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# Characterization and Pathogenicity of Fungal Pathogens Associated with Root Diseases of Citrus in Oman

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## Abstract

A study was conducted to characterize fungal and oomycete pathogens associated with root diseases of acid lime and sweet lime in Oman. A survey which covered 17 administrative districts and 166 farms showed prevalence of dieback symptoms in 8.8% of acid limes and in 15.9% of sweet limes. Some of the acid limes (3.0%) and sweet limes (3.5%) were also found to suffer from gummosis at the trunk base. Isolations from bark and root samples obtained from diseased acid lime and sweet lime trees showed association of 19 fungal species with these plants, with *Lasiodiplodia hormozganensis, L. theobromae* and *Fusarium solani* being the most common. When the most common fungi were inoculated on acid lime and sweet lime seedlings, *L. hormozganensis*, *L. theobromae*, *Neoscytalidium dimidiatum* and *F. solani* reproduced wilt, decline or gummosis symptoms on the inoculated plants. Isolations from 14 nursery seedlings of acid lime and sweet lime showed association of 12 fungal species, which implies that nurseries act as a main source for some citrus pathogens in Oman. The study reports for the first time sweet lime as a host for *L. hormozganensis* and *N. dimidiatum*. It also reports for the first time the presence of six new fungi to Oman. © 2014 Friends Science Publishers

**Keywords:** Mexican lime; Dieback; Wilt; Molecular characterization of fungi

## Introduction

Citrus species are among the most widely cultivated crops in different parts of the world (Wali *et al.*, 2013). Oman, which lies in the eastern part of the Arabian Peninsula, is characterized by arid and hot climate in most times of the year. Acid lime (*Citrus aurantifolia* S.) ranks the fourth crop in terms of production after date palms, mangoes and bananas in Oman. Other important citrus species include sweet lime (*C. limettioides* Tan.), orange (*C. sinensis* Osbeck), mandarin (*C. reticulata* Blanco) and grapefruit (*C. paradisi* Macf). Acid lime is grown in most parts of Oman with production being concentrated in Batinah region. Sweet lime is also grown in different parts of Oman, with Samael district being of great importance in sweet lime production.

Despite heavy reliance on citrus in Oman, especially acid lime, citrus species suffer from a number of disease problems, which include witches' broom disease of lime, *Citrus tristeza virus*, citrus gummosis and citrus viroids (Moghal *et al.*, 1993; Al-Sadi *et al.*, 2012a, b; Al-Harthi *et al.*, 2013). Preliminary surveys in Oman indicated association of dieback and wilt symptoms with some citrus trees in Oman (Moghal *et al*., 1993). However, little is still known about distribution of these disease symptoms in different citrus growing regions in Oman.

Previous studies have provided evidence for transmission of fungal pathogens of citrus via several means, including propagative material of citrus (Graham and Timmer, 1994). In Oman, farmers obtain citrus seedlings from government or private nurseries. Previous studies have provided evidence that citrus seedlings imported via private nurseries from different parts of the world could act as a source of *Citrus tristeza virus* (Al-Sadi *et al.*, 2012a). It is however not clear whether these nurseries could also potentially distribute citrus seedlings, which are affected by fungal pathogens.

*Phytophthora parasitica, P. citrophthora, P. palmivora* and *P. citricola*, *L. theobromae*, *Phomopsis citri* and *F. solani* are among the fungal pathogens most commonly associated with gummosis, dieback, wilt and other root diseases in citrus (Whiteside *et al.*, 1988; Graham and Timmer, 1994; Vernière *et al.*, 2004). Despite reports from other parts of the world, it is not clear whether pathogens associated with root diseases of citrus in other countries could also associate with root diseases of citrus under arid conditions of Oman.

The main objective of this study is to characterize fungal pathogens associated with root diseases in acid lime and sweet lime in Oman. Specific objectives include: (1) to characterize incidence of dieback associated with acid lime and sweet lime in Oman; (2) to characterize fungal

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pathogens associated with citrus and to examine their pathogenicity on citrus seedlings; and (3) to characterize the potential of nurseries as sources of fungal pathogens of citrus in Oman. Knowledge in these areas may help establish management strategies for this disease in Oman and other areas with similar climatic conditions.

## Materials and Methods

### Survey and Collection of Samples

Survey was carried out during December 2009 to May 2011 in 17 administrative districts (wilayats) located in 7 geographical regions in Oman (Fig. 1). During the survey, a total of 166 citrus-growing farms were visited, with at least 5 randomly surveyed farms per district. The survey was in the winter, with the exception of 5 districts (Buraimi, Mahadha, Salalah, Taqa and Marbat), which were surveyed in the summer of 2010. Acid lime and sweet lime were the two target species in the survey because of their importance in the Sultanate (MAF, 2005). During the survey, the incidence of dieback was recorded in acid lime and sweet lime grown in farms by determining the percentage of plants showing dieback symptoms. Samples of roots and bark were collected from acid lime and sweet lime developing symptoms of dieback. A total of 86 bark samples and 86 root samples were collected from acid lime trees while 65 bark samples and 70 root samples were collected from sweet lime trees.

A survey was also conducted in nurseries in Al Seeb area in March and June, 2011, in order to investigate pathogens associated with citrus seedlings originating from different countries. A total of 14 citrus seedlings (acid lime and sweet lime) suffering from dieback symptoms were collected.

### Pathogens Associated with Dieback

Isolations were carried out from root and bark samples, which were collected during the survey. Isolations were established from a total of 86 root and 86 bark samples collected from acid lime trees and 70 root and 65 bark samples collected from sweet lime trees.

Three different media were used for detecting pathogens associated with root and bark samples. The first media, denoted as PDA-R, was made up of 2.5% potato dextrose agar (PDA) amended with 10 mg $L^{-1}$ rifampicin. The second media, denoted as CMA-PARP, consisted of 1.5% corn meal agar (CMA) amended with 10 mg $L^{-1}$ pimaricin, 200 mg $L^{-1}$ ampicillin, 10 mg $L^{-1}$ rifampicin and 100 mg $L^{-1}$ pentachloronitrobenzene (Jeffers and Martin, 1986). The third media, denoted as CMA-PARPH, was made up of CMA-PARP and 50 mg $L^{-1}$ hymexazol.

All root and bark samples were washed with water to remove soil. The samples were cut into small pieces (5-10 mm) and disinfected with sodium hypochlorite (1%) for 2 minutes. Then, the pieces were rinsed with sterile distilled water and blotted dry on sterile filter paper. After that, root and bark pieces were placed on the three different media (PDA-R, CMA-PARP and CMA-PARPH) using two Petri-dishes for each sample and 4 pieces per Petri-dish. The plates were incubated at 25˚C up to 10 days. Fungal growth out of the samples was excised and transferred to new PDA-R plates. Pure cultures were obtained using mycelium tip culture (Al-Sadi *et al.*, 2011b).

Isolations were also carried out from samples of root and bark collected from nursery seedlings. Isolations were established from a total of 10 root and 10 bark samples collected from acid lime and 4 root and 4 bark samples collected from sweet lime. The isolation of fungi from root and bark samples of nursery seedlings was as explained previously.

### Identification of Fungal Species

Preliminary identification of fungi was based on morphological characteristics (Plaats-Niterink, 1981; Barnett and Hunter, 1998; Leslie and Summerell, 2006). However, identify of the isolated species was confirmed using sequences of the internal transcribed spacer region of the ribosomal DNA (ITS rDNA) following a modified protocol of Al-Sadi *et al.* (2011a).

About 80 mg of freeze dried mycelium was grinded using sterile sand. DNA extraction was as per a modified protocol of Lee and Taylor (1990) as described by Al-Sadi *et al*. (2011b). The ITS rDNA region was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The polymerase chain reaction (PCR) mixture consisted of PuReTaq Ready-To-Go PCR Beads, 0.4 µM ITS1 primer, 0.4 µM ITS4 primer, 2 µL of DNA and Milli-Q water up to a final volume of 25 µL. Samples were heated at 95˚C for 10 min followed by 35 cycles of denaturation at 95˚C for 30 s, annealing at 55˚C for 30 s and extension at 72˚C for 90 s. The final extension was performed at 72˚C for 10 min. Successful amplification of the ITS region was checked by running 5 µL of each reaction mixture on a 1.5% agarose gel in 0.5x Tris-borate-EDTA buffer (TBE) on a gel electrophoresis cell at 120 volts for 40 min (Fig. 2). The PCR products were purified from primers and dNTPs. Samples were then sent for sequencing in Macrogen, Korea using the primers ITS1 and ITS4.

The forward and backward ITS sequences of each isolate were first aligned together and edited using ChromasPro version 1.41. The resulting ITS sequence for each isolate was compared to other sequences of similar species which are deposited in NCBI (National Centre for Biotechnology Information) using BLAST search. Phylogram was constructed using the Kimura 2 parameter evolutionary model (Mega 5) (Tamura *et al*., 2011). Bootstrap 60% majority-rule consensus trees were generated using 1000 replications.

**Aggressiveness Test**

In order to test if the isolated fungi are of the pathogenic species on acid limes and sweet limes, the following experimental approach was followed. Pathogenicity tests were conducted only for pathogens which have an isolation frequency of more than 5% from bark or root samples. The isolated fungi were tested for pathogenicity on their original host species. To do that, *Bionectria ochroleuca, Conidiobolus coronatus, Fusarium equiseti, F. solani, Lasiodiplodia hormozganensis* and *L. theobromae* were examined for pathogenicity on acid lime seedlings while *B. ochroleuca, Cochliobolus hawaiiensis, F. solani, L. hormozganensis, L. theobromae, Neoscytalidium dimidiatum, Phoma* sp. and *Rhizoctonia* sp. were inoculated on sweet lime seedlings.

Six to twelve-month old acid lime and sweet lime seedlings, which develop no apparent symptoms of gummosis, dieback or weak growth were used for inoculation. An injury was made at the base line of the seedlings (7 × 7 mm) followed by inserting 5 mm mycelial plug obtained from the edge of 5-6 day-old culture of each isolate grown on potato dextrose agar (PDA) (Al-Sadi *et al*., 2010). Moistened sterile cotton was used to wrap the plug to avoid desiccation of the mycelial plug. Seedlings were placed in a glasshouse under 25°C in a completely randomized design. The aggressiveness test was done for each isolate, with 5 replicate seedlings per isolate. Where available, 2-4 different fungal isolates of the same species were used for inoculation and the aggressiveness test was repeated at least once. Inoculated seedlings were irrigated once every two days. Seedlings were observed for the development of gum, dieback or death on a weekly basis for 3-4 months. Isolations were established from seedlings developing any of the above symptoms in order to confirm association of the inoculated fungi with the observed symptoms. The isolations were done as explained previously.

## Results

**Survey and Collection of Samples**

Survey in different parts of Oman showed that dieback symptoms are widespread in acid lime trees and sweet lime trees in various districts in the country. Symptoms of the disease were characterized by weak growth associated with loss of leaves from the top of the branches towards the bottom and in some severe cases death of whole branches. Incidence of the disease was 8.8% in acid lime trees and 15.9% in sweet lime trees (Table 1). Symptoms of dieback were observed in 64.6% of acid lime farms and in 87% of sweet lime farms.

Gummosis symptoms were also observed in some farms. Typical symptoms of the disease in the field are in the form of gum which exudates from the lower 0.5- 1.0 m of the trunk and sometimes from branches. Incidence of this disease was 3% in acid in trees and 3.5% in sweet lime trees (data not presented).

**Pathogens Associated with Acid Limes and Sweet Limes**

Isolations from 86 bark and 86 root samples obtained from symptomatic acid limes yielded 11 fungal pathogens. *F. solani* was found to be the most common pathogen in roots of acid lime trees followed by *L. theobromae* and *L. hormozganensis* (Table 2). *Bipolaris nodulosa, B. spicifera, B. ochroleuca, Ceratobasidium cornigerum, C. coronatus, F. equiseti, N. hyalinum* and *Pythium acanthophoron* were found to have a low rate of recovery from roots and barks of the affected acid lime trees.

Isolations from 65 bark and 70 root samples obtained from symptomatic sweet lime trees yielded 12 different fungi. *F. solani, L. hormozganensis* and *L. theobromae* were found to have the highest rates of isolation from barks and roots of the affected trees, with the other pathogens having a low rate of isolation (Table 2).

Molecular characterization of *L. hormozganensis* showed that it is separated from *L. theobromae*, a closely related species, by a very high bootstrap support (100%) (Fig. 3).

**Pathogens Associated with Nursery Seedlings**

Isolation from citrus seedlings in nurseries showed association of 12 fungal species with the seedlings. *Fusarium solani* and *L. theobromae* were recovered from acid lime and sweet lime seedlings and were found to be the most common fungi in root and bark samples (Table 3). Other fungi included species of *Alternaria, Aspergillus, Bionectria, Cochliobolus, Coniothyrium, Exserohilum, Fusarium, Pythium, Rhizoctonia* and *Rhizomucor* (Table 3).

**Aggressiveness Test**

Inoculation of acid lime seedlings with 6 fungal species and with PDA plugs produced variable effects on the lime seedlings within 4 months of inoculation. *L. theobromae* resulted in production of gum and dieback symptoms in 10 and 40% of the inoculated seedlings, respectively (Table 4). *L. hormozganensis* resulted in dieback symptoms in 38% of the seedlings. *Fusarium solani* was less aggressive. It induced mortality in 9% of the inoculated seedlings. None of the other fungal species or the control produced symptoms on the inoculated acid lime seedlings (Table 4). *L. theobromae, L. hormozganensis* and *F. solani* were re-isolated from the symptomatic lime seedlings.

Inoculating sweet lime seedlings with 8 fungal species showed that *L. theobromae* is the most aggressive. It induced gummosis and dieback in 10 and 25% of the inoculated seedlings, respectively (Table 5). *L. hormozganensis, F. solani* and *N. dimidiatum* induced dieback and mortality in 50, 15 and 13% of the inoculated seedlings, respectively. Neither the control nor the other

**Table 1:** Incidence and distribution of dieback in acid lime and sweet lime in Oman

| Citrus sp. | No. of surveyed farms (% affected) | No. of surveyed trees (% symptomatic) | Distribution* |
|---|---|---|---|
| Acid lime | 149 (64.6) | 9432 (8.8) | BH, BK, BU, DB, IB, MA, SO, SH, SU, SM, NZ, QR, MH, MD, SL, MR, TQ |
| Sweet lime | 77 (87) | 2137 (15.9) | MH, BK, SU, SM, SH, BH, DB |

*indicates districts in which symptoms were detected, where BH is Bahla, BK is Barka, BU is Buraimi, DB is Dibba, IB is Ibra, MA is Madha, MD is Mudhaibi, MH is Mahadha, MR is Marbat, NZ is Nizwa, QR is Qurayat, SH is Shinas, SL is Salalah, SM is Samael, SO is Sohar, SU is Suwaiq and TQ is Taqa

**Table 2:** Frequency (%) of isolation of fungal and oomycete species from bark, root and soil samples of acid lime and sweet lime trees affected by dieback symptoms

| | Acid lime* | | Sweet lime* | |
|---|---|---|---|---|
| Pathogens | Bark (86) | Root (86) | Bark (65) | Root (70) |
| *Bionectria ochroleuca* | 21 | 16 | 19 | 20 |
| *Bipolaris nodulosa* | 5 | 0 | 5 | 0 |
| *Bipolaris spicifera* | 0 | 5 | 0 | 0 |
| *Cochliobolus hawaiiensis* | 0 | 0 | 19 | 0 |
| *Conidiobolus coronatus* | 0 | 5 | 0 | 0 |
| *Ceratobasidium cornigerum* | 0 | 5 | 0 | 0 |
| *Fusarium acuminatum* | 0 | 0 | 0 | 4 |
| *F. brachygibbosum* | 0 | 0 | 5 | 0 |
| *F. equiseti* | 11 | 16 | 5 | 8 |
| *F. solani* | 63 | 89 | 33 | 52 |
| *Lasiodiplodia hormozganensis* | nt | 28 | nt | 24 |
| *Lasiodiplodia theobromae* | 68 | 14 | 67 | 24 |
| *Macrophomina phaseolina* | 0 | 0 | 0 | 4 |
| *Nigrospora oryzae* | 0 | 0 | 5 | 0 |
| *Neoscytalidium dimidiatum* | 0 | 0 | 0 | 8 |
| *Neoscytalidium hyalinum* | 0 | 4 | 0 | 0 |
| *Phoma* sp | 0 | 0 | 14 | 0 |
| *Pythium acanthophoron* | 0 | 4 | 0 | 0 |
| *Rhizoctonia* sp | 0 | 0 | 0 | 12 |

*The sample size for the different soil, bark and root samples ranged from 53-86 samples obtained from 23 acid lime farms and 26 sweet lime farms. Frequency of recovery of pathogens is presented as percentage of farms with the pathogens. (nt) means not tested

**Table 3:** Frequency (%) of isolation of different fungal and oomycete species from bark and root samples of nursery citrus seedlings

| | Acid lime (Oman)* | | Sweet lime (Oman, Lebanon)* | |
|---|---|---|---|---|
| Pathogens | Bark (10) | Root (10) | Bark (4) | Root (4) |
| *Alternaria brassicae* | 10 | 0 | 0 | 0 |
| *Aspergillus calidoustus* | 0 | 0 | 25 | 25 |
| *Bionectria ochroleuca* | 10 | 10 | 0 | 0 |
| *Cochliobolus hawaiiensis* | 10 | 0 | 0 | 25 |
| *Coniothyrium aleuritis* | 0 | 0 | 25 | 0 |
| *Exserohilum rostratum* | 0 | 10 | 0 | 0 |
| *Fusarium equiseti* | 20 | 0 | 0 | 0 |
| *F. solani* | 10 | 10 | 25 | 50 |
| *Lasiodiplodia hormozganensis* | nt | 6 | nt | 15 |
| *L. theobromae* | 10 | 4 | 25 | 10 |
| *Pythium aphanidermatum* | 0 | 10 | 0 | 0 |
| *Rhizoctonia solani* | 10 | 0 | 0 | 0 |
| *Rhizomucor variabilis* | 10 | 0 | 0 | 0 |

*Countries within brackets indicate origin of the citrus seedlings while numbers within brackets indicate the sample size (number of seedlings). (nt) means not tested

fungal species induced symptoms on any of the inoculated

**Table 4:** Aggressiveness of fungal species on acid lime seedlings

| Fungal species | Gummosis | Dieback |
|---|---|---|
| Control | 0 | 0 |
| *Bionectria ochroleuca* | 0 | 0 |
| *Conidiobolus coronatus* | 0 | 0 |
| *Fusarium equiseti* | 0 | 0 |
| *F. solani* | 0 | 9 |
| *Lasiodiplodia hormozganensis* | 0 | 32 |
| *L. theobromae* | 10 | 40 |

Data, presented as percentage of diseased seedlings, represent average of at least two separate trials for each fungal pathogen

**Table 5:** Aggressiveness of fungal species on sweet lime seedlings

| Fungal species | Gummosis | Dieback |
|---|---|---|
| Control | 0 | 0 |
| *Bionectria ochroleuca* | 0 | 0 |
| *Cochliobolus hawaiiensis* | 0 | 0 |
| *Fusarium solani* | 0 | 15 |
| *Lasiodiplodia hormozganensis* | 0 | 50 |
| *L. theobromae* | 10 | 25 |
| *Neoscytalidium dimidiatum* | 0 | 13 |
| *Phoma* sp. | 0 | 0 |
| *Rhizoctonia* sp. | 0 | 0 |

Data, presented as percentage of diseased seedlings, represent average of at least two separate trials for each fungal pathogen

seedlings up to 4 months of inoculation (Table 5).

## Discussion

Survey in different parts of Oman showed that dieback symptoms are common in most acid lime and sweet lime farms. Although the overall incidence of the disease was 8.8% in acid limes and 15.9% in sweet limes, the disease was observed in 64.6% and 87% of acid lime and sweet lime farms, which were surveyed, respectively.

Isolations from barks, roots and soil samples of acid lime and sweet lime trees affected by dieback followed by pathogenicity tests have shown that four fungal species are pathogenic on acid limes and sweet limes. These include *L. theobromae*, *L. hormozganensis*, *N. dimidiatum* and *Fusarium solani*. In pathogenicity tests, *L. theobromae* and *L. hormozganensis* induced dieback and mortality in the inoculated acid lime and sweet lime seedlings. *N. dimidiatum* induced dieback and mortality in sweet lime seedlings and *F. solani* induced dieback and mortality in acid lime and sweet lime seedlings.

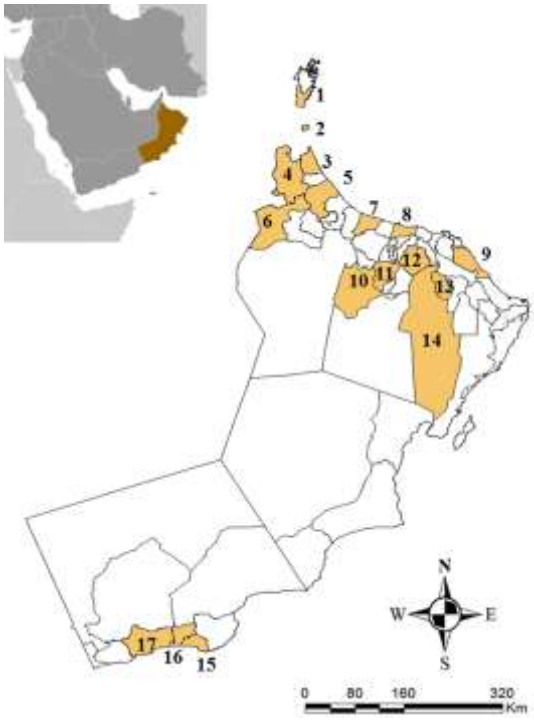


**Fig. 1:** A map showing the main districts which were surveyed. The districts are as follows: 1-Dibba, 2-Madha, 3-Shinas, 4-Mahadha, 5-Sohar, 6-Buraimi, 7-Suwaiq, 8-Barka, 9-Quarayat, 10-Bahla, 11-Nizwa, 12-Samael, 13-Ibra, 14-Mudhaibi, 15-Marbat, 16-Taqa and 17-Salalah

*L. theobromae* is most commonly associated with dieback and is consistently isolated from various tissues (twigs, bark, vascular tissue and fruits) of affected plants (Mullen *et al.*, 1991; Moghal *et al.*, 1993; Mohali *et al.*, 2005). *L. theobromae* has been reported to cause gummosis of *Jatropha podagrica* in China (Fu *et al.*, 2007) and root rot and collar rot disease on *J. curcas* in India (Latha *et al.*, 2009). Moreover, *L. theobromae* was isolated from citrus trees with dieback and gummosis symptoms in the Aragua valleys of Venezuela in 1996 (Ferrari *et al.*, 1996).

*Fusarium* spp. are known to be vascular pathogens and have been reported to associate with several plant species including Citrus (Timmer, 2000; Bashir and Tahira, 2012; Yaseen and D'Onghia, 2012). *N. dimidiatum* has also been found to associate with two re-grafted citrus orchards (*Citrus sinensis* (L.) Osbeck) on sour orange rootstock in Italy (Polizzi *et al*., 2011). *L. hormozganensis* has been reported as a pathogen of acid lime and mango (Abdollahzadeh *et al.*, 2010; Al-Sadi *et al.*, 2013). Our study reports for the first time sweet lime as a natural host for *L. hormozganensis* and *N. dimidiatum.*

Collection of samples from nurseries have shown that some of the citrus seedlings in nurseries could be potential sources of citrus pathogens. Most of the isolated fungi were similar to these which were recovered from acid lime and sweet lime trees in the field. Previous studies have shown

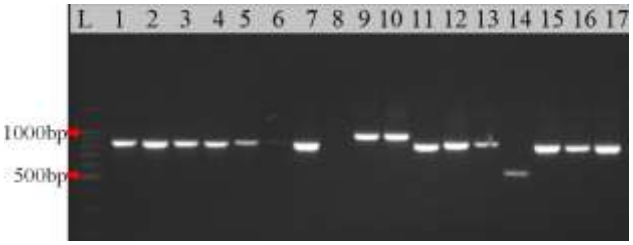


**Fig. 2:** Gel electrophoresis showing PCR amplification of 17 fungal isolates using primers ITS1 and ITS4. These samples were purified and subjected to sequencing in both directions

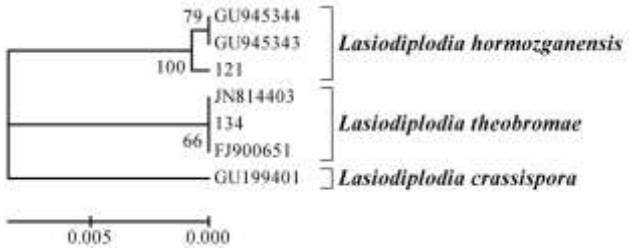


**Fig. 3:** Phylogram representing the relationship of Lasiodiplodia isolates (#121 and #134) from Oman to *L. hormozganensis,* and *L. theobromae* based on the combined EF 1α-ITS rDNA sequence data. Numbers within the tree represent the bootstrap values (values above 60% are indicated; 1000 replications). The tree was rooted to *L. crassispora*

that citrus seedlings imported from different countries could act as sources of citrus pathogens (Park *et al.*, 2003; Al-Sadi *et al.*, 2012a; Al-Harthi *et al.*, 2013). Governmental sectors should therefore implement strict quarantine measures as to prevent introduction of serious disease problems into our country.

In short, the study is the first to report sweet lime as a host for *L. hormozganensis* and *N. dimidiatum*. It also appears to report for the first time the occurrence of 6 new fungal species in Oman. These are *Bipolaris nodulosa, Ceratobasidium cornigerum, C. coronatus, N. dimidiatum, N. hyalinum* and *Pythium acanthophoron*. Since pathogenicity tests were not conducted for some of these fungi, future studies may consider evaluating pathogenicity of these fungi on citrus species in order to investigate the problems they may cause. Giving the fact that dieback of citrus is a complex problem and can be caused by other pathogenic organisms and environmental conditions, future studies may be required to address other factors responsible for dieback in Oman.

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