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Characterization and Pathogenicity of Phytopathogenic Fungi Associated with *Pinus pinea* in Northeastern Tunisia: Implications for Forest Health in the Mediterranean Basin

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Abstract

Phytopathogenic fungi are significant contributors to dieback in various plant hosts. During surveys in Tunisian forests, *Pinus pinea* trees were observed exhibiting symptoms such as dieback, leaf spot, necrosis, trunk cankers, and canopy wilt, though these occurrences remain underreported. This study aimed to identify the causal agents of *P. pinea* disease in Northeastern Tunisia. Three fungal morphotypes were isolated from symptomatic branches and identified as *Diplodia seriata*, *Heterotruncatella spartii*, and *Pestalotiopsis biciliata* through morphological characterization and molecular analysis of the internal transcribed spacer (ITS) region. Pathogenicity tests demonstrated the aggressiveness of all three isolates. These findings highlight the potential threat posed by these fungi to the health of pine forests in the Mediterranean region. Further research is needed to better understand the impact of these pathogens on stone pine trees and to develop effective biological control strategies.

Keywords – Dieback – *Diplodia seriata* – *Heterotruncatella spartii* – *Pestalotiopsis biciliata* – *Pinus pinea*

Introduction

In the Mediterranean Basin, forest trees, including pine species, are the most explored natural resources, giving us various beneficial services, making this ecosystem crucial for socio-economic development (EFI 2020). In Tunisia, *Pinus pinea* (stone pine) covers an area of around 21.165 ha (El Khorchani 2010). It is among the most widely used forest species in the reforestation program after *Pinus halepensis* (Aleppo pine) (Sghaier et al. 2006). Since 2017, pine trees have decreased due to abiotic and biotic factors, including drought, pests, viruses, and fungi. Forest disease is arising because of the impact of climate change, mainly global warming, followed by a decrease in its

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defence ability and the establishment of favourable conditions for infection by biotic pathogens agents (De Sousa et al. 2008). The severity of tree diseases caused by pests is expected to increase with drought (Gautam et al., 2013). Various fungal pathogens can cause severe disease and persist in latency inside the living tissue of their plant hosts (Franceschini et al. 2005). The most widespread and aggressive species mainly belong to Amphisphaeriaceae and Botryosphaeriaceae (Deidda et al. 2012). Few studies focus on diagnostic methods for detecting phytopathogenic fungi linked to pine tree dieback in Tunisia, North Africa. Hence, the objective of the present work was to characterize the causal agents associated with stone pine diseases.

Materials & Methods

Sampling and fungal isolation

In total, 43 stone pine trees were examined in Northeastern Tunisia (Nabeul Forest, 36.30'.406" N; 10.38'.780"E). Ten symptomatic branches were collected from each declining *P. pinea* tree and transferred to the laboratory for fungal isolation. Samples (2 × 2 mm) taken from necrotic tissues of each branch were surface-disinfected and placed in potato dextrose agar (PDA) plate containing streptomycin sulphate (0.05 g/l) and subsequently incubated at 25°C in darkness for three days. Cultures were purified by the hyphal tip technique and incubated under the conditions described above (Hlaiem et al. 2020).

Fungal identification

The culture and microscopic characteristics of the obtained isolates were recorded according to Phillips et al. (2007) and Maharachchikumbura et al. (2017). Based on morphological features, one isolate from each morphotype was chosen for molecular analysis. Total DNA from fresh colony grown on PDA was extracted using an innuPREP Plant DNA Kit according to the manufacturer's instructions (Analytik Jena AG, Germany). The ITS rDNA region was amplified using the universal primer pairs: ITS1/ ITS4 (White et al. 1990). PCR products were sent for Sanger sequencing at the Laboratory of the Interdepartmental Center for Biotechnology Services of Agricultural, Italy. New sequences were edited with FinchTV v1.4.0 (http://geospiza.com/ finchtv) and compared with those deposited in GenBank through BLAST searches (http://ncbi. nih.gov/; accessed on February, 2024).

Phylogenetic Analysis

The ITS sequences were aligned using ClustalX v. 1.83 (Thompson et al. 1997). The ITS sequences obtained in this study were supplemented with further sequences retrieved from GenBank through BLAST searches. Phylogenetic trees were generated using the Neighbour-Joining (NJ) method with MEGA v. 6 (Tamura et al. 2013). New sequences in this study were deposited in the National Center for Biotechnology Information (NCBI) (https://ncbi.nlm.nih.gov/) under accession numbers.

Pathogenicity test

Pathogenicity assays were achieved by inoculating the fungal pathogens on 3-year-old *P. pinea* seedlings grown in plastic pots (five seedlings for each fungal pathogen inoculation) at the nursery in natural conditions using Hlaiem et al. (2021) methods: briefly, a mycelial plug (3–4 mm²), taken from the growing colony on PDA of the isolate, was placed in a shallow wound on the stem of each seedling. Five control seedlings of pine species were inoculated with PDA plugs in similar wounds. The inoculation points were covered with Parafilm for a week.

Results & Discussion

Symptoms of dieback

In the field, 67% of the investigated *P. pinea* trees revealed a branch canker. The frequently noticeable symptoms include defoliation, yellowish brown needles, canopy wilt, shoot blight,

necrotic lesions in wood, and branch cankers (Fig. 1). Accordingly, similar symptoms have been previously described on kermes oak shrubs in Greece (Tsopelas et al. 2010), on stone pine trees orchards in Portugal (Silva et al. 2020), and on Aleppo pine trees in Tunisia (Hlaiem et al. 2022).

Fig. 1 – Disease symptoms on *Pinus pinea*. a Symptomatic trees with defoliation and canopy wilt. b Necrotic lesions detected in naturally infected branches.

Molecular identification

Morphological characterization of the three representative isolates was carried out by analysis of the internal transcribed spacer regions (ITS). BLAST searches exhibited that the isolate TN.3 of the first morphotype showed 99% similarity with *Diplodia seriata*, including the ex-epitype strain CBS 112555 (Phillips et al. 2007, Alves et al. 2014) (Fig. 2). The isolate TN.8 of the second morphotype exhibited a high degree of similitude (99%) with *Heterotruncatella spartii* strain CBS 143894 (Maharachchikumbura et al. 2017). The isolate TN.13 of the third morphotype showed 100% identity with *Pestalotiopsis biciliata* strain CBS 124463 (Maharachchikumbura et al. 2017) (Fig. 3). The obtained sequences were deposited in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and are available under the accession numbers: MT711987, MK418461 and MN847720, respectively.

Cultural and microscopic characterization

The obtained isolates were divided into three morphotypes based on colony morphology on PDA and conidia aspect. Colonies of the first morphotype were primarily white fluffy with abundant mycelium. Later, it moderately became grey to olivaceous-grey with age. Conidia were ellipsoid, brown, and aseptate (Fig. 4a, d). This morphotype showed typical features of *Diplodia seriata*, which per the description reported by Phillips et al. (2007). The colonies of the second morphotype were flat off-white at first and later turned to pale luteous. Conidia were fusoid to ellipsoid, straight to slightly curved, with three septa. The two medial cells appeared dark brown, while both apical and basal cells were hyaline; the apical cell had 3 or 5 appendages (Fig. 4b, e). These morphological traits were consistent with the characterization of *Heterotruncatella spartii* (Maharachchikumbura et al. 2017). The third morphotype exhibited white cottony colonies and moderately dense mycelium, producing black acervuli. Conidia were fusiform with four septa; the three medial cells were olivaceous, and the apical and basal cells were hyaline with 2–3 apical and two basal appendages (Fig. 4c, f). These morphological characteristics were congruous with the description of *Pestalotiopsis biciliata* (Maharachchikumbura et al. 2017).

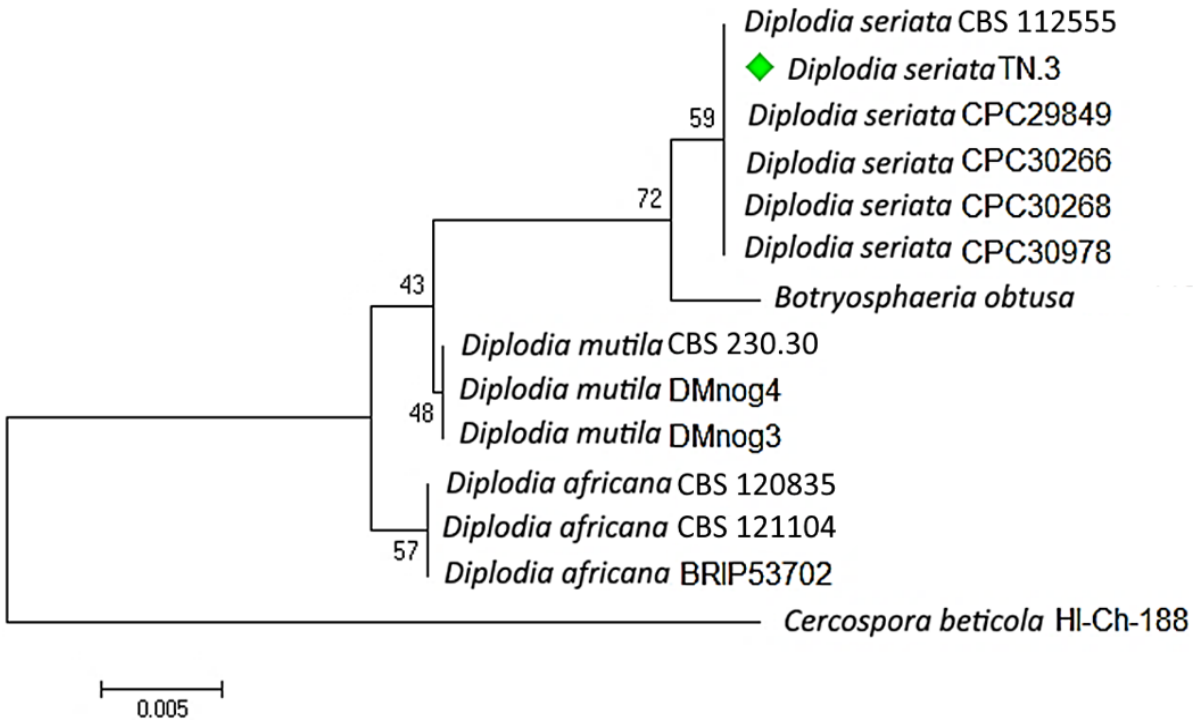

Fig. 2 – Phylogenetic tree obtained from the ITS sequence data of *Diplodia seriata*. Bootstrap support values from 1000 replicates are indicated on the nodes. The tree was rooted to *Cercospora beticola* (HI-Ch-188). The scale bar indicates 0.005 substitutions per site.

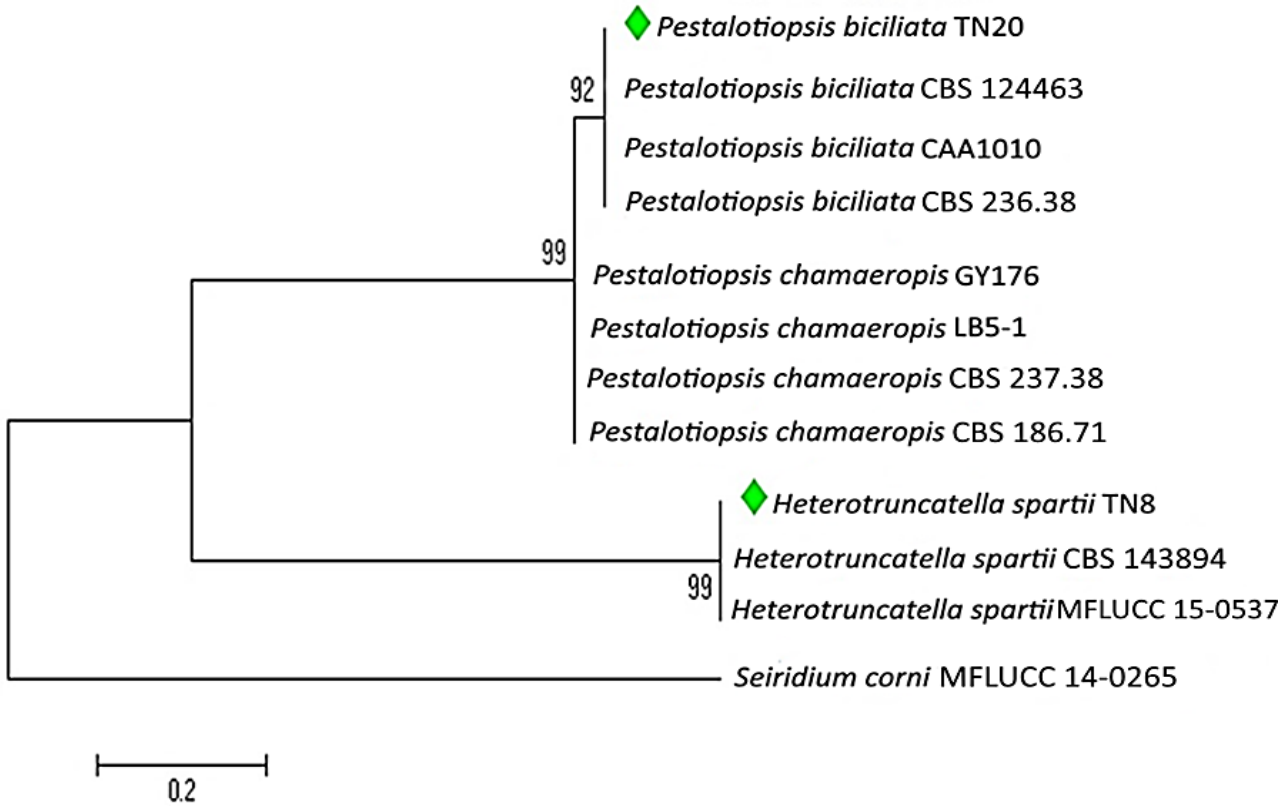

Fig. 3 – Phylogenetic tree obtained from the ITS sequence data of *Pestalotiopsis biciliata* and *Heterotruncatella spartii*. Bootstrap support values from 1000 replicates are indicated on the nodes. The tree was rooted to *Seiridium corni* (MFLUCC 14-0265). The scale bar indicates 0.005 substitutions per site.

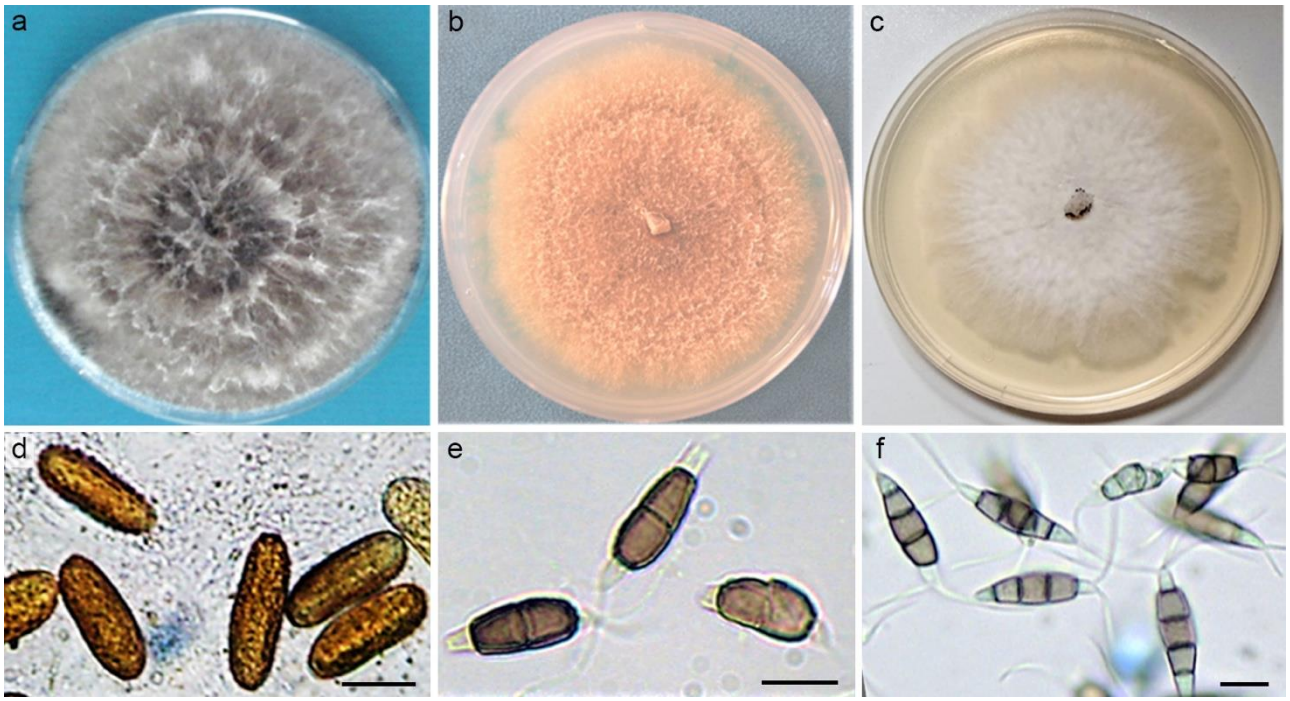

Fig. 4 – Morphological characteristics at 25°C for seven days on PDA. a *Diplodia seriata* colony. b *Heterotruncatella spartii* colony. c *Pestalotiopsis biciliata* colony. d brown aseptate conidia of *D. seriata*. e conidia with two concolorous median cells of *H. spartii*. f conidia with three concolorous median cells of *P. biciliata*. Scale Bars =10 μm.

Fig. 5 – Pathogenicity test results after four weeks post-inoculation on *Pinus pinea* seedling. a inoculated with *Diplodia seriata* (isolate TN.3). b inoculated with *Heterotruncatella spartii* (isolate TN.8). c inoculated with *Pestalotiopsis biciliata* (isolate TN.13). d asymptomatic control seedling.

Pathogenicity test

The three species, *D. seriata* (TN.3), *H. spartii* (TN.8), and *P. biciliata* (TN.13) were assessed for their pathogenicity toward healthy seedlings *P. pinea*. Four weeks after inoculation, brown lesions on bark and wood tissues extending from inoculation points were noticed, confirming the aggressiveness of all evaluated fungi. Stem lesions measured 6.36±0.19 cm, 5.6 ±0.35 cm, and 5.3±0.5 cm in length for *D. seriata*, *H. spartii* and *P. biciliata*, respectively (Fig 5a, b, c). Control seedlings remain symptomless (Fig. 5d). Results revealed the same symptoms as detected in the field conditions. Identical findings regarding the aggressivity of *D. seriata* on grapevine have been reported in Spain by Luque et al. (2009) and on Aleppo pine in Tunisia by Hlaiem et al. (2022). Moreover, *P. biciliata* was recognized as a pathogen on *Eucalyptus* spp. (Morales-Rodriguez et al. 2019) in Italy, and this pathogen has been reported to cause dieback on *Quercus coccifera* and

Pistacia lentiscus in Tunisia (Hlaiem et al. 2022). Recently, *H. spartii* has been described as a pathogen on *Pinus sylvestris* in China (Wang et al. 2022).

Conclusion

This study has identified *Diplodia seriata*, *Heterotruncatella spartii*, and *Pestalotiopsis biciliata* as the fungal pathogens responsible for dieback symptoms in *Pinus pinea* trees in Northeastern Tunisia. Morphological characterization and molecular analysis of the internal transcribed spacer (ITS) region confirmed the identification of the isolates, and pathogenicity tests demonstrated the aggressiveness of all three species. The symptoms observed in both field and inoculation experiments, including branch cankers, shoot blight, and necrotic lesions, are consistent with previously reported dieback in other conifer species across the Mediterranean region.

The findings highlight the potential threat these pathogens pose to the health of *P. pinea* forests in Tunisia and the broader Mediterranean Basin. Given the rapid disease progression and the role of these fungi in forest decline, continuous monitoring and proactive management strategies are essential. Additionally, the global spread of these pathogens underscores the importance of further research to develop effective biological control methods to mitigate their impact under the pressures of climate change.

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