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Temperature impact on mycelial growth of *Diplodia* spp. causing Botryosphaeria canker in Tunisian forest host species

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Abstract

Environmental stress factors such as freezing, heat, drought, and injury can predispose forest trees and shrubs to infection by fungi. Among these factors, temperature is one of the most critical drivers of climate change, influencing the occurrence of the phytopathogenic fungi. Global warming can substantially affect the development of fungal diseases and alter host defense mechanisms. Taxa belonging to Botryosphaeriaceae, including *Diplodia* spp., are highly destructive fungal pathogens that cause Botryosphaeria canker, leading to branch lesions and trunk cankers in a wide range of forest species. In this study, four *Diplodia* species (*D. africana*, *D. pseudoseriata D. scrobiculata*, and *D. seriata*), known as aggressive pathogens of numerous plant hosts, were evaluated *in vitro* to determine their optimum temperature for mycelial growth. Growth on Potato Dextrose Agar (PDA) showed that these pathogens grew well between 20°C and 30°C, with optimal growth at 25°C. However, growth was inhibited at 5 °C and 40°C, except for *D. seriata*, which was able to grow at 40°C. Mycelial growth differed significantly among isolates across temperatures ($P < 0.0001$). These findings improve our understanding of the role of temperature on the biology of *Diplodia* spp. and highlight their ability to acclimatize to different thermal conditions. Consequently, rising temperatures may significantly increase the severity and spread of Botryosphaeria canker in forest ecosystems.

Keywords – Fungal phytopathogens – Mycelial growth – Temperature.

Introduction

The risk of forest dieback is increasing worldwide. Most events observed over the past 15 years can be clearly attributed to climate change, drought, and high temperatures. Future climate scenarios suggest that these phenomena will intensify even more rapidly, while fires associated with global warming and dieback are expected to exacerbate the ecological consequences (Vennetier 2012). In Mediterranean countries, forest decline and degradation are mainly linked to the impacts of climate change and/or anthropogenic pressures (Ben Jamâa et al. 2005, Nsibi et al. 2006). Plant damage in these regions is primarily attributed to warming and drought, which cause physiological disturbances such as water stress, sap blockage, and nutrient depletion to trees

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(Nebeker et al. 1993, Allen et al. 2010). The decline of Mediterranean forest species is therefore generally due to the combined effects of global warming and drought, which weaken tree defenses and create favorable conditions for infection by pests and fungi (De Sousa et al. 2008; Pautasso et al. 2012, Gautam et al. 2013, Allen et al. 2010).

In particular, drought caused by elevated temperatures predisposes plants to both abiotic and biotic stress, especially infection by pathogenic fungi, which are major stressors affecting forest health (Desprez-Loustau et al. 2006, Frankel et al. 2012). Increasing temperature extremes have already had a severe impact on the health of forest stands (Sarris et al. 2011 Girard et al. 2012 Sanchez-Salguero et al., 2012), further amplifying the severity of plant diseases caused by viruses, bacteria, insects, and fungi (Pautasso et al. 2012, Gautam et al. 2013).

The objective of this study was to investigate the effect of temperature on mycelial growth of four known *Diplodia* species to understand the correlation between increasing temperature and the development of phytopathogenic fungi.

Materials & Methods

Fungal isolates

Field surveys conducted in Northern (Bizerte forest) and Northeastern (Nabeul forest) Tunisia showed a severe withering affecting forest species. Symptoms of disease observed included cankers, necrotic lesions on branches, yellowish brown leaves, and numerous pycnidia on the surface of infected branches often associated with gummy exudates. From each symptomatic tree, ten branches showing cankers were collected, put in labeled plastic bags and transferred to the laboratory for pathogen isolation. Subsequently, fragments were taken from the margin between necrotic and healthy tissues of infected branches. Samples were then surface-sterilized before being placed in Petri dishes containing potato dextrose agar (PDA) and incubated in the darkness at 25 °C for 3 days (Hlaiem et al. 2021). Pure cultures were obtained by plating a small piece of mycelium from the margin of each growing colony on PDA and incubating them under the same conditions as described above. Four *Diplodia* species were obtained from branch cankers of declining *Pistacia lentiscu*s, *Retama raetam*, *Pinus halepensis*, and *Tetraclinis articulata* trees, respectively, collected from the investigated Tunisian forests (Table 1): *Diplodia africana* TN.102, *D. pseudoseriata* TN.80, *D. scrobiculata* TN.44, and *D seriata* TN.27. The isolates were identified by means of morphological characters and molecular analysis to species level following the methods described by Hlaiem et al. (2021). The aggressiveness of all the obtained *Diplodia* isolated has been confirmed utilizing pathogenicity tests (Hlaiem et al., 2019a, b; Hlaiem et al., 2020; Hlaiem et al., 2021, 2022). Pathogenic isolates were stored in sterile distilled water at 4 °C and maintained by regular subculturing on Potato Dextrose Agar medium (PDA) as required (Figure 1).

Table 1 Isolates used in this study and their corresponding GenBank accession numbers.

Forest	Host	Isolate	GenBank accession numbers		
			ITS	*EF1-α*	TUB
Nabeul	*Tetraclinis articulata*	*Diplodia seriata* TN.27	MN850391	-	-
Bizerte	*Pinus halepensis*	*D. scrobiculata* TN.44	MK170175	OM428167	MT164530
	Retama raetam	*D. pseudoseriata* TN.80	MN123532	MN125371	MN125372
	Pistacia lentiscus	*D. africana* TN.102	MK230889	MK746133	MK746134

Temperature effect on mycelial growth

All fungal isolates were grown on PDA and subcultured onto fresh medium. The effect of temperature on mycelial growth of *Diplodia africana* TN.102, *D. pseudoseriata* TN.80, *D. scrobiculata* TN.44, and *D. seriata* TN.27 was evaluated *in vitro* on PDA at different incubation temperatures (5, 10, 15, 20, 25, 30, 35, and 40 °C). Five replicates were carried out for each trial. Radial growth of *Diplodia* colonies was measured at 3 days and 6 days after incubation. The percentage of growth was calculated using the following formula: GR (%) = (Dc ÷ Dp) ×100 according to Abouzkhar et al. (2023), where GR is Growth Rate, Dc is the average colony diameter, and Dp is the diameter of the plate.

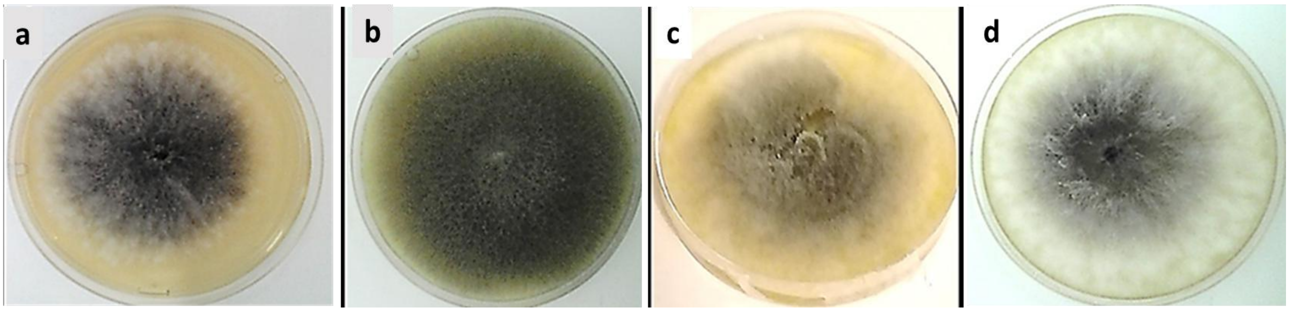

Fig. 1 – Culture characteristics of *Diplodia* isolates grown on PDA at 25C for 6 days: (a) *Diplodia africana* TN.102, (b) *D. pseudoseriata* TN.80, (c) *D. scrobiculata* TN.44, and (d) *D seriata* TN.27.

Data analysis

One-way analysis of variance (ANOVA), followed by Duncan's multiple range test using SPSS version 20, was conducted to evaluate the effect of temperature on the mycelial growth of the *Diplodia* isolates (TN.27, TN.44, TN.80, and TN.102).

Results & Discussion

After 3 days of incubation, *D. scrobiculata* exhibited the slowest growth, while *D. seriata* showed the fastest growth (Table 2). Statistical analysis revealed significant differences in mycelial growth among the four isolates at 10°C ($F_{(4,10)}$ = 12.258; P= 0.001), 20°C ($F_{(4,10)}$ = 6.389; P= 0.008), 25°C ($F_{(4,10)}$ =5.774; P= 0.011), 30°C ($F_{(4,10)}$ =13.939; P<0.0001), 35°C ($F_{(4,10)}$ = 15.771; P<0.0001) and 40°C ($F_{(4,10)}$ = 20.150; P<0.0001). No significant difference was observed at 5°C ($F_{(4,10)}$ = 3.359; P = 0.055) and 15°C ($F_{(4,10)}$ = 2.726; P = 0.090).

After 6 days of incubation, *D. scrobiculata* continued to show slower growth, while *D. seriata* maintained the fastest growth (Table 2). Significant differences in mycelial growth were observed at 10C ($F_{(4,10)}$ = 18.362; P<0.0001), 15C ($F_{(4,10)}$ = 21.277; P<0.0001), 20C ($F_{(4,10)}$ =12.266 ; P= 0.001), 25C ($F_{(4,10)}$ =42.258; P<0.0001), 30C ($F_{(4,10)}$ =53.544; P<0.0001), 35C ($F_{(4,10)}$ = 16.258; P<0.0001) and 40C ($F_{(4,10)}$ =31.247 ; P<0.0001). No significant difference was observed at 5°C ($F_{(4,10)}$ = 1.352; P = 0.317). The optimal mycelial growth temperature for all four isolates was around 25°C. Temperatures at the extremes (5°C and 40°C) inhibited the growth of all species except for *D. seriata*, which could still grow at 35°C and even at 40°C, but with a growth rate lower than the other temperatures (Table 2).

The results demonstrate that the optimum growth temperature ranged from 20 to 30°C for the four tested isolates with different mycelial growth rates depending on the species, in accordance with previous research conducted on Botryosphaeriaceae species (Agustí-Brisach et al. 2019, López-Moral et al. 2022). Overall, these results indicate that mycelial growth of the *Diplodia* isolates is strongly influenced by both incubation time and temperature. After 6 days of incubation, mycelial growth had increased significantly for almost all the isolates, as compared to 3 days post-incubation. Additionally, most species showed negligible growth below 5°C or above 40°C, consistent with previous reports that these fungi are mesophilic, growing optimally between 20 and 28°C (Urbez-Torres et al. 2010; Michailides et al. 2010, Qiu et al. 2016, Zlatković et al. 2016). These findings corroborate earlier studies (Urbez-Torres et al. 2010, Pitt et al. 2013, Qiu et al. 2016) showing that *Diplodia* and related species grow best at moderate temperatures and are largely inhibited by extremes. Martos (2008) reported that the minimum growth temperature of several Botryosphaeriaceae species in different culture media was approximately 15°C, while the maximum temperature was around 35°C. However, mycelial growth varies among species.

Table 2 Mycelial growth (cm) of our isolates incubated on PDA at different temperatures (°C) after 3 and 6 days.

Incubation period (days)	Isolate(s)	Temperatures (°C)							
		5	10	15	20	25	30	35	40
3	**TN.27**	0,90±0,03^{a}	2,36±0,14^{b}	3,60±0,07^{b}	5,13±0,1^{c}	6,13±0,1^{b}	5±0,12^{c}	2,90±0,23^{b}	1,80±0,11^{c}
	TN.44	0,66±0,06^{ab}	1,16±0,12^{a}	1,80±0,11^{a}	3,43±0,1^{a}	5,43±0,1^{b}	4±0,21^{b}	2,03±0,06^{b}	0,76±0,06^{ab}
	TN.80	0,80±0,07^{a}	2±0,03^{b}	3,40±0,07^{ab}	3,83±0,2^{a}	5,83±0,1^{b}	4,23±0,1^{b}	2,46±0,10^{bc}	1,03±0,06^{b}
	TN.102	0,63±0,06^{ab}	1,26±0,10^{a}	3,15±0,08^{ab}	4,90±0,3^{bc}	5,46±0,1^{b}	3,80±0,1^{b}	2,95±0,02^{a}	0,80±0,07^{ab}
6	**TN.27**	0,93±0,05^{a}	3,16±0,06^{c}	5,13±0,17^{c}	6,36±0,2^{c}	8,30±0,1^{d}	7,43±0,1^{d}	6,06±0,16^{b}	2,53±0,14^{c}
	TN.44	0,83±0,02^{a}	1,50±0,12^{a}	2,63±0,16^{a}	4,70±0,1^{a}	6,70±0,1^{b}	5,03±0,1^{b}	1,25±0,14^{a}	0,73±0,06^{a}
	TN.80	0,90±0,03^{a}	2,70±0,10^{c}	4,86±0,17^{c}	5,76±0,1^{b}	8,06±0,0^{d}	6,86±0,1^{d}	5,4±0,15^{b}	1,4±0,15^{b}
	TN.102	0,86±0,06^{a}	2,30±0,11^{b}	3,80±0,15^{b}	6,06±0,0^{bc}	7,26±0,1^{c}	5,96±0,14^{c}	3,46±0,17^{a}	0,76±0,06^{a}

Within each column, values followed by the same letters are not significantly different based on Duncan's Multiple Range test at $P < 0.05$.

Statistical analysis in our study confirmed significant differences in mycelial growth among the species tested at different temperatures. *Diplodia* isolates were capable of growing between 15 and 30°C, with the exception of *D. seriata*, which was able to grow at 35°C and even at 40°C, but slower than at optimal temperature. These results highlight the ability of *D. seriata* to tolerate higher temperatures (> 35°C) and to exhibit the fastest growth under all tested conditions (10 – 40 °C), which may explain its wide distribution and its capacity to infect diverse forest species. Its acclimatization is likely facilitated by the Mediterranean climate of Northern Tunisia, characterized by mild winters and dry, hot summers (Donat et al. 2014). These data confirm the hypothesis that the development of this pathogen could be favored by raising temperatures (Batista et al. 2023). *Diplodia seriata* has been reported on 35 host plants and is frequently isolated from grapevine cankers in California, demonstrating high growth across a broad temperature range (Urbez-Torres et al. 2010). This fungal species consistently exhibits rapid growth across temperatures, with an optimum at approximately 25°C, consistent with findings by Urbez-Torres et al. (2006) and Diaz et al (2022), who reported an optimum between 25 and 27.6°C. Linaldeddu et al. (2011) reported that the optimal temperature for *D. africana* was 25°C, with slow mycelial growth at 10°C. Accordingly, growth is slowed or even inhibited at 5°C and 35°C. *Diplodia africana* grows better at 25°C than at 20°C, which aligns with our results. In contrast, *D. scrobiculata* exhibited slow growth across all temperatures tested. Nivault (2017) reported that *D. scrobiculata*, *D. intermedia,* and *D. rosulata* displayed the lowest mycelial growth among *Diplodia* species and failed to grow at 35°C after six days. Nevertheless, the broad temperature range supporting growth in Botryosphaeriaceae species highlights their adaptability to diverse environmental conditions, contributing to the ubiquitous distribution of this family across various climatic regions (Phillips et al. 2013).

Conclusion

Our findings highlighted that temperature is crucial for mycelial growth of *Diplodia* fungi and it affects the pathogens' colonization of the plant host, which ultimately leads to Botryosphaeria canker development and disease. This study contributes to understanding the combined impact of global warming and phytopathogenic fungi on forest diseases in Tunisia. Our results suggest that rising temperatures may influence the severity of Botryosphaeria canker disease in various forest species. Identifying optimal temperatures for fungal growth can help anticipate periods of heightened disease risk and enable timely management interventions.

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