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ORIGINAL ARTICLE

# Fungal pathogens associated with twig canker of shrub species in Tunisia: Considering the effect of the factors correlated

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## Abstract

Decline phenomena of shrub species such as *Quercus coccifera* and *Retama raetam* have occurred throughout Tunisian forests since 2012. These evergreen shrubs have long been regarded for their medicinal and ecological interests. Therefore, their preservation as valuable forest resources is of great interest. However, information regarding aetiology of this disease is still scarce. Hence, the aim of this study was to identify and characterize the causal agents associated with disease symptoms in two Tunisian forests. Thirty-eight isolates were obtained from symptomatic *Q. coccifera* and *R. raetam* twigs. Morphological characterization and phylogenetic analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene cluster and partial sequence of the translation elongation factor 1-alpha gene (*tef1-α*) allowed the identification of three *Diplodia* species namely *Diplodia africana*, *D. seriata* and *D. pseudoseriata*. Our findings revealed that the incidence of *Diplodia* species was significantly correlated to the altitude, the temperature and the rainfall. Pathogenicity test showed that all *Diplodia* isolates are pathogenic. However, *D. africana* revealed to be the most aggressive species toward *R. raetam*. These findings were the first record of *D. seriata* as fungal pathogen associated with *Q. coccifera* dieback and *D. pseudoseriata* and *D. africana* on *R. raetam* in Tunisia.

**KEYWORDS**
characterization, *Diplodia*, disease, ecological factors, pathogenicity, shrub species

## 1 | INTRODUCTION

Climate change alters forest ecosystems and is predicted to increase forest damage (Seidl et al., 2017) mainly in Mediterranean regions in which decline is taking place due to drought, fires and anthropic pressure (Allen et al., 2010). The combined effect of these factors decreases the defence ability of plants and favours their vulnerability to diseases and attacks by pathogens and pests (De Sousa et al., 2008). Various diseases are widespread throughout the Mediterranean basin affecting a wide variety of vegetation including oak, spruce and beech pine species in Spain, France, Italy (Di Filippo et al., 2010) and North Africa including Morocco and Tunisia (El-Badri & Abadieb, 2000; Hlaiem et al., 2019, 2020, 2022). Fungal pathogens are the most common pathogens causing plant disease and severe damages on a variety of forest tree species (Slippers et al., 2007; Úrbez-Torres, 2011). They are primarily recognized as endophytes in symptomless tissue of woody plants and persist dormant until the onset of stress conditions (Pérez et al., 2010). Withering, necrosis and cankers in the vascular system are commonly observed and are probably the most

important symptoms since they caused a rapid dieback of the plant and often results in the eventual decline and death of the host (Phillips et al., 2013). Furthermore, Botryosphaeriaceae family with different life cycles, such as saprophytic, endophytic and mainly, plant pathogens (Machado et al., 2019) are able to cause a variety of plant diseases (Slippers & Wingfield, 2007). Moreover, numerous *Botryosphaeria* species including *Diplodia* spp. have been shown to cause under drought stress disease, decline and, in severe cases, plant death (Galarneau et al., 2019; Qiu et al., 2016). In the Northern and Southern Mediterranean Hemispheres, *D. sapinea* and *D. scrobiculata* have been recognized as pathogens of *Pinus* species (Bihon et al., 2010). *Diplodia seriata* was associated with various diseases on forest trees around the world (Farr & Rossman, 2020) and *D. africana* on *Juniperus phoenicea* in Italy (Linaldeddu et al., 2011). Recently, *D. seriata* and *D. africana* have been reported on *Pistacia lentiscus* in Tunisia (Hlaiem et al., 2020).

A severe and widespread withering affecting shrub species have been observed since 2012 in Bizerte and Nabeul forests. Mediterranean shrub species, mainly *Quercus coccifera* and *Retama raetam* grow abundantly from North-African countries to Syria (Al-Tubuly et al., 2011; Canellas & San Miguel, 2000) allowing soil fixation, enhancing restoration of the vegetation especially after fire (Canellas & San Miguel, 2000). Over the centuries, these species have been used in traditional medicine to treat various diseases due to their anti-microbial, antioxidant and antiviral activities (Edziri et al., 2010; Sorrentino et al., 2017). In view of the high value of these shrub species, with high economic and medicinal interests, substantial attention should be given to the dieback and decline events that have occurred in Bizerte and Nabeul forests. In this context, this work aims to explore the factors associated with these decline events. Accordingly, the main objectives of our research were to: (i) evaluate the incidence of dieback; (ii) identify the fungal pathogens associated with *Q. coccifera* and *R. raetam* dieback and their occurrence and (iii) study the relationships between their occurrence and the ecological factors.

## 2 | MATERIALS AND METHODS

### 2.1 | Study area and phytosanitary status

The study was carried out in March 2017, in the Northeastern (forest of Nabeul) and in the Northern (forest of Bizerte) Tunisia. Characteristics of the two sites are summarized in Table 1. Latitude, longitude, altitude, temperature (minimum and maximum) and rainfall (minimum and maximum) were provided by the General Directorate of Forestry (GDF) of Tunisia. The vegetation consists of Mediterranean maquis (Ezzine et al., 2015; General Directorate of Forestry (GDF), 1995; Schoenenberger et al., 1971) with shrubs (1–2 m high); composed mainly by *Tetraclinis articulata* L., *P. lentiscus* L., *Erica arborea* L., *Olea europeae* L., *Q. coccifera* L. and *R. raetam* L. The two latter have been the aim of this study. Disease incidence was calculated as the following formula: $DR\,\% = n \div N \times 100$, with DR (dieback rate), $n$ (number of symptomatic plants) and $N$ (total number of examined plants).

### 2.2 | Fungal isolation and morphological characterization

Ten twigs showing cankers and vascular necrosis were collected from symptomatic shrubs of each species (*Q. coccifera* and *R. raetam*) at each site and transferred to the laboratory. Fragments of 3 × 3 mm were taken from the margin between necrotic and healthy tissues of twigs for pathogen isolation. Samples were then surface-sterilized in 70% ethanol for 2 min and rinsed three times in sterilized water before being placed in Petri dishes containing potato dextrose agar (PDA) supplemented with streptomycin sulphate (0.05 g/L) and incubated in the darkness at 25°C for 3 days (Franceschini et al., 2005). Pure cultures were obtained by plating a small piece of mycelium from the margin of each colony grown on PDA and incubating them under the same conditions described above. Sporulation was induced on PDA amended with autoclaved pine needles at 25°C under continuous near-ultraviolet light (Linaldeddu et al., 2011). *Diplodia* species were identified based on morphological traits (colour and texture) of 7-day-old cultures and conidial morphology, shape and size according to Phillips et al. (2013). The isolation frequency (IF %) has been assessed by means of cultural morphology features. It was calculated as following: $IF\,(\%) = 100 \times (N_i \div N_t)$ with $N_i$ (number of fragments colonized by the fungus) and $N_t$ (total number of plated fragments) Franceschini et al. (2005).

### 2.3 | Molecular characterization

Based on morphological characterization, one isolate was selected from each obtained morphotype for molecular analysis. In brief,

**TABLE 1** Ecological parameters of the two investigated study sites.[a]

| Site | Latitude | Longitude | Altitude (m) | Temperature (°C) | | Rainfall (mm) | | Bioclimate |
|---|---|---|---|---|---|---|---|---|
| | | | | Min | Max | Min | Max | |
| Bizerte | 37°.17′.48″ | 10°0′2″ | 41 | 11 | 31 | 178 | 473 | Subhumid in warm winter |
| Nabeul | 36°.30′.406″ | 10°.38.780′ | 226.5 | 15 | 20 | 350 | 450 | Semi-arid superior to mild winter |

[a]Ecological data is collected from the General Directorate of Forestry (GDF) of Tunisia.

approximately 100 mg of dry weight mycelium were collected and used for DNA extraction according to the manufacturer's instructions of DNA extraction commercial Kit « innu PREP Plant DNA Kit » (Analytik Jena AG). The internal transcribed spacer region (ITS rDNA) and a fragment of the translation elongation factor 1-α gene (*tef1-α*) were amplified using the universal primer pairs ITS1/ITS4 (White et al., 1990) and EF1-688F/EF1-986R (Alves et al., 2008; Carbone & Kohn, 1999).

PCR reaction was performed in a total volume of 25 μL containing 1 μL of genomic DNA template (50 ng/μL), 1× buffer de PCR Master Mix 2.5 μL of $MgCl_2$ (25 mM), 0.2 μL of dNTPs (10 mM), 1 μL of each primer (10 μM), 0.2 μL of *Taq* DNA polymerase (5 Units/μL) (Dream *Taq*) and 19.1 μL dd$H_2O$. Amplification conditions were conducted as described previously by Alves et al. (2008). PCR products were electrophoresed on a 1.5% agarose gel, then stained with GelRed and visualized with UV transilluminator. The size of PCR products was estimated by comparison with a DNA ladder 100 bp plus. Amplified products were sequenced. Sequences were edited with FinchTV v1.4.0 (Geospiza, Inc.; http://www.geospiza.com/finchtv) and compared with those deposited in GenBank through BLASTn searches (Table 2). Newly ITS and *tef1-α* sequences were deposited in GenBank under the accession numbers mentioned in Table 3.

### 2.4 | Phylogenetic analysis

The ITS and *tef1-α* sequences obtained in this study were combined, then supplemented with further reference sequences of *Diplodia* spp. retrieved from GenBank which have been selected based on their high similarity with our query sequences (Table 2) and they were aligned using ClustalX v. 1.83 (Thompson et al., 1997) and uninformative terminal regions were excluded from the analysis. Phylogenetic tree was generated under Neighbour-Joining (NJ) using MEGA version 6 and evaluated with 1000 bootstrap replicates (Tamura et al., 2013).

### 2.5 | Pathogenicity assay

Pathogenicity tests were conducted in vitro according to Moral et al. (2017) methodology by inoculating isolates on 20 healthy twigs collected from the two host species [*Q. coccifera* ($n = 5$, isolate TN.19; $n = 5$, isolate TN.86) and *R. raetam* ($n = 5$, isolate TN.35; $n = 5$, isolate TN.80)]. Briefly, after removal of leaves, twigs were surface sterilized with ethanol (70%) and wounded by a sterilized scalpel. For each isolate, a mycelial plug (6 mm diameter) was taken from the margin of an actively growing 7-day-old colony and placed on the wounds of the twigs. The inoculation point was covered with cotton wool soaked in sterile water and wrapped with Parafilm. Ten control twigs were inoculated with sterile PDA plugs [*Q. coccifera* ($n = 5$); *R. raetam* ($n = 5$)]. All twigs were kept at room temperature in the laboratory (22–26°C) and monitored weekly for necrosis development during 30 days.

### 2.6 | Statistical analysis

All statistical analyses were performed using SPSS v.20. Generalized linear model (GLM) was performed to two dependent variables: (1) dieback rate and (2) isolation frequency. Shrub species and sites were given as fixed factors. The same procedure was applied to necrosis length as dependent variable. Isolates and sites defined fixed factors. Correlation analysis between isolation frequency of *Diplodia* spp. and ecological parameters (Altitude, temperature and rainfall) (Table 1) were estimated using Pearson correlation tests.

## 3 | RESULTS

### 3.1 | Incidence of dieback disease

Field surveys conducted on *Q. coccifera* and *R. raetam* shrubs showed disease symptoms in the two forests. Symptoms included cankers, shoot blight, necrotic lesions on twigs, vascular discoloration in wood, yellowish brown leaves, numerous pycnidia on the surface of infected twig often associated with gummy exudates (Figure 1). The dieback rate (DR) was 38% and 42% for *Q. coccifera* and *R. raetam* in Nabeul forest, and 44% and 39% for *Q. coccifera* and *R. raetam* in Bizerte forest, respectively. Moreover, no dead shrub was observed. The GLM test indicated no significant differences of DR neither among sites nor among shrub species. The interaction term was also not significant.

### 3.2 | Morphological characterization

A total of 55 isolates was obtained including *Diplodia* spp. ($n = 38$), *Penicilium* sp. ($n = 9$), *Mucor* sp. ($n = 5$) and *Aspergillus* sp. ($n = 3$). The majority of isolates were fast-growing, initially white produced aerial and abundant mycelium. Then, gradually became grey to olivaceous-grey and finally turn to black with age (Figure 2a–c). Preliminary, based on colony colour on PDA and conidia shape and size, isolates were showed typical traits of *Diplodia* species which is consistent with the description of previous studies (Damm et al., 2007; Linaldeddu et al., 2011; Pérez et al., 2010; Phillips et al., 2007). In presence of sterilized pine needles, all *Diplodia* isolates produced dark brown to black pycnidia, unilocular and thick-walled. Conidiophores reduced to hyaline conidiogenous cells, cylindrical, thin-walled and smooth and produced a single conidium at the tip. Based on their conidial morphological appearance (Figure 2d–f), three groups have been observed. The first group ($n = 21$ isolates) was characterized by hyaline conidia that turned brown at maturity. These conidia were smooth aseptate, ellipsoidal to ovoid, apex obtuse, widest in the middle, base truncate or rounded with thick melanised cell walls and 17–27.0 × 9.5–12.5 μm in size. The second group ($n = 10$ isolates) is characterized by hyaline conidia, aseptate with obtuse apex and

TABLE 2 Fungal isolates included in the phylogenetic analysis.

| Strain | Species | Host | Country | GenBank accession numbers | |
|---|---|---|---|---|---|
| | | | | ITS | tef1-α |
| CBS120835[a] | *Diplodia africana* | *Prunus persica* | South Africa | EF445343 | EF445382 |
| CBS121104 | *D. africana* | *P. persica* | South Africa | EF445344 | EF445383 |
| BRIP53702 | *D. africana* | *Pinus muricata* | Australia | MH057169 | MH102232 |
| **TN.35** | ***D. africana*** | ***Retama raetam*** | **Tunisia** | **MN841978** | **MT159337** |
| CBS393.84[a] | *D. sapinea* | *Pinus nigra* | Netherlands | DQ458895 | DQ458880 |
| CBS109725 | *D. sapinea* | *P. patula* | South Africa | DQ458896 | DQ458881 |
| BL189 | *D. sapinea* | *Corylus avellana* | Italy | KX833082 | KX833083 |
| CBS141915 | *D. sapinea* | *Eriobotrya japonica* | Italy | KT956270 | KU378605 |
| **TN.19** | ***D. seriata*** | ***Quercus coccifera*** | **Tunisia** | **MN096735** | **MN104954** |
| **TN.86** | ***D. seriata*** | ***Q. coccifera*** | **Tunisia** | **MN318471** | **MN373270** |
| CBS119049 | *D. seriata* | *Vitis vinifera* | Italy | DQ458889 | DQ458874 |
| CBS112555[a] | *D. seriata* | *V. vinifera* | Portugal | AY259094 | AY573220 |
| BL130 | *D. seriata* | *F. angustifolia* | Italy | KF307723 | HQ629958 |
| CBS121425 | *D. seriata* | *P. persica* | South Africa | EF445299 | EF445365 |
| CAA502 | *D. seriata* | *Fraxinus ornus* | Portugal | KJ361842 | KJ361836 |
| CPC28088 | *D. seriata* | *Citrus reticulata* | Spain | MW413849 | MW419167 |
| **TN.80** | ***D. pseudoseriata*** | ***Retama raetam*** | **Tunisia** | **MN123532** | **MN125371** |
| UY788 | *D. pseudoseriata* | *Eucalyptus* sp. | Uruguay | EU080927 | EU863181 |
| UY671 | *D. pseudoseriata* | *Eucalyptus* sp. | Uruguay | EU080922 | EU863179 |
| CPC27963 | *D. pseudoseriata* | *C. sinensis* | Portugal | MW413834 | MW419152 |
| CBS124906[a] | *D. pseudoseriata* | *Blepharocalyx salicifolius* | Uruguay | EU080927 | EU863181 |
| CBS109944 | *D. scrobiculata* | *Pinus greggii* | Mexico | DQ458899 | DQ458884 |
| CBS113423 | *D. scrobiculata* | *P. greggii* | Mexico | DQ458900 | DQ458885 |
| CBS118110[a] | *D. scrobiculata* | *P. resinosa* | USA | AY253292 | AY624253 |
| CBS 112549[a] | *D. corticula* | *Quercus suber* | Portugal | AY259100 | AY573227 |
| CBS112547 | *D. corticula* | *Q. ilex* | Spain | AY259110 | DQ458872 |
| BL10 | *D. corticula* | *Q. ilex* | Italy | JX894191 | JX894210 |
| CBS121887[a] | *D. olivarum* | *Olea europaea* | Italy | EU392302 | EU392279 |
| BL96 | *D. olivarum* | *Pistacia lentiscus* | Italy | KX833078 | KX833079 |
| CPC27856 | *D. olivarum* | *C. sinensis* | Malta | MW413833 | MW419151 |
| CBS133852[a] | *D. quercivora* | *Q. canariensis* | Tunisia | JX894205 | JX894229 |
| CBS116470[a] | *D. rosulata* | *Prunus africana* | Ethiopia | EU430265 | EU430267 |
| CBS 112553[a] | *D. mutila* | *V. vinifera* | Portugal | AY259093 | AY573219 |
| CBS 230.30 | *D. mutila* | *Phoenix dactylifera* | USA | DQ458886 | DQ458869 |
| **Out group** | | | | | |
| CBS 447 | *Guignardia philoprina* | *Taxus baccata* | Netherlands | FJ824768 | FJ824773 |
| CPC15875 | *Cercospora* sp. | *Euphorbia* sp. | Mexico | JX143731 | JX143490 |
| CBS111166 | *Mycosphaerella* sp. | *Eucalyptus cladocalyx* | South Africa | JX901773 | JX901664 |

*Note*: The sequences generated in this study are indicated in bold.

[a]Ex-type.

truncate ends becoming brown at maturity and measuring 22.5–30 × 9.5–14.5 µm. Conidia of the last group ($n = 7$ isolates) were aseptate, hyaline, thick-walled, smooth, subcylindrical to oblong-elliptical, with rounded ends, hyaline after discharge from pycnidia and 17–33 × 11 to 16 µm in size.

### 3.3 | Fungal incidence

*Diplodia* spp. was isolated at the two investigated sites with different frequencies ranging from 62% in Nabeul to 78% in Bizerte. The isolation frequency of *Diplodia* species was 35% and 43%

from *Q. coccifera* and 27% and 35% from *R. raetam* respectively in Nabeul and Bizerte forests. The highest frequency of *Diplodia* spp. was noted for *Q. coccifera* in Bizerte forest. *Diplodia* spp. were the most frequently isolated fungi with relatively high frequency than other saprophytic fungi, namely *Penicilium* sp. (IF = 16%), *Mucor* sp. (IF = 9%) and *Aspergillus* sp. (IF = 5%). The GLM test carrying out on shrub species revealed highly significant differences of *Diplodia* frequency between the two forests ($F_{(1,16)} = 28.366$, $p < .001$) but not between the two shrub species. The interaction term was significant.

**TABLE 3** Correlations between *Diplodia* spp. frequency and ecological parameters.

| | Isolation frequency of *Diplodia* spp. (FI%) | |
|---|---|---|
| | Correlation coefficient (*r*) | Significance probability (*p*) |
| Ecological parameters | | |
| Altitude | −.764** | <.001 |
| Rainfall min | −.764** | <.001 |
| Rainfall max | .764** | <.001 |
| Temperature min | −.764** | <.001 |
| Temperature max | .764** | <.001 |

**Highly significant at $p < .01$.

## 3.4 | Correlation analysis

The Pearson test showed that *Diplodia* frequency was positively correlated to the maximum temperature ($r = .764$; $p < .001$) and the maximum rainfall ($r = .764$; $p < .001$). However, it was negatively correlated to the altitude ($r = -.764$; $p < .001$) (Table 3).

## 3.5 | Molecular characterization and phylogenetic analysis

PCR amplifications of the ITS and *tef1-α* regions gave products of approximately 560 and 340 bp, respectively. These new sequences (Table 4) were deposited in GenBank and are available under accessions numbers (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed for *Diplodia* spp. to build the topology of the tree of the combined gene regions (ITS and *tef1-α*). The combined ITS and *tef1-α* dataset of *Diplodia* spp. included 37 taxa (34 in-group and 3 out-group) and contained 812 characters (including gaps). *Guignardia philoprina* (CBS447), *Mycosphaerella* sp. (CBS111166) and *Cercospora* sp. (CPC15875) presented the out-group. This process allowed us to identify three *Diplodia* distinct species belonging to three species (Figure 3). BLAST searches in GenBank showed that ITS and *tef1-α* sequences of *Diplodia* isolates had 98%–100% homology with the ex-type or epitype strains of *D. africana* (CBS 120835), *D. pseudoseriata* (CBS124906) and

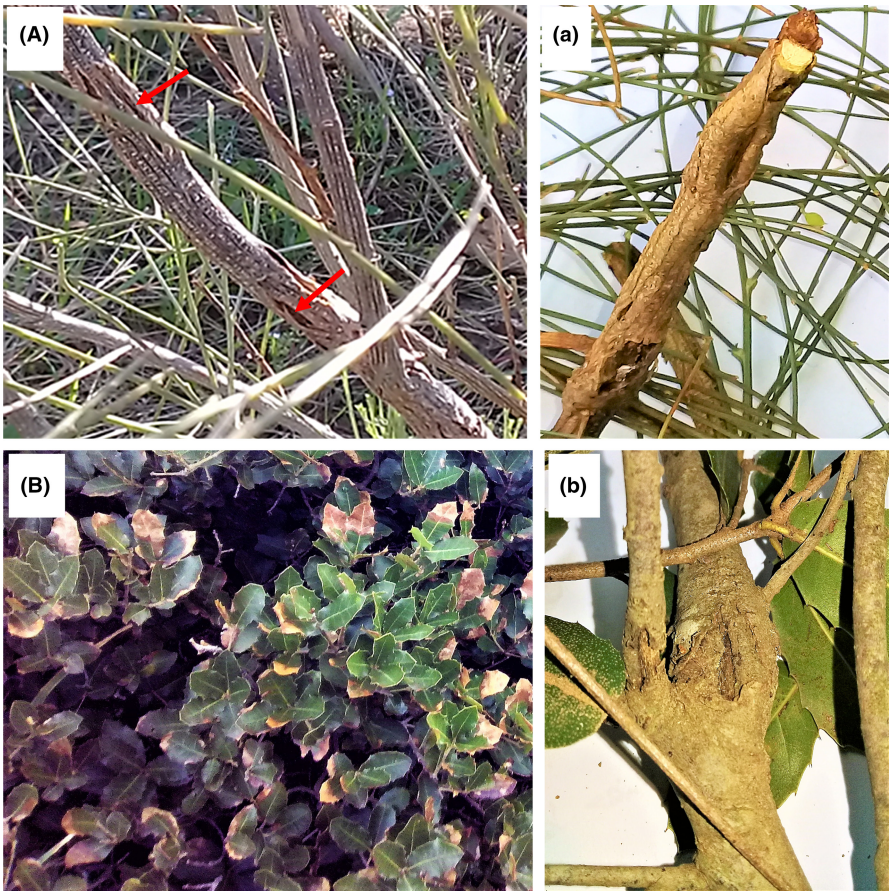


**FIGURE 1** Dieback symptoms observed in naturally infected *Retama raetam* (A) and *Quercus coccifera* shrubs (B), Cankers on twigs of *Retama raetam* (a) and *Quercus coccifera* (b).

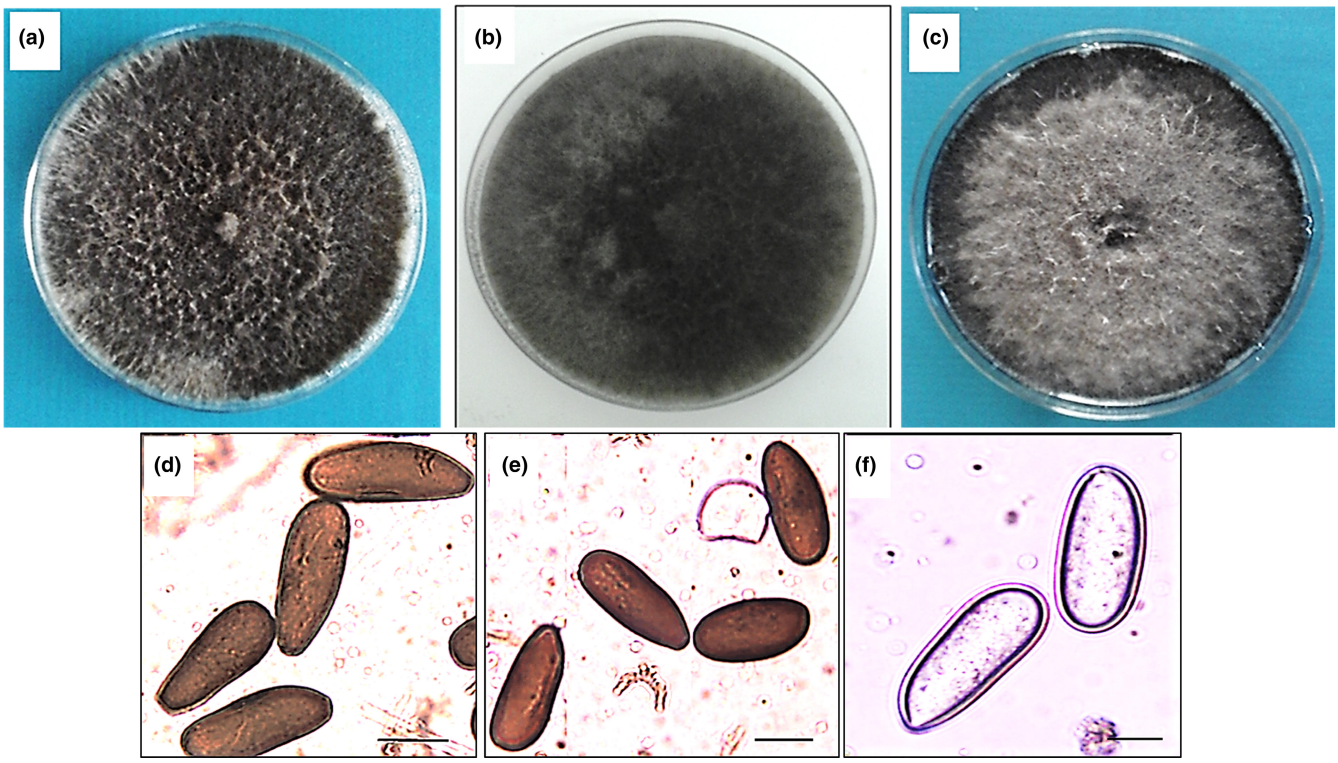


FIGURE 2 Morphological characteristics of *Diplodia* colony on PDA after 10 days growth at 25°C (a–c) and mature aseptate conidia (d, e). Scale bars: 10 μm.

TABLE 4 Identity of the isolates, GenBank accession numbers of the sequences and necrosis length.

| Sites | Hosts | Isolates | GenBank accession numbers | | Necrosis length (cm)* |
|---|---|---|---|---|---|
| | | | ITS | tef1-α | |
| Nabeul | Q.c | *D. seriata* (TN.19) | MN096735 | MN104954 | $3 \pm 0.00^{a}$ |
| | R.r | *D. africana* (TN.35) | MN841978 | MT159337 | $5.66 \pm 0.39^{b}$ |
| | | Control | – | – | $0.9 \pm 0.3^{c}$ |
| Bizerte | Q.c | *D. seriata* (TN.86) | MN318471 | MN373270 | $4 \pm 0.00^{a}$ |
| | R.r | *D. pseudoseriata* (TN.80) | MN123532 | MN125371 | $5 \pm 0.00^{b}$ |
| | | Control | – | – | $1.1 \pm 0.0$ |

Abbreviations: Q.c, *Quercus coccifera*; R.r, *Retama raetam*.

[a,b,c]Within each column, values followed by the same letters are not significantly different based on Duncan's Multiple Range test at $p < .05$.

*Mean values correspond to the sum of both upward and downward extent of vascular necrosis (5 repetitions per isolate) measured from the point of inoculation ± SE.

*D. seriata* (CBS 112555). The molecular characterization confirmed the identity of the four selected isolates TN.35 as *D. africana*, TN.19 and TN.86 as *D. seriata* and TN.80 as *D. pseudoseriata*.

### 3.6 | Pathogenicity assay

Thirty days after inoculation, cankers, dark brown discolouration of the bark and wood tissues extending above and below the inoculation point in all inoculated excised twigs were appeared confirming the pathogenicity of all the tested isolates toward their host shrub species (*Q. coccifera* and *R. raetam*). Artificially obtained symptoms confirm the potential of all fungal species to cause necrosis on excised twigs. However, the GLM test revealed that this pathogenicity varied significantly among fungal isolates ($D_1 = 17.193$, $p < .001$) but not among forests. The interaction term was significant ($D_3 = 3.884$, $p = .030$). *Diplodia africana* isolate was the most aggressive species causing the largest lesions on *R. raetam* excised twigs (Table 3). Disease symptoms were absent on control twigs. All isolates were successfully re-isolated from the margins of the necrotic lesions and their identity was confirmed based on morphological and cultural characters.

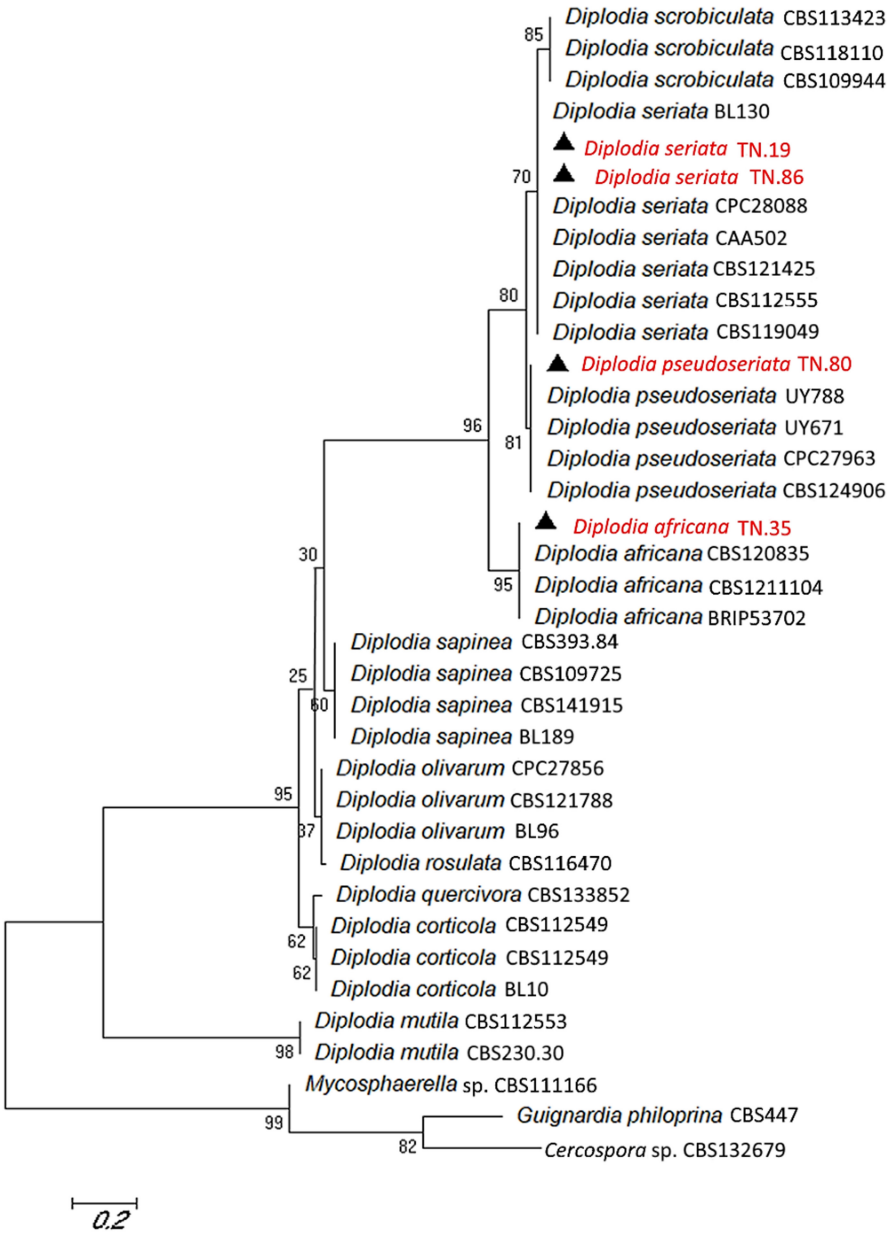


**FIGURE 3** Phylogenetic tree obtained from combined ITS and *tef1-α* sequence data of *Diplodia* spp. Bootstrap support values (%) from 1000 replications are shown at the nodes. The scale bar indicates 0. Substitutions per site. The tree is rooted to *Guignardia philoprina* CBS 447, *Cercospora* sp. and *Mycosphaerella* sp.

## 4 | DISCUSSION

The last decades, stress and inadequate plant growth conditions frequently, give rise to the expression of diseases associated with Botryosphaeriaceae species (Pour et al., 2020; Slippers & Wingfield, 2007). This work presents the first study of *Diplodia* species associated with *Q. coccifera* and *R. reatam* dieback in Tunisia. During our surveys, disease symptoms including twig dieback, branch cankers and vascular discoloration of the wood were observed on the two shrub species. Similar symptoms were previously described a range of hosts in Australia, Greece, Italy and Spain (Linaldeddu et al., 2011; Moral et al., 2017; Phillips et al., 2007; Slippers et al., 2004; Tsopelas et al., 2010).

Based on morphological characteristics, the fungal isolates were firstly identified as *Diplodia* genus (Damm et al., 2007; Pérez et al., 2010; Phillips et al., 2007). In fact, *Diplodia* spp. contribute with other factors namely temperature, prolonged drought periods and ecological parameters to cause forest trees dieback (Hlaiem et al., 2021). Given *Diplodia* species primarily occurred as endophyte then turn on pathogen under stress conditions. A significant correlation was noted between the maximum temperature and *Diplodia* isolation frequency in the two study sites as also noted by Calzarano et al. (2018) in Italy. Furthermore, a positive correlation between isolation frequency of *Diplodia* and rainfall has been revealed. Calzarano et al. (2018) and Kraus et al. (2019) reported similar findings in Italy and in Germany, respectively. In fact, conidia which are recognized as the dominant dispersal form of *Diplodia* species are splash-borne dispersed by heavy rainfall over relatively short distances arriving to susceptible wounds (Úrbez-Torres, 2011). Studies of Yangui et al. (2021) supported a correlation as well between the ecological parameters (altitude, temperature and rainfall) and the occurrence of the pathogen associated with *Quercus suber*

in northwestern Tunisia. Accordingly, ecological factors may be the origin of the higher incidence of *Diplodia* species in the two Tunisian forests. Indeed, Botryosphaeriaceae family including *Diplodia* species, could trigger a shift from the latent to the pathogenic phase acting on the host species (Desprez-Loustau et al., 2006). Furthermore, the impact of the pathogens in relation to climatic conditions has been well documented in parts of southern Europe (Piskur et al., 2011) and in South Africa (Van der Linde et al., 2012). Hence, high temperature could improve plant stress and generate optimal conditions for the development of Botryosphaeria dieback (Pour et al., 2020; Slippers & Wingfield, 2007). Consequently, the potential impact of the *Diplodia* species, which is a widespread pathogen present as an endophyte in numerous plant communities in various parts of the world, might be exacerbated (Desprez-Loustau et al., 2006).

Molecular identification allowed us to characterize *D. seriata* from *Q. coccifera*, *D. africana* and *D. pseudoseriata* from *R. raetam*. Furthermore, *D. seriata* has been isolated from more than 250 plant hosts including almond, pistachio and walnut trees (Chen et al., 2014; Gramaje et al., 2012; Inderbitzin et al., 2010). Moreover, *D. africana* has been isolated from symptomatic *Prunus* species in South Africa (Damm et al., 2007) and from Holm Oak in Italy (Seddaiu et al., 2019). In addition, *D. pseudoseriata* has been described from avocado stems in Chile (Valencia et al., 2019).

On the other hand, pathogenicity assay confirmed the ability of the three identified *Diplodia* species to induce lesions in all inoculated excised twigs. In fact, phytopathogenic fungi mainly arise by way of natural openings or wounds (Slippers et al., 2013). *Diplodia africana* induced the largest average lesions, whereas *D. seriata* caused the lowest ones. Accordingly, *D. africana* has been reported to cause various diseases on stone fruit trees in South Africa (Van Niekerk et al., 2004) on juniper (Linaldeddu et al., 2011) in Italy. Mohammadi et al. (2013) indicated that *D. seriata* is ranked among the moderately pathogen fungal species. Likewise, Pérez et al. (2010) describe *D. pseudoseriata* as pathogen on native *Myrtaceae* trees in Uruguay. To the best of our knowledge this paper reports *D. seriata*, as a new pathogen on *Q. coccifera* and *D. pseudoseriata* and *D. africana* on *R. raetam* shrubs in Tunisia.

## 5 | CONCLUSION

This gives rise to an appraisal of the combined impact of abiotic factors (altitude, temperature and rainfall) and canker pathogens on shrubs decline in Tunisia. Moreover, our findings highlighted that *Diplodia* species were involved in twig canker, presents a potential threat and appear to be the main phytopathological problem in Tunisian forests. Given the serious damages that cause fungal species on shrubs species, the definition of suitable sustainable development strategies has become of primary importance in order to overcome the increasing spread of forest decline phenomena in Tunisia.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests. All authors of this manuscript are aware with the content of the article and have approved of its submission to this journal.

## DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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## REFERENCES

Allen, C. D., Macalady, A. K., Chenchouni, H., Bachelet, D., McDowell, N., Vennetier, M., Kitzberger, T., Rigling, A., Breshears, D. D., Hogg, E. H., Gonzalez, P., Fensham, R., Zhang, Z., Castro, J., Demidova, N., Lim, J. H., Allard, G., Running, S. W., Semerci, A., & Cobb, N. (2010). A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *Forest Ecology and Management*, *259*(4), 660–684.

Al-Tubuly, R. A., Auzi, A. A., Al-Etri-Endi, A. A., Nahar, L., & Sarker, S. D. (2011). Effects of *Retama raetam* (Forssk) Webb & Berthel. (Fabaceae) on the central nervous system in experimental animals. *Archives of Biological Sciences*, *63*(4), 1015–1021.

Alves, A., Crous, P. W., Correia, A., & Phillips, A. J. L. (2008). Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. *Fungal Diversity*, *28*, 1–13.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2010). *Diplodia scrobiculata* found in the southern hemisphere. *Forest Pathology*, *41*, 175–181.

Calzarano, F., Osti, F., Baránek, M., & Di Marco, S. (2018). Rainfall and temperature influence expression of foliar symptoms of grapevine leaf stripe disease (esca complex) in vineyards. *Phytopathologia Mediterranea*, *57*, 488–505. https://doi.org/10.14601/Phytopathol_Mediterr-23787

Canellas, I., & San Miguel, A. (2000). Biomass of root and shoot systems of *Quercus coccifera* shrublands in Eastern Spain. *Annals of Forest Science*, *57*(2000), 803–810.

Carbone, I., & Kohn, L. M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia*, *91*(3), 553–556.

Chen, S. F., Morgan, D. P., Hasey, J. K., Anderson, K., & Michailides, T. J. (2014). Phylogeny, morphology, distribution, and pathogenicity of Botryosphaeriaceae and Diaporthaceae from English walnut in California. *Plant Disease*, *98*, 636–652.

Damm, U., Crous, P. W., & Paul, H. F. (2007). *Botryosphaeriaceae* as potential pathogens of *Prunus* species in South Africa, with descriptions of *Diplodia africana* and *Lasiodiplodia plurivora* sp. nov. *Mycologia*, *99*(5), 664–680. https://doi.org/10.1080/15572536.2007.11832531

De Sousa, E., El Antry, T. S., Atay Kadiri, Z., & Abourouh, M. (2008). Problématique des subéraies dans le bassin méditerranéen. *Annales de la Recherche forestière au Maroc*, *39*, 63–73.

Desprez-Loustau, M. L., Marçais, B., Nageleisen, L. M., Piou, D., & Vannini, A. (2006). Interactive effects of drought and pathogens on forest trees. *Annals of Forest Science*, *63*, 597–612.

Di Filippo, A., Alessandrini, A., Biondi, F., Blasi, S., Portoghesi, L., & Piovesan, G. (2010). Climate change and oak growth decline: Dendroecology and stand productivity of a Turkey oak (*Quercus cerris* L.) old stored coppice in Central Italy. *Annals of Forest Science*, *67*(7), 706.

Edziri, H., Mastouri, M., Cheraif, I., & Aouni, M. (2010). Chemical composition and antibacterial, antifungal and antioxidant activities of the flower oil of *Retama raetam* (Forssk.) Webb from Tunisia. *Natural Product Research*, *24*, 789–796.

El-Badri, N., & Abadieb, M. (2000). Observations sur la dynamique du développement du *Diplodia mutila* Fr. apud Mont. sur le chêne-liège, *Quercus suber* L., au Maroc. Observations on the dynamic of *Diplodia mutila* Fr. apud Mont. Development on the cork-oak, *Quercus suber* L., in Morocco. *Cryptogamie Mycologie*, *21*(4), 235–248.

Ezzine, O., Manuela, B., Claire, V., Stefan, S., Said, N., & Ben Jamâa, M. L. (2015). Host use in *Orgyia trigotephras* (Erebidae, Lymantriinae) during outbreak: Effects on larval performance and egg predation. *Annals of Forest Science*, *2015*(72), 561–568. https://doi.org/10.1007/s13595-015-0484-7

Farr, D. F., & Rossman, A. Y. (2020). Fungal Databases, U.S. National Fungus Collections, ARS, USDA. https://nt.ars-grin.gov/fungaldatabases/

Franceschini, A., Linaldeddu, B. T., & Marras, F. (2005). Occurrence and distribution of fungal endophytes in declining cork oak forests in Sardinia (Italy). *IOBC/WPRS Bull*, *28*, 67–74.

Galarneau, E. R. A., Lawrence, D. P., Travadon, R., & Baumgartner, K. (2019). Drought exacerbates Botryosphaeria dieback symptomsin grapevines and confounds host-based molecular markers of infection by Neofusicoccum parvum. *Plant Disease*, *103*, 1736–1745.

General Directorate of Forestry (GDF). (1995). Forêt domaniale De Béni Oualid. Plan d'aménagement 1996–2015. Direction générale des forêts. CRDA de Nabeul SOGET Maghreb.

Gramaje, D., Agustí-Brisach, C., Pérez-Sierra, A., Moralejo, E., Olmo, L., Mostert, L., Damm, U., & Armengol, J. (2012). Fungal trunk pathogens associated with wood decay of almond trees on Mallorca (Spain). *Persoonia*, *28*, 1–13.

Hlaiem, S., Yangui, I., Della Rocca, G., Barberini, S., Danti, R., & Ben Jamâa, M. L. (2021). *Diplodia* species causing dieback on *Pinus Pinea*: Relationship between disease incidence, Dendrometric and ecological parameters. *Journal of Sustainable Forestry*, *42*, 59–76. https://doi.org/10.1080/10549811.2021.1944879

Hlaiem, S., Yangui, I., Della Rocca, G., Barberini, S., Danti, R., & Ben Jamâa, M. L. (2022). First report of *Pestalotiopsis biciliata* causing dieback on *Quercus coccifera* and *Pistacia lentiscus* in Tunisia. *Canadian Journal of Plant Pathology*, *44*(4), 534–541. https://doi.org/10.1080/07060661.2022.2032831

Hlaiem, S., Zouaoui Boutiti, M., & Ben Jamâa, M. L. (2019). First report of shoot blight caused by *Diplodia scrobiculata* on *Pinus halepensis* in Tunisia. *Journal of Plant Pathology*, *101*(4), 1237. https://doi.org/10.1007/s42161-019-00293-8

Hlaiem, S., Zouaoui-Boutiti, M., Yangui, I., & Ben Jamâa, M. L. (2020). Identification and pathogenicity of *Diplodia seriata* and *Diplodia africana* related to lentisk dieback in Tunisia. *Archives of Phytopathology and Plant Protection*, *53*, 99–111. https://doi.org/10.1080/03235408.2020.1728018

Inderbitzin, P., Bostock, R. M., Trouillas, F. P., & Michailides, T. J. (2010). A six-locus phylogeny reveals high species diversity in Botryosphaeriaceae from California almond. *Mycologia*, *102*, 1350–1368.

Kraus, C., Voegele, R. T., & Fischer, M. (2019). The esca complex in German vineyards: Does the training system influence occurrence of GLSD symptoms. *European Journal of Plant Pathology*, *155*, 265–279. https://doi.org/10.1007/s10658-019-01769-0

Linaldeddu, B., Scanu, B., Maddau, L., & Franceschini, A. (2011). *Diplodia africana* causing dieback disease on *Juniperus phoenicea*: A new host and first report in the northern hemisphere. *Phytopathologia Mediterranea*, *50*, 473–477.

Machado, A. R., Custódio, F. A., Cabral, P. G. C., Capucho, A. S., & Pereira, O. L. (2019). *Botryosphaeriaceae* species causing dieback on Annonaceae in Brazil. *Plant Pathology*, *68*, 1394–1406. https://doi.org/10.1111/ppa.13060

Mohammadi, H., Gramaje, D., Banishashemi, Z., & Armengol, J. (2013). Characterization of *Diplodia seriata* and *Neofusicoccum parvum* associated with grapevine decline in Iran. *Journal Agriole Science Technology*, *15*, 603–616.

Moral, J., Agusti-Brisach, C., Perez-Rodriguez, M., Xavier, C., Carmen Raya, M., Rhouma, A., & Trapero, A. (2017). Identification of fungal species associated with branch dieback of olive and resistance of table cultivars to *Neofusicoccum mediterraneum* and *Botryosphaeria dothidea*. *Plant Disease*, *101*, 306–316. https://doi.org/10.1094/PDIS-06-16-0806-RE

Pérez, C. A., Wingfield, M. J., Slippers, B., Altier, N. A., & Blanchette, R. A. (2010). Endophytic and canker-associated Botryosphaeriaceae occurring on non-native eucalyptus and native Myrtaceae trees in Uruguay. *Fungal Diversity*, *41*, 53–69.

Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald, J. Z., & Crous, P. W. (2013). The Botryosphaeriaceae: Genera and species known from culture. *Studies in Mycology*, *76*, 51–167.

Phillips, A. J. L., Crous, P. W., & Alves, A. (2007). *Diplodia seriata*, the anamorph of "*Botryosphaeria obtusa*". *Fungal Diversity*, *25*, 141–155.

Piskur, B., Pavlic, D., Slippers, B., Ogris, N., Maresi, G., Wingfield, M. J., & Jurc, D. (2011). Diversity and pathogenicity of *Botryosphaeriaceae* on declining Ostrya carpinifolia in Slovenia and Italy following extreme weather conditions. *European Journal of Forest Research*, *130*(2), 235–249. https://doi.org/10.1007/s10342-010-0424-x

Pour, F. N., Ferreira, V., Félix, C., Serôdio, J., Alves, A., Duarte, A. S., & Esteves, A. C. (2020). Effect of temperature on the phytotoxicity and cytotoxicity of Botryosphaeriaceae fungi. *Fungal Biology*, *124*, 571–578.

Qiu, Y., Steel, C. C., Ash, G. J., & Savocchia, S. (2016). Effects of temperature and water stress on the virulence of *Botryosphaeriaceae* spp. causing dieback of grapevines and their predicted distribution using CLIMEX in Australia. *Acta Horticulturae*, *1115*, 171–181.

Schoenenberger, A., Albert, E., Dimanche, P., Franclot, A., & Marion, J. (1971). *Premiers enseignements des arboretums forestiers* (p. 178). Programme des Nations Unis pour le développement. ONU.

Seddaiu, S., Sechi, C., Ruiu, P. A., & Linaldeddu, B. T. (2019). First report of canker and dieback caused by *Diplodia africana* on holm oak in Italy. *The American Phytopathological Society (APS)*, *103*(10), 2670. https://doi.org/10.1094/PDIS-05-19-1062-PDN

Seidl, R., Thom, D., Kautz, M., Martin-Benito, D., & Peltoniemi, M. (2017). Forest disturbances under climate change. *Nature Climate Change*, *7*, 395–402.

Slippers, B., Boissin, E., Phillips, A. J. L., Groenewald, J. Z., Lombard, L., Wingfield, M. J., Postma, A., Burgess, T., & Crous, P. W. (2013). Phylogenetic lineages in the Botryosphaeriales: A systematic and evolutionary framework. *Studies in Mycology*, *2013*(76), 31–49.

Slippers, B., Fourie, G., Crous, P. W., Coutinho, T. A., Wingfield, B. D., Carnegie, A., & Wingfield, M. J. (2004). Speciation and distribution of *Botryosphaeria* spp. on native and introduced eucalyptus trees in Australia and South Africa. *Studies in Mycology*, *50*(2), 343–358. https://edepot.wur.nl/44555

Slippers, B., Smit, W. A., Crous, P. W., Coutinho, T. A., Wingfield, B. D., & Wingfield, M. J. (2007). Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome and stone fruit trees in South Africa and other regions of the world. *Plant Pathology*, *56*, 128–139.

Slippers, B., & Wingfield, M. J. (2007). Botryosphaeriaceae as endophytes and latent pathogens of woody plants: Diversity, ecology and impact. *Fungal Biology Reviews*, *21*, 90–106.

Sorrentino, E., Succi, M., Tipaldi, L., Pannella, G., Maiuro, L., Sturchio, M., Coppola, R., & Tremonte, P. (2017). Antimicrobial activity of gallic acid against food-related pseudomonas strains and its use as biocontrol tool to improve the shelf life of fresh black truffles. *International Journal of Food Microbiology*, *2*(266), 183–189. https://doi.org/10.1016/j.ijfoodmicro.2017.11.026

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, *25*(24), 4876–4882.

Tsopelas, P., Slippersb, B., Gonou-Zagouc, Z., & Wingfieldb, M. J. (2010). First report of Diplodia corticola in Greece on kermes oak (*Quercus coccifera*). *Plant Pathology*, *59*, 805. https://doi.org/10.1111/j.1365-3059.2009.02244.x

Úrbez-Torres, J. R. (2011). The status of Botryosphaeriaceae species infecting grapevines. *Phytopathologia Mediterranea*, *50*, 5–45.

Valencia, A. L., Gil, P. M., Latorre, B. A., & Rosales, I. M. (2019). Characterization and pathogenicity of Botryosphaeriaceae species obtained from avocado trees with branch canker and dieback and from avocado fruit with stem end rot in Chile. *Plant Disease*, *103*(5), 996–1005. https://doi.org/10.1094/PDIS-07-18-1131-RE

Van der Linde, J. A., Roux, J., Wingfield, M. J., & Six, D. L. (2012). Die-off of giant euphorbia trees in South Africa: Symptoms and relationships to climate. *South African Journal of Botany*, *83*, 172–185.

Van Niekerk, J. M., Crous, P. W., Fourie, P. H., & Hallen, F. (2004). DNA phylogeny, morphology and pathogenicity of *Botryosphaeria* species on grapevine. *Mycologia*, *96*, 781–788.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols. A guide to methods and applications* (pp. 315–322). Academic Press.

Yangui, I., Ben Jamaâ, M. L., Zouaoui Boutiti, M., Vettraino, A. M., Vannini, A., & Messaoud, C. (2021). Occurrence of *Biscogniauxia mediterranea* in cork oak stands in Tunisia. *Phytoparasitica*, *2021*(49), 131–141.

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