



Influence of the dendrometric and ecological factors on the occurrence of *Alternaria* spp.: Fungal pathogens associated with Tunisian stone pine dieback.

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Abstract

The process of forest decline involves dynamic interactions between numerous biotic and abiotic factors and the plant host. This dieback observed on forest species is one of the main effects of water stress causing mainly by global warming. Stone pine trees (*Pinus pinea* L.) were surveyed in the Tunisian Nabeul forest to monitor the disease symptoms as well as trunk canker, branch necrosis, and canopy transparency. The objective of this study was to determine the cause of the decline in *P. pinea* Tunisian forest and assess the factors affecting its incidence. Correlations between the pathogen incidence, ecological parameters, and pine tree dendrometric characteristics were assessed. Using genetic identification, two distinct species of *Alternaria* were identified: *Alternaria* sp1. and *Alternaria* sp2. A significant relationship between the trunk circumference, the ecological parameters and the *Alternaria* occurrence on stone pine trees is apparent.

Key words – dendrometric parameters – ecological factors –fungi– *Pinus pinea*.

Introduction

Plant health is being negatively impacted by climate change because it causes plants to become maladapted to their existing habitats (Ramsfield et al. 2016). However, little is known about the potential effects of climate change on forest health (Seidl et al. 2017). The frequency of abiotic stress events is expected to rise with longer and more severe droughts or increased rainfall, making tree hosts more susceptible to pathogen and insect pest attacks (Hadziabdic et al. 2021). The dynamic process of plant disease involves a reciprocal relationship between a pathogen that is closely linked to the environment and a susceptible host. Furthermore, changes in the species composition of forest trees are another effect of climate change. The phytosanitary forests status has been impacted by the continual emergence of the fungal pathogens (Avila-Quezada et al. 2018). Likewise, fungi have engendered irreversible modifications on forest trees (Hadziabdic et al. 2021). The consequences of phytopathogenic fungi ranging from moderate infection to host mortality (Doehlemann et al. 2017). Some symptoms related with fungal infections include cankers, spots, necrosis, rots, blights, wilts, and damping-off (Jayawardena et al. 2019). Through infection of

pathogenic fungi endangers the health and viability forest ecosystems (Fisher et al. 2020). The effects are more severe on pathogen aggressiveness (Avila-Quezada et al. 2018). As a result, the environment can affect plant growth and vulnerability, pathogen proliferation as well as plant-pathogen interactions (Gaumann 1950). Fungal pathogens already have a clearly detrimental impact on plants, and climate change will exacerbate the situation (Gautam et al. 2013). According to Allen et al. (2010), among the greatest vulnerable regions to the influence of the changing climate is the Mediterranean area, particularly with regard to pollution, high temperature, and frost (Allen et al. 2010). In light of the anticipated climate change, a number of fungal pathogens affect forest trees by lowering their defenses, producing illnesses, causing tree mortality, and changing the composition of the forest (Ivanová & Berandovičová 2010). Despite the fact that climate change including global warming poses a threat to forest trees, since 2017 numerous *Pinus* species have been detected widespread withering in the forests of Tunisia (Hlaiem et al. 2021).

Due to its ecological and socioeconomic advantages, this coniferous evergreen tree is the most appreciated forest tree in Tunisia. Hence, the objective of the current investigation was to identify the fungal pathogens responsible for *P. pinea* decline and assess the relationship between fungal presence, dendrometric parameters, and ecological factors.

Materials & Methods

Field surveys

Investigations were conducted in costal dunes of Nabeul forest in the Northeastern Tunisia (36.30'.406" N; 10.38'.780" E; alt. 226.5 m) conforming to the semi-arid climate defined by annual temperatures of between (15- 20°C) and annual rainfall (350- 450 mm). The research area of approximately eight hectares contains an innumerable forest species. *P. pinea* trees dominate the vegetation, which also includes species of shrubbery. The objective of this investigation was the stone pine exhibiting symptoms indicative of decline. Throughout the investigation, total height (m), trunk circumference (m) at 1.3 m, and crown width (m) were measured for each tree. According to Hlaiem et al. (2021), each decline tree was assessed using the following classes: (C₀) absence of symptoms; (C₁) = (1 - 25%); (C₂) = (26 - 50%); (C₃) = (51 - 75%); and (C₄) = (76 - 100%). The phytosanitary status of stone pine trees was appraised based on the visual identification of disease symptoms. The following formula was used to assess dieback rate (DR): $DR \% = n \div N \times 100$, with, n (number of infected tree) and N (total number of investigated tree).

Isolation of fungi and morphological identification

Ten branches exhibiting disease symptoms were taken from each affected tree and brought to the laboratory for fungal isolation according to the method of Hlaiem et al. (2021). A small fragment of mycelium was removed from the edge of each developing colony, transferred to PDA media, and cultured for seven days at 25°C in the dark in order to produce pure cultures. One week after incubation on PDA at 25°C in darkness, colonies were then evaluated based of their conidial and cultural characteristics (Wikee et al. 2011). To identify fungal species, all collected isolates were first morphological characterization in accordance with descriptions of Woudenberg et al. (2013) taxonomic references. The Hlaiem et al. (2021) formula was used to determine the isolation frequency (IF). $IF (\%) = (N_i / N_t) \times 100$; with N_i (number of fragments removed from the diseased branches colonized by the fungus) and N_t (total number of planted fragments recovered from the symptomatic branches)

Molecular and phylogenetic analyses

Two typical isolates (TN.1 and TN.14) were chosen for molecular analysis after being morphologically characterized. Colonies were developed on PDA under the same conditions as mentioned above. Subsequently, the DNA of the scraped fungal mycelia obtained from surface of the agar was extracted using a commercial Plant DNA Kit. The internal transcribed spacer (ITS) region of the ribosomal DNA was then amplified using the two primers ITS1 and ITS4 (White et al.

1990). The PCR reaction was carried out in accordance with Alves et al. (2008). The new sequences were aligned with ClustalX v. 1.83 (Thompson et al. 1997) after being coupled with other sequences that were obtained from GenBank. Using MEGA 6.0 software, a phylogenetic tree was constructed using the maximum likelihood approach (Tamura et al. 2013).

Statistical data

The Pearson correlation coefficient was used to evaluate the relationships between the dendrometric parameters, the ecological factors (altitude, temperature, and rainfall), and the isolation frequency of the fungal pathogens. SAS software (version 9.0) was used to perform the statistical analysis.

Results & Discussion

Dendrometric characteristics and phytosanitary status

The results exhibited that the 43 investigated stone pine trees, aged 87 years, are characterized by a crown width equal to 5.40 ± 0.13 m, a trunk circumference of 1.8 ± 0.05 m, and a total height 3.6 ± 0.08 m. The dieback rate of the examined trees was 67%. The disease symptoms, which include defoliation, canopy wilt, and shoot blight, manifest as light brown needles, branch cankers, and brown necrosis on woody tissues that enlarge continuously (Figure 1). The initial symptoms of dieback were sunken, limited necrosis, and a lesion on the branch, subsequently the damage finally resulted in tree death. At a rate of 45%, trees of the decline class (C_2) predominate, followed by (C_3), (C_1), and (C_4) trees with a rate of 35%, 15%, and 5%, respectively. According to our research, disease symptoms are identical to those described by Bagherabadi & Zafari (2022) on walnut trees in Iran, by Laidou et al. (2007) on cotton trees in Greece, and by Hlaiem et al. (2023) on kermes oak in Tunisia. In addition, *Alternaria* species were recognized to be associated with practically all conifer species decline (Danelia et al. 2021).



Fig. 1 – Dieback symptoms of the stone pine trees: infected trees with defoliation and canopy wilt.

Morphological characterization

A total of 60 isolates, including *Alternaria* spp. (n = 32), *Aspergillus* spp. (n = 13), *Penicillium* spp. (n = 10), and other saprobic fungi (n = 5), were isolated one week after incubation. A comparatively high isolation frequency (IF = 53.33%) was reported for the majority of isolates revealed olive green to black colonies with a consistent white border. The colonies had a light-brown band around their black lower surface. The cottony aerial hyphae changed from being colorless to being light brown. Concatenated conidia were either single or in chains, the conidial body was 19.5–39.70 μm \times 8.75–18.95 μm (average 22 \times 13 μm , n = 25), generally ellipsoid and obclavate, with 1-3 longitudinal and 1-2 transverse septa that are slightly constricted close to multiple septa. Initially yellow-brown, the conidia eventually turned black-brown. *Alternaria* was recognized by the morphological features. (Figure 2). Our findings highlighted that *Alternaria* spp. are the most frequently isolated fungi. Cultural and conidial characteristics of isolates were in accordance with the description of *Alternaria* species (Simmons 2007).

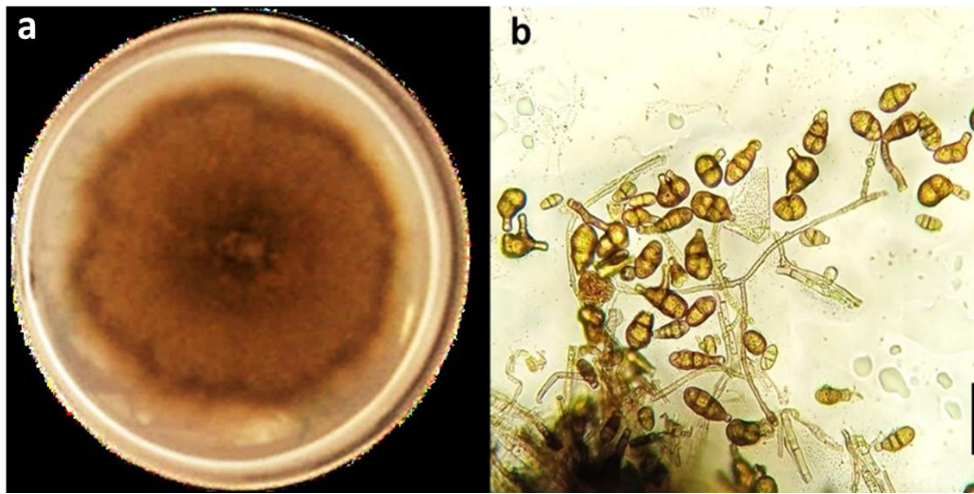


Fig. 2 – Morphological features of *Alternaria* spp. (a) colony incubated on PDA at $25 \pm 2^\circ\text{C}$ for 5 days and (b) multicell conidia (40 \times). Scale bars: 10 μm .

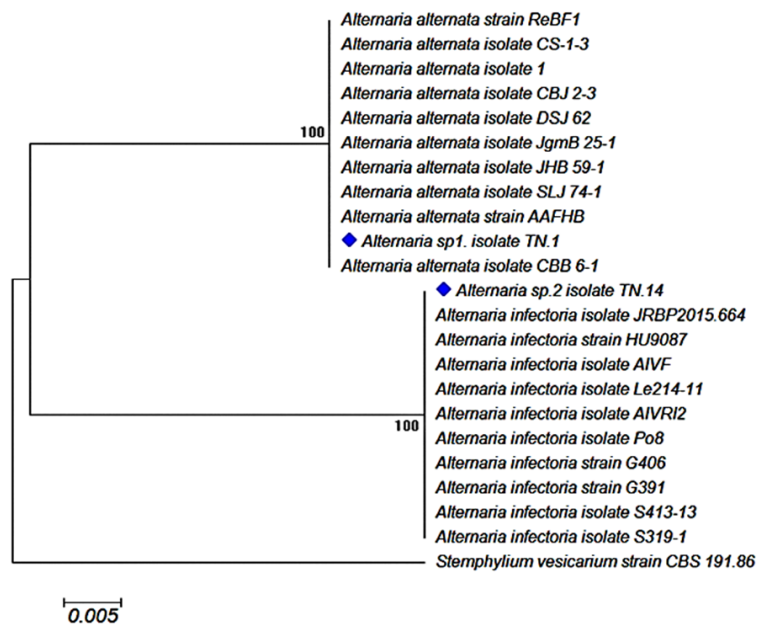


Fig. 3 – Maximum likelihood (ML) phylogenetic tree generated utilizing the data set of ITS rDNA of *Alternaria* spp. The tree is rooted to *Stemphylium vesicarium* strain CBS 191.86. Bootstrap support values (%) from 1000 replications are indicated at the nodes.

Molecular identification and phylogenetic analysis

Recognition of *Alternaria* isolates was established by molecular analysis. In PCR reactions, a 580 bp DNA fragment (ITS rDNA) was amplified. The two isolates, TN.1 and TN.14, demonstrated 100% similarity with *Alternaria* sections *alternaria* (CBB 6-1) and *infectoria* (HU908 strain), according to the phylogenetic tree (Figure 3). The new sequences were submitted in GenBank and are accessible by the accession numbers (TN.1: MN843956; TN.14: MN847721). Furthermore, based on nucleotide sequence data, approximately 300 species have been identified, and their taxonomic position is crucial to pertinent alterations (Woudenberg et al. 2013). The occurrence of *Alternaria* species, particularly the *Alternaria* sections *infectoria* and *alternaria*, was revealed by molecular identification of pathogenic fungi isolated from symptomatic branches of *P. pinea* trees (Lawrence et al. 2013).

Alternaria occurrence and correlations analysis

The Pearson test revealed a positive correlation between *Alternaria* isolation frequency (IF = $48.72 \pm 0.53\%$) and altitude, maximum temperature, minimum rainfall, and trunk circumference of *P. pinea* trees. Nevertheless, neither the total height nor the crown width showed any significant correlation with its incidence (Table 1). According to Matovu et al. (2013), fungal disease occurrence and severity are influenced by ecological factors in Uganda. The same results were reported in Germany by Kraus et al. (2019) and in India by Fagodiya et al. (2022). Additionally, a positive influence of temperature was described also by Escuredo et al. (2011). Likewise, previous studies reporting that the occurrence of *Alternaria* disease could be influenced by temperature, rainfall, and wind speed (Bhat et al. 2015, Chen et al. 2020). Accordingly, Vaquera et al. (2014) corroborate that the high temperature boosts the growth rate of *A. arborescens* the causal agent of tomato stem canker in Argentina. Furthermore, it has been established that global climate change may also alter the degree of pathogenicity of various fungi (Linnakoski et al. 2017). In this regard, several studies confirmed a relationship between fungal pathogens linked to *Quercus suber* and *Pinus pinea* disease and ecological parameters such as rainfall, temperature, and altitude in Tunisia (Hlaiem et al. 2021, Yanguai et al. 2021).

Table 1 Correlations between *Alternaria* spp. isolation frequency, ecological parameters, and dendrometric variables.

	Frequency of isolation of <i>Alternaria</i> FI (%)	
	Correlation coefficient (r)	Significance probability (P)
Dendrometric variables (m)		
Crown width	0.236 ^{ns}	0.055
Trunk circumference	0.422^{**}	<0.001
Total height	-0.169 ^{ns}	0.055
Ecological parameters		
Altitude	0.407^{**}	0.001
Rainfall min	0.407^{**}	0.001
Rainfall max	-0.407^{**}	0.001
Temperature min	-0.407^{**}	0.001
Temperature max	0.407^{**}	0.001

^{ns}: no significant, ^{**}: highly significant (at $\alpha < 0.05$)

This study demonstrated that tree size has an impact on *Alternaria* species incidence in addition to ecological factors. The largest trees are actually more susceptible to *Alternaria* infection, as demonstrated by the positive correlation detected between the trunk circumference and the isolation frequency of *Alternaria* spp. This is consistent with the findings of Hlaiem et al. (2021) and Bihon et al. (2010), which showed that fungal diseases are more common in older trees. Accordingly, the geographical location of a host-plant can have an effect on the abundance, the

diversity, and the pathogenicity of the fungi (Higgins et al. 2007). Generally, fungi typically remain dormant in healthy trees for extended periods. They may thrive as opportunistic pathogens, attacking weaker plant hosts during severe conditions like dryness and warm weather (Vannini et al. 2009). Hence, these taxa may lead to a switch from latent to pathogenic stage, affecting host species, particularly during global warming scenarios.

Conclusion

Two isolates from two sections of *Alternaria* are reported here for the first time as the new fungal pathogens responsible for the stone pine disease worldwide, particularly in Tunisia. This research offers important information for further investigation of these isolates. Nevertheless, additional ecological and epidemiological studies are required to comprehend the relationship between environmental variables and the fungal pathogen response.

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