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ORIGINAL PAPER

First Record of *Leptoxyphium madagascariense* Causing Sooty Mould on Mango (*Mangifera indica* L.) in Egypt

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Abstract
Mango (*Mangifera indica* L.), known as the king of tropical fruits, is classified as an evergreen tree; belonging to the Anacardiaceae family that is widely cultivated in tropical and subtropical regions as a fruitful tree. It is widely cultivated in Egypt due to its high nutritional value, attractive fruits and delicious taste.
During the fall seasons of 2022 and 2023, unknown fungal growth was observed on the upper surfaces of mango leaves as a black coating layer with a velvety appearance (sooty mould). This layer forms along the leaf surface, on about 5-20% of mango trees growing in some orchards in Ismailia Governorate (Ismailia and Al-Qantarah Sharq County) and the Nubaria region Beheira Governorate. The associated fungus was isolated and identified as *Leptoxyphium madagascariense* (OQ345924) based on the morphological and molecular analysis. A pathogenicity study was performed according to Koch's postulates.
To our knowledge, this study is the first record of *Leptoxyphium madagascariense* as an associated agent of sooty mould on mango (*Mangifera indica* L.) tree in Egypt.
Keywords: Mango, *Mangifera indica* L., Sooty mould, Symptoms, Molecular and Morphological Identification, ITS-rDNA region, *Leptoxyphium madagascariense*.

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1. Introduction

Mangifera indica L. (Mango) is a tropical and subtropical tree belonging to the *Anacardiaceae* family, believed to be native to Southeast Asia, particularly in eastern India and Bangladesh. It is considered one of the most important and popular types of fruits grown in Egypt. Mango was introduced to Egypt during the reign of Muhammad Ali Pasha in 1825. It is grown commercially as a fruit tree because it produces edible fruits, which are a rich source of vitamins, minerals, and total soluble solids (Yahia *et al*. 2023). Numerous plant pathogens infect the mango tree all over the world especially in warm and humid climates causing various diseases (Prakash 2003, Ploetz 2003 and Ploetz, 2004).

In recent years, a fungal disease called sooty mould has been identified affecting mango trees. Sooty mould is caused by a wide range of non-phytopathogenic fungi that may appear within a single season or can persist for more than one season on wood (Daliyamol *et al.* 2021). It appears as a black layer on various parts of the plant, such as leaves, twigs and fruits, as well as the branches of numerous evergreen and deciduous trees and shrubs (Chomnunti, *et al.* 2014). These fungi feed manily on insect dew. Honeydew is a sweet, transparent, sticky substance secreted as waste by piercing-

sucking insects. Honeydew drops on tree leaves, covering the leaves and twigs with a sticky, sugary layer. The main component of honeydew is polysaccharides and it also contains small amounts of other nutritional compounds (Shukla *et al*., 2017). The sooty moulds grow on this and produce a thin superficial network of dense, dark hyphae. A heavy layer of black mould may accumulate on the leaves over several seasons.This layer blocks light, reducing the host plants' ability to photosynthesize. Sooty mould also predisposes to the emergence of more diseases due to increased humidity inside the canopy due to the mucous hypha retaining moisture for long periods (Chomnunti *et al*., 2014 and Shukla *et al*., 2017).

Most sooty mould species belong to the family *Capnodiaceae* of the order *Capnodiales*; class *Dothideomycetes* (Chomnunti *et al*., 2011 and 2014). Fungi belonging to this family produce separate, dark brown outer hyphae, which aggregate and form a thin web of mycelium on the surface of plants. Anamorph and teleomorph stages are usually found either on the same plants or on separate plants. In addition, the teleomorph stage for a few of them is not detected (Chomnunti *et al*., 2011 and 2014). The genus *Leptoxyphium* is a member of the *Capnodiaceae* family and is the asexual form in this family (Yang *et al*., 2014). *Leptoxyphium* genus is characterized mainly by superficial and irregular networks of mycelium resulting from spirally coiled hyphae, which expand into a funnel-shaped form with a terminal conidiogenous region and conidia ellipsoidal, hyaline, unicellular and become guttulate when mature (Chomnunti *et al*., 2011 and Yang , *et al*., 2014). There are many publications indicating that fungi belonging to the genus *Leptoxyphium*, commonly known as sooty mould fung, were isolated from the leaves of many species of trees and plants (Yang *et al*., 2014; Shankar *et al*., 2019 and Daliyamol *et al*., 2021). *Leptoxyphium madagascariense* Cheewangkoon & Crous isolated from leaves of *Eucalyptus camaldulensis* from the island of Madagascar has been described by Cheewangkoon *et al*. (2009). However, there is no report on *Leptoxyphium madagascariense* on mango leaves in Egypt or the world yet. Accordingly, this study aimed to describe and identify this fungus based on its morphological and molecular characteristics as a causative agent of sooty mould on mango in Egypt.

2. Materials and Methods

2.1. Isolation and Identification of fungi associated with mango sooty mould

To determine the causative agent of this phenomenon, leaves showing symptoms of sooty mould were collected from mango trees growing in some orchards in Ismailia Governorate (Ismailia and Al-Qantarah Sharq County) and the Nubaria region Beheira Governorate during the fall seasons in 2022 and 2023. Samples were kept in plastic bags and transferred to the laboratory within 24 hours. Isolation trials were carried out according to the method adopted by Dhingra and Sinclair (1985). Single spore isolation was performed by the spore suspension method as described by Puturak *et al*. (2014). To obtain pure fungal cultures, hyphae tips were taken from each developing colony and transferred to medium plates of potato dextrose agar (PDA) modified with the antibiotic streptomycin sulfate at a concentration of 100 mg/L. The morphological characteristics of the isolated fungus were examined based on the description provided by Abdel-Sater *et al*. (2018). The surface color of the colony was recorded, and microscopic examination was performed using an optical microscope for a 7-day-old colony. The mycelium and spore properties were examined for at least 30 measurements of the latter structure. The obtained fungal cultures were maintained at 5 °C for further studies .

2.2. Molecular Characterization

2.2.1. DNA extraction

To confirm initial identification based on morphological characteristics, the modified Dellaporta method protocol (Dellaporta *et al*., 1983) was used to extract the genomic DNA from the fungal isolate.

2.2.2. Polymerase chain reaction (PCR) and Nucleotide sequencing

PCR was performed with ITS4 and ITS5 following the described method by White *et al*., (1990). The internal transcribed spacer (ITS), was amplified using the ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′), primer pair as follows. The amplified PCR product was sequenced at the Macrogen Inc. Sequencing Service (Seoul, Korea). The sequence comparison was performed by search tool of the basic local alignment (BLAST, http://blast. ncbi. nlm. nih. gov/) with the nucleotide sequences available in the NCBI GenBank database (https:// www. ncbi. nlm. nih. gov/ genba nk/) to identify the closest available reference sequences. The ITS rDNA sequences of various species in the family *Capnodiaceae* were restored from GenBank for phylogenetic analyses. Then, the sequence of the obtained representative isolate was deposited in GenBank.

2.3. Pathogenicity Test

A pathogenicity study was carried out according to Koch's postulates. The fungal isolate was grown on potato dextrose agar (PDA) medium at 25°C for one week. Leaves of ten healthy 1-year-old seedlings of *M. indica* (var. Sukkary), germinated from seeds, were disinfected with 0.1% NaOCl, cleaned with water and allowed to dry. Leaves were divided into two groups: The first included leaves inoculated after being wounded by piercing them in different places with a sterile needle and then sprayed with a suspension of viable spores (1×10^6 conidia/ml); the second included spore suspension sprayed at the concentration mentioned before evenly on healthy unwounded leaves. Another five apparently healthy leaves were sprayed with aseptic filtered water as a check **(**Chen *et al*., 2022). The seedlings were covered separately with plastic bags to keep moisture for 48 hours and kept in the greenhouse conditions for 15 days.

2.4. Histopathological studies

Anatomical studies were carried out in order to study the relation between the plant tissue and the fungus. Samples of the wounded and unwounded leaves of mango var. Sukkary were taken 15 days after artificial inoculation as follows:

Samples were cut into small pieces (1 cm^2). The wounded and unwounded parts were immersed in formalin acetic acid alcohol solution (FAA) to kill and fix the tissues. Dehydration was carried in increasing series of ethanol, then clearified in xylol and finally embedded in paraffin wax (58-60 ^oC) according to the method described by Johansen (1940). Cross sections (15 µm thick) were carried out by the rotary microtome. The sections were fixed on a series of glass slides with Hoples adhesive. The sections were passed through regular xylol-alcohol concentration down to alcohol. Staining of sections was done using safranin and light green pigment. The sections were cleared in xylol and mounted in Canada balsam. Finally, the sections were examined microscopically.

3. Results

3.1. Disease Symptoms

Symptoms of sooty mould appeared on the upper surfaces of mango leaves. As a black coating layer with a velvety appearance. This layer is formed over the entire surface of the leaf, where the honeydew secreted by the piercing-sucking insects falls (Fig. 1A and B).

3.2. Isolation and Morphological Identification

Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium were inoculated with the fungal isolate and incubated at 25°C.The surface color of the 7-day-old colony was dark grey-brown, and produced numerous dark surface synnemata, with whitish masses of conidia (Fig. 2A). This fungus is mainly characterized by the presence of irregular superficial hyphae of septate mycelium, from which arise spirally twisted, unbranched hyphae called conidiomata, known as synnemata, aggregated or single, upright to slightly curved and consist of three parts: (1) thick base dark brown; (ii) The cylindrical portion, consisting of composed stalk of parallel, unbranched synnematous hyphae and occasionally consist of two to three synnemata on a single hyphal strand, 200-300 x 8-11µm; (n = 30) (iii) funnel-shaped hyphal apex, up to 50 µm tall, and 60 µm broad; (n = 30) (Fig. 2B and C). Conidiophores are cylindrical, subulate and septate, clustered in the top part of synnematous hyphae, which diverged near the apex and the width of the hyphae is (3–4.5 µm), septate, little dense-walled and flared at the top with rounded tops. Single-celled hyaline conidia, 1–3 guttules, rod shape, with rounded ends, 4.5–5 x 3–3.5 µm (n = 30) (Fig.2D).

3.3. Molecular Characterization

Another search was performed by GenBank BLAST and showed that the ITS sequence was up to 100% similar to *Leptoxyphium madagascariense* (MH 863407.1 and NR_137731.1) and 99.27% similarity with the strain type *L. madagascariense* (MG323879.1) on sugar cane juice, isolated from Assiut, Egypt (Table, 1). The phylogenetic tree was constructed using ITS sequences as shown in (Table 1 and Figure 3), the isolate obtained in this study was compared with isolates from other known sooty mould species in *Capnodiales* for example. *L. madagascariense* NR_137731, GQ 303277, MH863407 and MG323879 (100, 100, 100 and 99% sequence similarity, respectively), *L. kurandae* (99%), *L. glochidion* (99%), L. *fumago* (99%) and *Fumiglobus pieridicola* (91%), with no gap or only one gap in the isolated case MG323879. The *L. madagascariense* sequence OQ345924 formed a clade together with sequences from three *L. madagascariense* isolates (GQ303277, NR_137731 and MH863407) (Fig. 3). Furthermore, the ITS sequence of the representative isolate obtained has been deposited in GenBank (accession number OQ345924). Based on the morphological and molecular characterization carried out in this study, the sooty mould fungus isolated from mango leaves was identified as *L. madagascariense* Cheewangkoon & Crous, a sooty mould not previously reported on mango in Egypt.

3.4. Pathogenicity Test

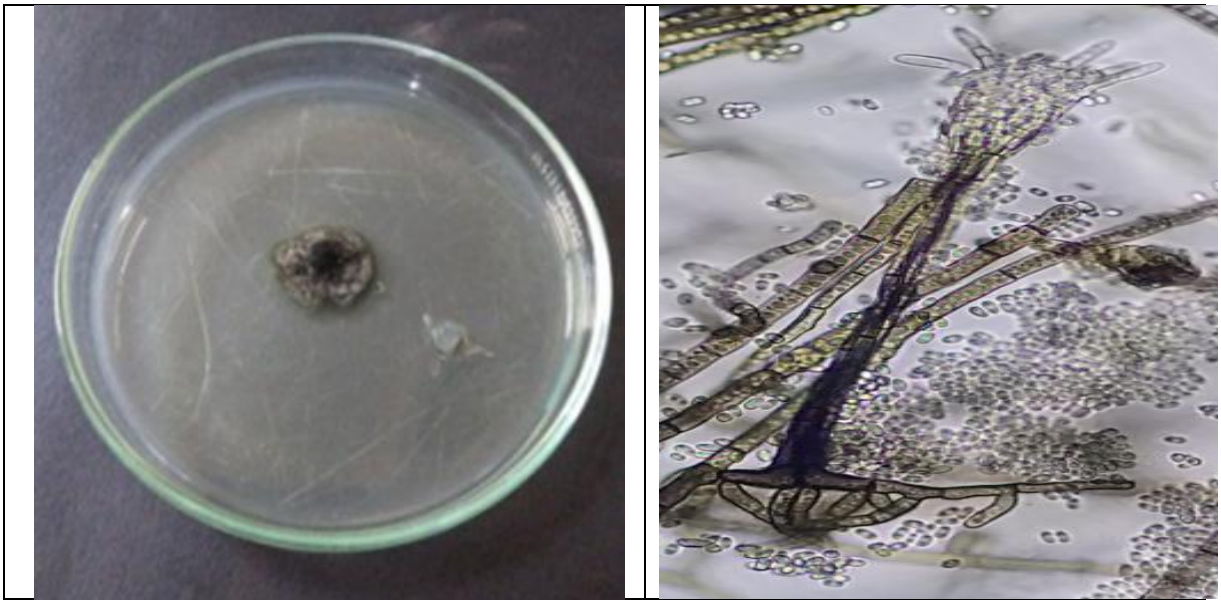
During the test period, the examined leaves of the seedlings were without disease symptoms, and no obvious damage was observed on the seedlings, and the control plants remained without symptoms.

3.5. Histopathological studies

Sections prepared in the inoculated leaves showed no penetration or infection sites. Also, the fungus did not enter in the leaf tissues, but was found only on the leaf surface

(A)	(B)

Figure 1: Mango sooty mould caused by the fungus *Leptoxyphium madagascariense*.
(a) Typical symptoms of sooty mould initiation and appearance of superficial mycelium on leaves. (b) The appearance of honeydew on the leaves



A

B

C

D

Figure 2: Macroscopic and microscopic characteristics of sooty mould colony caused by *Leptoxyphium madagascariense* OQ345924.
(A) Upper surface of the colony on potato dextrose agar (PDA), one week old.
(B) Synnematous conidiophores, X 400.
(C) Final part of the conidiophore releases spores, X 400.
(D) Hyaline conidia formed on culture plates, X 400.

Table 1. GenBank accession number, closest related species , maximum identity (%), number of bp analyzed, gaps, host, and reference for closest matches in GenBank database of *L. madagascariense* strain EGYARC1 isolated from mango leaves in Egypt (accession GenBank number OQ345924, length of base pairs = 420).

GenBank accession number	Closest related species	Maximum identity (%)	N° of bp analyzed	Gaps	Host	Reference
NR_137731	*Leptoxyphium madagascariense*	420/420(100%)	627	0/420(0%)	*Eucalyptus camaldulensis*	Cheewangkoon, *et al*., 2009
GQ303277	*Leptoxyphium madagascariense*	420/420(100%)	627	0/420(0%)	*E. camaldulensis*	Cheewangkoon, *et al*., 2009
MH863407	*Leptoxyphium madagascariense*	420/420(100%)	544	0/420(0%)	-----	Vu, *et al*., 2019
MG323879	*Leptoxyphium madagascariense*	407/410(99%)	545	1/410(0%)	sugarcane juice	Abdel-Sater, *et al*., 2018
JF951150	*Leptoxyphium kurandae*	416/420(99%)	689	0/420(0%)	*Eucalyptus* sp.	Crous, *et al*., 2011
KF826942	*Leptoxyphium kurandae*	391/395(99%)	519	0/395(0%)	Insect gut	Kajale, *et al.*, 2015
KP992873	*Leptoxyphium kurandae*	386/390(99%)	503	0/390(0%)	Kenaf	Choi, *et al.*, 2015
MH756173	*Leptoxyphium kurandae*	362/367(99%)	497	0/367(0%)	*Thespesia populnea* (L.) Sol. ex Correa	Sripriya, *et al*., 2019
KF982310	*Leptoxyphium kurandae*	416/420(99%)	539	0/420(0%)	*Psidium guajava*	Yang, *et al*., 2014
KM226890	*Leptoxyphium kurandae*	387/390(99%)	504	0/390(0%)	*Hibiscus rosa-sinensi*	Park, *et al*., 2014
KF982307	*Leptoxyphium glochidion*	416/420(99%)	541	0/420(0%)	*Glochidion wrightii*	Yang, *et al*., 2014
NR_155316	*Leptoxyphium glochidion*	416/420(99%)	541	0/420(0%)	*G. wrightii*	Yang, *et al.*, 2014
KX289331	*Leptoxyphium fumago*	416/420(99%)	7962	0/420(0%)	---	Kellner, *et al*., 2016
KF263961	*Fumiglobus pieridicola*	366/404(91%)	540	9/404(2%)	*Pieris japonica*	Bose, *et al*., 2014

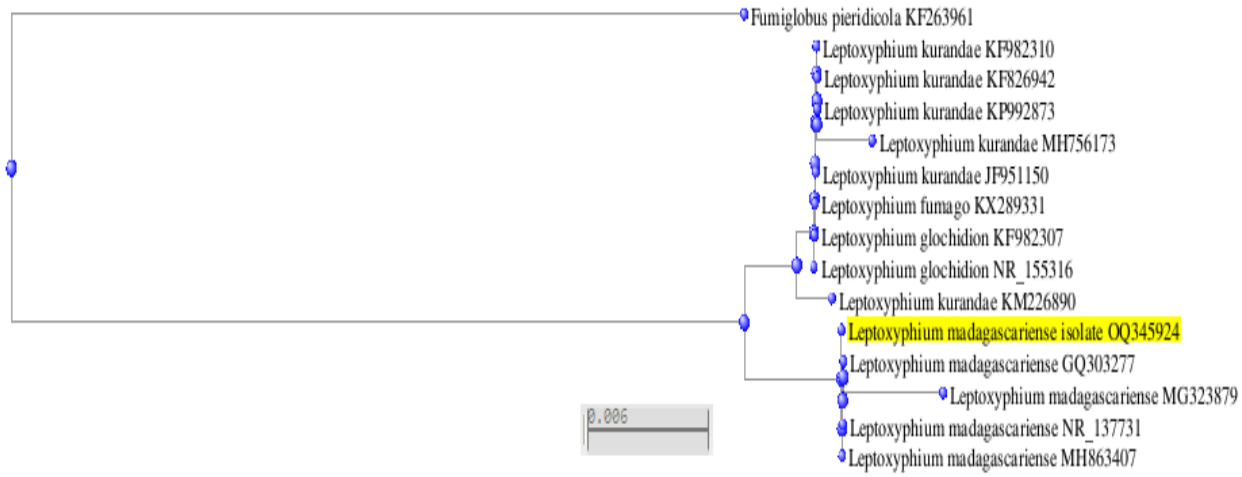


Figure 3. Phylogenetic tree of *Leptoxyphium madagascariense* isolate OQ345924 with the related species of *Leptoxyphium*. As shown in the yellow shaded square notes the sequence obtained from the current study. *Fumiglobus pieridicola* KF263961 was used as an outgroup.

4. Discussion

Sooty mould is caused by a wide range of non-parasitic, non-phytopathogenic fungi that can persist within one season or persist for more than one season on branches (Crous *et al*., 2009). This study aimed to find out the cause of sooty mold on mango leaves. Symptoms of sooty mould were a thin, black, velvety coating on the upper surfaces of mango leaves, covering the whole leaf, and sometimes it may appear in flakes. In advanced cases, the canopy turns completely black, with mould appearing on the whole surface of the leaves and branches. Finally, the leaves curl and wilt when exposed to periods of drought. During the pathogenicity test, the leaves of mango seedlings inoculated with the fungus were asymptomatic, and no symptoms of sooty mould were observed on the seedling. This can be explained by the fact that the fungi that cause sooty mould reproduce on the "honeydew" and spread on the plant canopy, making it black, and the severity of the incidence depends on the secretion of sugar by the insects (Misra *et al*., 2012). Although the sooty mould is caused by saprophytic fungi, it does not cause any direct damage to the plant. But it is indirect damage, as it affects the performance of the photosynthesis process inside plant by preventing sun's rays from reaching the chloroplasts, and thus affects plant growth (Scott, 2008).

This leads to an economic loss resulting from a lack of flowers and thus a decrease in yield (Chomnunti *et al*., 2014). The genus *Leptoxyphium* is typical of sooty moulds found in a wide range of hosts, but it is non-pathogenic because its mycelium lives on the leaf surface and does not enter the host's tissues for nutrition but takes it from the "honeydew". Consequently, the damage is indirect because the fungus grows and affects the plant's vital processes by reducing the area of the leaves that are effective in photosynthesis (Misra *et al.*, 2012). The genus *Leptoxyphium* is a member of the *Capnodiaceae* family, and includes saprophytic fungi commonly associated with insects that secrete honeydew. The honeydew is an essential food source for the growth of sooty mould (Chomnunti *et al*., 2011). Whereas, Daliyamol *et al*., (2021) confirmed that *Leptoxyphium* sp. is fungus closely associated with insect feeding which infesting coconut palm leaves in coconut gardens from Kerala, India. Also, members of the genus *Leptoxyphium* perhaps derive their food from extrafloral nectarines as *L. kurandae* has been isolated from the sooty mould of leaves of Chinese hibiscus (*Hibiscus rosa-sinensis*) and kenaf. Where there was a relationship between sooty mould and extrafloral nectarines (Park *et al*. 2014 and Choi *et al*. 2015). Interestingly, *L. kurandea* was recovered from the gut of an insect (dusky cotton bug) from India (Kajale *et al*. 2015). Therefore, it can be assumed that the members of the genus *Leptoxyphium* are not host-specific. Whereas, six species of the genus *Leptoxyphium* have been recorded in India on many crops such as; *L. axillatum, L. bahiense, L. fumago, L. graminum, L. longispora* and *L. zeae* (Kajale *et al*. 2015). *L. madagascariense* Cheewangkoon & Crous has also been described from the island of Madagascar, on *E.camaldulensis* leaves (Cheewangkoon *et al*. 2009).

Different fungal species have been reported to cause sooty mould in mangoes which include *Capnodium roseum* (Hamid and Jalaluddin, 2006), *Trichomerium foliicola* (Chomnunti, *et al.*, 2012), *Capnodium mangiferae* (Rebolledo-martínez, *et al.*, 2013) and *Meliola mangiferae* (Parida *et al.* 2019). In addition, the fungus *L. fumago* was reported on mango leaves in India (Misra *et al.*, 2012). In Egypt, the only reported case of *Leptoxyphium madagascariense* was its isolation from sugarcane juice in Assiut, Egypt (Abdel-Sater *et al*., 2018). However, the presence of *L. madagascariense* on mango has not been reported so far; hence, our current study is the first to indicate the association of this fungus with mango in Egypt.

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