the Al Rustaq and Dan’k areas. Thus, within 1 year, from April 1986 to April 1987, the disease clearly had progressed within the Al Murayr-Saham area and trees with witches'-brooms were detected for the first time outside this area.

Surveys done in April 1987 indicated that WBDL was still restricted to the Sultanate of Oman. No evidence of the disease could be found in the UAE, at Kalba and Fujairah, immediately north of the border with the Sultanate of Oman. In 1989, however, clear-cut symptoms of the disease were seen in the UAE at Hatta and Fujairah. Samples collected in the UAE in 1990 by M. Taher of the Food and Agriculture Organization (FAO), Rome, were analyzed by us and found positive by electron microscopy and immunofluorescence. The results indicate that WBDL has reached the UAE, as expected in view of the rapid spread of the disease in the Sultanate of Oman.

**Experimental Transmission of the WBDL MLO**

Five witches'-brooms from WBDL-affected lime trees were collected in April 1986 in the Liwa area of the Sultanate of Oman and brought to Bordeaux. Shoots from each witches'-broom were grafted under quarantine conditions onto small-fruited acid lime seedlings that were kept at 32°C during the day and 27°C during the night. Within 6–12 months, all graft-inoculated lime seedlings developed symptoms similar to those observed in the field on adult trees: proliferation of auxiliary buds and production of small to very small leaves that eventually dry but remain attached for some time on the dead seedling (Fig. 4A). The infected lime seedlings died within 2 years after inoculation. The presence of MLOs in the inoculated seedlings was confirmed by electron microscopy. For graft transmission of WBDL to citrus other than lime, symptomatic shoots from infected lime seedlings were side-grafted onto seedlings of sweet orange (*Citrus sinensis* (L.) Osbeck 'Madame Vinous'), cirtance (*Poncirus trifoliata* (L.) Raf. × *C. sinensis* 'Troyer'), and sour orange (*C. aurantium* L.). Only one of many Troyer cirtance seedlings developed symptoms 6 months after inoculation; symptoms appeared on only one shoot of the seedling, a lateral shoot that grew immediately below the site of insertion of the lime shoot used as the graft inoculum (Fig. 4B). The symptomatic cirtance shoot had short internodes and minute leaves. Electron microscopy and immunofluorescence confirmed the presence of the WBDL.

![Image](image-url)

**Fig. 2.** Small-fruited acid lime trees affected by witches'-broom disease: (A) A few witches'-brooms (arrows) in early stage and (B) many in advanced stage of disease. (C) Final stage preceding death. (D) Small, pale green leaves on a witches'-broom (right) compared with a normal shoot and leaves. (E) Witches'-brooms with dry leaves.
MLO in the symptomatic shoot. These observations indicate that Troyer citrange is susceptible to the WBDL MLO but that apparently the MLO is not transmitted as readily from the grafted lime shoot to the Troyer citrange receptor plant as to lime seedlings, which take 6-12 months after graft inoculation for symptoms to appear.

The lime shoot inoculated on the Troyer citrange seedling (Fig. 4B) grew into a well-developed witches'-broom by the time the citrange shoot had become symptomatic. The other graft-inoculated Troyer citrange seedlings and the sweet and sour orange seedlings had not shown symptoms 1 year after inoculation, even though the infected lime shoots used for graft inoculation had produced large witches'-brooms with high numbers of MLOs in the sieve tubes. These experiments might mean that sweet orange and sour orange are resistant or tolerant to the WBDL MLO, but the MLO may not be translocated readily from the graft to the receptor plant.

When field dodder (Cuscuta campestris Yuncker) was used to transmit the WBDL MLO to periwinkle plants, dodder seeds were first germinated on moist cotton and established on healthy periwinkles (dodder grown from seed is free of MLOs). After about 15 days, when the dodder shoots were about 10 cm long, strands were attached to a lime seedling showing symptoms of WBDL. After the dodder had formed haustoria within the infected lime seedling, the strands between it and the periwinkle were cut. Within 1 week, dodder on the infected lime developed new strands, which then were connected to healthy periwinkle plants. The connections were maintained for at least 4 weeks, after which the plants were manually freed of dodder and kept in the greenhouse at 32 C during the day and 27 C during the night. The MLO was transmitted from infected periwinkle plants back to a lime seedling in the same manner.

The WBDL MLO was successfully transmitted to periwinkle plants via dodder. The symptoms developed on large old leaves 2 months after removal of the dodder strands. These leaves showed only a slight yellowing, but the lamina was distorted. The axillary buds of these leaves started to grow into thin, flexible shoots. The successive leaves that formed on these shoots became progressively smaller but showed no yellowing. When these shoots were top-grafted onto healthy periwinkle seedlings, they continued to grow and produced large witches'-brooms that required stakes to remain straight (Fig. 4E). The shoots of these witches'-brooms had very short internodes, and their numerous leaves were less than 1 cm long. The periwinkle plants carrying these witches'-brooms also began to show symptoms only 2-3 months after the top-graft inoculation; symptoms resembled those observed after dodder transmission. Occasionally, a few flowers appeared on symptomatic shoots. The flowers tended to be small and showed various degrees of virecence, from dashlike virecence on nearly normal petals (Fig. 4C) to uniformly green virulent flowers (Fig. 4D). In advanced stages of the disease, leaves and shoots progressively wilted and dried, until the plant itself was dead. Thus, WBDL was as severe on periwinkle as on lime.

Electron microscopy revealed the presence of numerous MLOs in the phloem of the symptomatic periwinkles. These MLOs were back-transmitted to a lime seedling via dodder. The symptoms observed on the dodder-inoculated lime seedling were identical to those observed on the initial graft-inoculated lime seedlings. As expected, MLOs were present in the back-inoculated lime seedling, and they reacted with the monoclonal antibodies specific to the WBDL MLO.

### Table 1. Electron microscopic detection of mycoplasmalike organisms (MLOs) in small-fruited acid lime trees with witches'-broom disease in the Sultanate of Oman

<table>
<thead>
<tr>
<th>Witches'-broom</th>
<th>Plant material</th>
<th>MLOs in sieve tubes&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large, with small normal leaves</td>
<td>Tips of shoots with small leaves</td>
<td>+</td>
</tr>
<tr>
<td>From tree in advanced stage of</td>
<td>Midribs of leaves 0.5-2 cm long</td>
<td>2+</td>
</tr>
<tr>
<td>decline</td>
<td>Midribs of leaves 3-4 cm long</td>
<td>-</td>
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<tr>
<td></td>
<td>Midribs of leaves 1-2 cm long</td>
<td>-</td>
</tr>
<tr>
<td>With mainly large normal leaves</td>
<td>Midribs of leaves 0.5-1 cm long</td>
<td>4+</td>
</tr>
<tr>
<td>With relatively large leaves</td>
<td>Midribs of leaves &lt;0.5 cm long</td>
<td>5+</td>
</tr>
<tr>
<td>Well developed, with leaves</td>
<td>Midribs of leaves 2 cm long</td>
<td>3+</td>
</tr>
<tr>
<td>1-2 cm long</td>
<td>Midribs of leaves 1-2 cm long</td>
<td>3+</td>
</tr>
<tr>
<td>Normal shoot from infected tree</td>
<td>Midribs of leaves 4-5 cm long</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>*</sup> + = A few MLOs, 5+ = tubes packed with MLOs; = no MLOs.

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**Fig. 3.** Electron micrographs of leaf midrib sections from a small-fruited acid lime tree affected by witches'-broom: (A) Phloem tissue packed with MLOs and (B) high magnification of MLOs. Scale bars = 0.5 μm.

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**Production and Use of Monoclonal Antibodies**

The availability of periwinkle plants infected with the WBDL MLO allowed us to produce monoclonal antibodies (MAbs) against the WBDL MLO using the general procedure that we have developed to obtain MAbs against phloem-restricted prokaryotes (11). Sixteen hybridomas secreting such MAbs were produced and used in indirect immunofluorescence assays (11) on cryosections of leaf midribs from healthy and infected citrus and periwinkle plants. The 16 (with immunoglobulin isotypes in parentheses) were: 1D12 (IgG<sub>2a</sub>), 1E2 (IgG<sub>1</sub>), 2B7 (IgG<sub>1</sub>), 2H3 (IgG<sub>1</sub>), 3A12 (IgG<sub>1</sub>), 3B1 (IgG<sub>2a</sub>), 4G12 (IgM), 7F12 (IgG<sub>1</sub>), 8C3 (IgG<sub>1</sub>), 9F12 (IgG<sub>1</sub>), 12A1 (IgG<sub>1</sub>), 15E5 (IgG<sub>1</sub>), 17B4 (IgG<sub>1</sub>), 17F8 (IgG<sub>1</sub>), 20A5 (IgM), and 20C7 (IgG<sub>1</sub>). All 16 MAbs reacted with the infected plants—symptomatic lime (Fig. 5A), Troyer citrange, and periwinkle—but not with healthy lime (Fig. 5B) and healthy periwinkle plants, or with periwinkle plants infected with the MLOs of apple proliferaion, clover phlyology, aster yelows, cabbage chloranthus, or tomato stolbur, or with periwinkle plants infected with Spiroplasma citri or the bacteriumlike organism associated with citrus greening
disease. MAs 1D12, 12A1, and 15E5 were used in western immunoblots, and each detected the same 19-kDa protein in periwinkle plants infected with the WBDL MLO but not in healthy periwinkles (Fig. 6).

Production of DNA Probes

DNA was purified from periwinkle plants infected with the WBDL MLO according to our recently developed technique (9). MLO DNA was cloned in pUC18 and amplified in Escherichia coli as described by Bonnet et al (2). Several recombinant plasmids had inserts that hybridized with the plants infected with the WBDL MLO but not with healthy plants (Fig. 7). Inserts P3, P4, and P5 reacted strongly with infected periwinkle plants and inserts P1 and P2 reacted faintly (Fig. 7).

Outlook

WBDL is the first graft-transmissible disease of citrus in which MLOs have been shown to be involved. Koch's postulates cannot be fulfilled at this time because MLOs, including the WBDL MLO, have not yet been obtained in pure culture. However, the successful graft inoculations and the dodder transmissions described here (including the back-transmission from periwinkle to lime) clearly indicate that the MLO present in the phloem of infected lime plants is the causal agent of the disease.

Another new disease of citrus, rubbery wood, recently described by Ashmat et al (1), is also associated with MLOs. Rubbery wood affects lemon and lime trees in India, but the symptoms differ from those of witches'-broom disease of lime. Relatedness between the two MLOs is being investigated with our MAs and DNA probes.

Until recently, relatedness between MLOs could be studied only by comparing the symptoms they induce on a common host plant, such as periwinkle. For instance, flower virescence is observed with certain MLOs but not with others. Because MLOs cannot be cultured, almost 20 years elapsed after their discovery in 1967 (6) before specific reagents became available. The hybridoma technology and the DNA recombinant methodology do not require pure MLO prepararations or cultures and have enabled preparation of MAs as well as DNA probes that are highly specific for a given MLO. In this way, the tomato stolbur MLO was detected by specific MAs not only in tomato, tobacco, pepper, eggplant, and celery, but also in bindweed (Convolvulus arvensis L.), a perennial plant in which the MLO is able to overwinter (8). Similarly, specific DNA probes have been prepared to detect the apple proliferation MLO in periwinkle, apricot, and plum (2). The witches'-broom MLO is one of the first for which both MAs and DNA probes have been produced.

WBDL was restricted to the Sultanate of Oman until 1989, when symptoms of the disease were seen in the UAE for the first time. MAs and DNA probes have been used to establish the identity between the MLO of WBDL in the Sultanate of Oman and that in the UAE. Lime trees are grown in other regions of the Arabian peninsula. In the southwestern part of Saudi Arabia, only lime trees are grown today; this is because sweet orange and mandarin (C. reticulata Blanco) trees have been wiped out by the Asian form of citrus greening disease (4). A similar situation exists in Yemen in the Taiz region, where the African form of greening is involved. In southern Iran, across the Persian Gulf, many lime trees are also grown, not only as scions but also as rootstocks. Even within the Sultanate of Oman, certain areas such as the Salala region in the south are still free from WBDL. Only strict quarantine measures and restricted movement of citrus plants will limit the extent of the disease.

WBDL is one of the most lethal diseases of lime. Only 4–5 years elapse between the appearance of the first witches'-broom and death of the tree. In the WBDL-affected area in Oman, only
lime is grown. No other citrus cultivars are present. Therefore, WBDL can be seen only on lime trees, and no conclusions can be drawn as to its host range in the field. Our experiments show that at least one other Rutaceae species is susceptible: Troyer citrange, a widely used rootstock throughout the world.

Whether additional citrus species can be affected remains to be seen.

Comparing the extent of the disease in the early 1980s with that in the late 1980s clearly indicates rapid spreading of the disease agent. Because the disease is associated with an MLO, it seems likely that an insect serves as the vector for the natural spread of the agent. The monoclonal antibodies against the WBDL MLO and the specific DNA probes that we have developed will be useful tools to help identify the insect vector(s) as well as the possible plant reservoir(s) of the agent. A similar approach has made possible the identification of the leafhopper vector of Spiroplasma citri (causal agent of citrus stubborn disease) in the Near East (7) as well as that of the stolbur MLO in southern France (unpublished). Identification of the insect vector of WBDL will undoubtedly contribute to better control of the disease.

WBDL seems to be an example of an apparently new disease that has suddenly occurred and is now destroying an age-old plant industry. From the lack of relatedness between the WBML MLO and the other MLOs tested (even though more MLOs must be examined), as well as the unusual symptoms on periwinkle, it seems that the WBML MLO is unique and has not yet been encountered elsewhere.

Acknowledgments

Without the contributions of the Ministry of Agriculture of the Sultanate of Oman and the Food and Agriculture Organization of the United Nations, this work would not have been possible. We thank D. L. Williamson, State University of New York at Stony Brook, and J. Stemke, University of Edmonton.

**Fig. 4.** (A) Stages of infection in lime seedlings grafted-inoculated with small-fruited acid lime tree MLO. (B) Troyer citrange graft-inoculated with MLO from small-fruited acid lime, showing symptomatic shoot (arrow) and lime shoot used as inoculum (star). (C and D) Periwinkle flowers showing MLO-induced virescence. (E) MLO transmission from an infected periwinkle plant to a periwinkle seedling by top-grafting, with abundant development of grafted shoot in witches'-broom.

**Fig. 5.** Indirect immunofluorescence assays on midrib sections from lime using monoclonal antibody 12A1: (A) Lime infected with witches'-broom MLO, indicated by green fluorescence in phloem tissue, and (B) healthy lime.

**Fig. 6.** Western immunoblotting of proteins extracted from (H) healthy and (I) infected periwinkle plants with monoclonal antibody 12A1 prepared against the MLO from plants affected with witches'-broom; (arrow) 19-kDa protein recognized by the antibodies.
Canada, for critical review of the manuscript. We also thank Colette Saillard for help in DNA blotting, Jeanne Gandar for doing the western blotting, and Patrick Bonnet for grafting the citrus plants.

**Literature Cited**


