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First Report on *Purpureocillium lilacinum* Infection of Indoor-Cultivated Morel Primordia

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Abstract: The cultivation of morel mushrooms (*Morchella* spp.) outdoors or in controlled indoor systems is a relatively new practice, and infections are beginning to be observed. Infection of indoor-cultivated *Morchella rufobrunnea* initials (primordia) occurred at our research facilities in Israel. The mushroom initials turned brown, were covered with a dense white mycelium of a foreign fungus and were disintegrated soon after. The isolation of a fungal contaminant from the infected mushroom revealed small colonies with a pinkish spore zone on potato dextrose agar medium. Molecular identification using partial large subunit 28S ribosomal DNA and rRNA internal transcribed spacer sequences identified the fungus as *Purpureocillium lilacinum*. Inoculation of Morchella colony on agar plat with the isolated fungus caused browning and inhibition of mycelial growth. Inoculation of a healthy primordium with *P. lilacinum* spores resulted in its browning and deterioration. This is the first report of an infection of indoor-cultivated mushroom and the first showing *P. lilacinum* as a pathogen of morels.

Keywords: *Morchella*; morel; mycoparasite; *Purpureocillium lilacinum*

Citation: Masaphy, S. First Report on *Purpureocillium lilacinum* Infection of Indoor-Cultivated Morel Primordia. *Agriculture* **2022**, *12*, 695. https://doi.org/10.3390/agriculture12050695

Academic Editor: Rosario Nicoletti

Received: 17 April 2022
Accepted: 13 May 2022
Published: 14 May 2022

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1. Introduction

Morchella species mushrooms (morels) (Ascomycota, Pezizales) are known for their delicate taste and aroma and for their wide range of antioxidant and health-related biological activities [1,2]. These edible mushrooms are mostly harvested from their natural habitat. For over a century, efforts have been made to cultivate them. However, due to the complex life cycle of this genus and a limited understanding of the factors affecting the fruiting process, the first report on indoor morel cultivation was only published in 1982 by Ower [3], followed by Ower et al. [4] in 1986. Several companies (e.g., Gourmet Mushrooms Inc.) then successfully grew it in the United States. In 2010, a successful indoor cultivation of *Morchella rufobrunnea* in a soilless system was achieved at our research facilities in Israel [5]. At the same time, reports of outdoor cultivation systems began to emerge, mainly in China, as summarized by Liu et al. [6] and Sambyal and Singh [7], showing controlled fruitification of *Morchella importuna*, *Morchella sextelata*, *Morchella eximia* and *Morchella conica*.

With the increasing cultivation of morels in artificial systems, infections began to appear. Commercial indoor cultivation of morels in the United States was completely abandoned due to bacterial contamination and reduced output [8], although recently, reports on indoor cultivation are reemerging [9]. In China, home to the world's largest outdoor cultivation of *Morchella* (over 1200 ha in 2015–2016), there have also been reports of infections caused by fungi, including *Fusarium incarnatum–equiseti* species complex [10], *Diploöspora longispora* [11], *Paecilomyces penicillatus* causing white mold disease and infecting cultivated *M. importuna* [12,13], as well as *Cladobotryum protrusum* [14], among other pathogens [15]. There are no sufficient studies regarding morel diseases in indoor cultivation systems to date.

In 2016, infected young fruiting bodies of morels were observed for the first time in the growing bed of our indoor cultivation facility. The growing bed surface was covered

with white mycelium, which also infected the young fruiting bodies (ascocarps of up to 2 cm in height), resulting in ageing and disintegration after severe browning. The aim of this work was to isolate and identify the contaminating fungus and to study its possible pathogenicity to the morel primordia.

2. Materials and Methods

2.1. Isolation of the Contaminating Fungus

The suspected pathogenic contaminating fungus was isolated from infected young fruiting bodies of *M. rufobrunnea* developing in a growth chamber at 17–20 °C and 90% air humidity. The contaminated ascomata were cut to reveal surface-underlying tissue and smeared aseptically on rose-bengal agar medium (Oxoid, Basingstoke, UK) to obtain separate, defined colonies and to limit the spread of the isolated filamentous fungal colonies [16]. The medium was supplemented with 200 mg/L chloramphenicol to prevent bacterial growth. Plates were incubated at 22 °C, in darkness. After 3 days, individual colonies from germinated spores were observed. Spores from the colonies were re-inoculated on rose-bengal-chloramphenicol medium to obtain colonies originating from single spores. The mycelium from these colonies was transferred to fresh potato dextrose agar (PDA, Difco) medium and regrown.

2.2. Microscopic Observation and Molecular Identification of the Contaminating Fungus

The morphological characterization of the isolated fungus was based on microscopic observation of 7-day-old colonies grown on PDA medium. A glass coverslip was overlaid on the surface of the colony and transferred to a microscope slide, where the fungus was stained with lactophenol cotton blue for microscopic observations.

For molecular identification of the fungus, fresh spores bearing mycelia were used. The surface part of the grown colony was removed from the agar medium, washed with sterile DW and centrifuged. Genomic DNA was extracted from the fungal pellet using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. DNA isolation was performed for three single colonies growing on PDA. The primer pairs LROR + LR6 and ITS1 + ITS4 were used to amplify the large subunit (LSU) ribosomal DNA region and the rRNA internal transcribed spacer (ITS) region, respectively, for phylogenetic analyses [17]. PCR amplification was carried out using a Flexigene thermocycler (Techne, UK) under the conditions described by Raja et al. [17] using 10 µL HY-Taq ReadyMix *2x (Hylabs, Rehovot, Israel), 0.5 µL of each primer, 2 µL DNA (50 ng/µL) and 7 µL nuclease-free water. PCR reactions involved 35 cycles at 94 °C for 1 min, 54° for 1 min and 72° for 1 min. Amplification products were sequenced by HyLabs (Israel). The sequences of the partial 28S LSU gene and the rRNA ITS region of the isolated contaminant, designated 1925-1, were submitted to GenBank (accession numbers OK178301 and OL911053, respectively). The partial 28S LSU gene and rRNA ITS region sequences were assembled and edited by SeqMan program.

2.3. Pathogenicity Test (Koch's Postulates)

The pathogenicity of the isolated *P. lilacinum* on *Morchella* was assayed by inoculation of either *Morchella* mycelial culture grown on PDA medium or the primordial stage of ascocarps produced in the growing system with *P. lilacinum* spores. In the first system, after 3 days of *M. rufobrunnea* mycelial growth at 22 °C on PDA medium, 2–5 µL of *P. lilacinum* spore suspension was applied on the colony surface. Changes in the colony morphology of *M. rufobrunnea* with continued incubation as above were monitored for 72 h. A control culture of healthy *M. rufobrunnea* without the isolated fungus was also grown. In the second assay, an aqueous suspension of 10^5 spores/mL from 7-day-old pre-cultured *P. lilacinum* was spray inoculated on freshly produced 3-day-old *M. rufobrunnea* primordia (3–8 mm long) in an indoor cultivation system. Phenotypic changes in the infected primordia were inspected. Three days after inoculation, the infected primordia were removed from the growing system and placed on PDA in a Petri dish to re-isolate the fungus and enhance

the pathogenicity test. In addition, the contaminating fungus was re-isolated directly from infected tissue of the primordia as described above in Section 2.1.

3. Results and Discussion

The infection of *M. rufobrunnea* was first observed in the growing room, with *Morchella* ascocarps already infected in their early stages of development (Figure 1). The surface of the growth substrate, as well as the initials and young ascocarps, were covered with white mycelium. The ascocarps ceased to increase in size, turned dark brown and disintegrated.

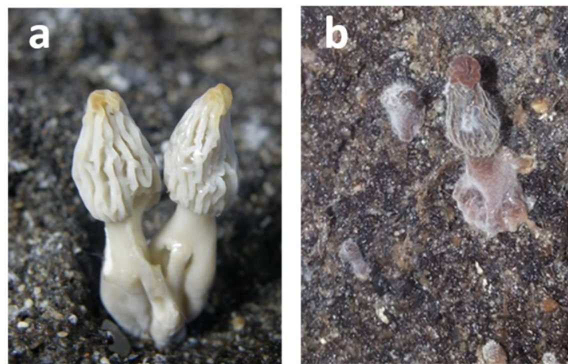

Figure 1. Healthy (**a**) and infected (**b**) young fruiting bodies of *M. rufobrunnea*.

The isolation of the contaminating fungus on rose-bengal medium and further transfer to PDA medium resulted in defined colonies with white mycelium at the edge and pinkish conidia at the center (Figure 2a). The morphological observation of the fungal colony showed typical conidiophores and phialides with chains of ellipsoid conidia (Figure 2b), in accordance with *P. lilacinum* characterization [18].

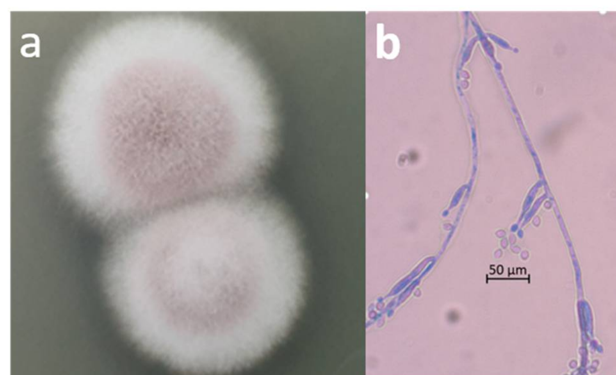

Figure 2. Colony of isolated fungus *P. lilacinum* 1925-1 on PDA (**a**) and conidiophores bearing ellipsoid conidia at 1000× magnification (**b**).

Molecular analysis yielded a 1000 bp partial sequence of the 28S ribosomal DNA region and a 591 bp sequence of the rRNA ITS region. Both sequences were aligned with several sequences from the GenBank database. For both regions, the highest similarity occurred with several *P. lilacinum* sequences. The ITS region of *P. lilacinum* 1925-1 showed 100% identity with the ITS region of range of *P. lilacinum* strains isolated from different substrates that were considered for species characterization by Luangsa-Ard et al. [18] (Table 1).

Table 1. Sequence homology of the rRNA ITS regions of isolate 1925-1 with previously identified *P. lilacinum* strains considered by Luangsa-Ard et al. [18].

NCBI Accession No.	Homology (%)	From
HQ842812	100	Nematoda
HQ842815	100	Other
HQ842816	100	Human
HQ842817	100	Entomogenous
HQ842819	100	Nematoda
HQ842820	100	Miscellaneous
HQ842821	100	Other
HQ842824	100	Human
HQ842825	100	Entomogenous
HQ842828	100	Human
HQ842838	100	Environmental
HQ842841	97.51	Environmental

After identifying the isolated fungus, we further confirmed its pathogenic activity against *M. rufobrunnea* using an in vitro agar plate system. When spores of the isolated fungus were applied on the surface of an already established 3-day-old *M. rufobrunnea* colony, the mycelium turned brown at the site of application within 1 day (Figure 3a). The browning process expanded with time, indicating toxic activity of the isolated fungus, which produced a white and dense mycelium on top of the *M. rufobrunnea* mycelium (Figure 3b, showing the culture 72 h after adding the isolated fungal spores). *M. rufobrunnea* mycelium, which was far (>1.5 cm) from the *P. lilacinum* inoculation point, continued its normal development, producing sclerotia (Figure 3b).

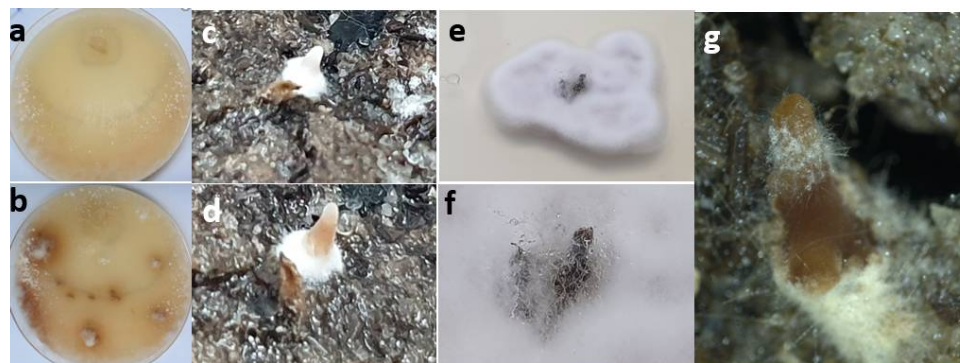

Figure 3. Pathogenicity assays of *P. lilacinum* 1925-1 against *M. rufobrunnea*. (**a**,**b**) *M. rufobrunnea* mycelial culture after inoculation of *P. lilacinum* spores on day 3 of growth (**a**) and 72 h later (**b**); (**c**,**d**) Young primordia in the growing system before (white, 3–8 mm long) (**c**) and 48 h after (**d**) inoculation with *P. lilacinum* spores; (**e**) Infected primordia with established *P. lilacinum* colony after transfer from the growing system to PDA medium; (**f**) Infected primordia covered by *P. lilacinum* hyphae on PDA; (**g**) Infected primordia on cultivation soil.

We then confirmed the pathogenicity of the isolated *P. lilacinum* against the developing *M. rufobrunnea* primordia in the mushroom growing system. The life cycle of Morel mushroom has been described by Volk and Leonard in 1990 [19], as well as in a work from our lab [20]. The primordial stage was chosen over the mature mushroom stage for the Koch's test because infection of the morels in the cultivation system was originally observed at this stage of ascocarp development. An aqueous spore suspension of *P. lilacinum* was

sprayed on the soil surface and primordia when they were still white and only 3–8 mm long (Figure 3c). Two days later, the primordial head turned brown and ceased to develop (Figure 3d). A few days later, white hyphae were observed to cover the young brown primordia (Figure 3g). Bbrown primordia were transferred to PDA medium, resulting in growth of a typical *P. lilacinum* colony on the infected primordium (Figure 3e), which eventually disintegrated under the isolated fungal hyphae (Figure 3f).

The results indicated that the isolated *P. lilacinum* 1925-1 spores could reproduce the disease symptoms, that is, browning of the mycelium and the developing ascocarp and further degradation of the *Morchella* tissue (Figure 3g), similar to those observed on the originally infected mushrooms from which *P. lilacinum* was isolated. The browning may reflect oxidative enzyme activity under stress conditions in the infected *Morchella*, similar to other cases of mushroom infection [21,22]. However, it should be noted that, in contrast to the current study, ascocarp browning was not prominent when *M. importuna* was infected with the pathogenic fungus *Diploöspora longispora* in an artificial inoculation assay [12].

The identified contaminating fungus *P. lilacinum* belongs to the phylum Ascomycota, subphylum Pezizomycotina, order Hypocreales, family Ophiocordycipitaceae [18]. This family contains many parasitic fungi. Originally designated *Paecilomyces lilacinus* (Thom) Samson, this fungus was later given the name *P. lilacinum* [18,23]. However, in several reports, it is described using its former designation of *P. lilacinus*, a biological control agent against root-knot nematodes and cotton aphids [24,25]. In a wide survey conducted by Luangsa-Ard et al. [18], *P. lilacinus* species were found to share sequence similarity with *P. lilacinum.*

Many recent reports have shown antifungal activity of *P. lilacinum* against a range of fungi, such as *Phytophthora infestans* [26], gray mold [27], *Verticillium dahliae* [28], *Sclerotinia sclerotiorum* [29] and green mold (*Penicillium digitatum*) [30], making it a candidate biocontrol agent against phytopathogenic fungi. Ali [31] reported that the filtrate of *P. lilacinum* inhibits *Stromatinia cepivora* mycelial growth and sclerotium formation. Moreover, treating soil with the filtrate caused a loss of *S. cepivora* sclerotial activity and significantly decreased disease incidence and severity. Whole-genome sequencing has additionally elucidated some of its mycoparasitic activities [32].

This is the first report of the isolation and identification of the fungus *P. lilacinum* on cultivated morel and, specifically, in indoor cultivation. A phenotypically similar disease of *M. importuna* cultivated outdoors was reported by He et al. [12], where the contaminating fungus was identified as *Paecilomyces penicillatus*. As in earlier publications, where the species name *P. lilacinus* was replaced by *P. lilacinum* [20,21], it is suggested that the *P. penicillatus* identified by He et al. [12] and the *P. lilacinum* reported in the present study may be related.

The results presented here show that *P. lilacinum*, a mycoparasitic fungus, attacks the vegetative mycelium, as well as young morel mushrooms. While *P. lilacinum* is naturally disseminated in the environment, it is also spread artificially for crop-pest management [33]. This is an important point when considering outdoor cultivation of morels, as it damages mushrooms, similar to the case of another mycoparasitic fungus, *Trichoderma*, from the same order (Hypocreales), which caused infections in the mushroom industry [34] while being used as a beneficial biocontrol agent for crops [35].

The same characteristics that define *P. lilacinum* as a good candidate for biocontrol of pathogenic fungi make it harmful to cultivated morel mushrooms. Further studies should be performed to prevent or control such contaminants. As with other indoor-cultivated mushrooms, treatments of all facilities, including growth substrates and the air, are required.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The author thanks Limor Zabari for technical assistance and the MIGAL research authority for support of the research.

Conflicts of Interest: The author declares no conflict of interest.

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