Plant Pathology & Quarantine 12(1): 124–132 (2022) ISSN 2229-2217
www.ppqjournal.org **Article**

Doi 10.5943/ppq/12/1/10

Characterization and pathogenicity of *Diplodia seriata* causing branch canker on *Pinus pinea* in Tunisia

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Hlaiem S, Yangui I, Ezzine O, Della Rocca G, Barberini S, Danti R, Ben Jamâa ML 2022 – Characterization and pathogenicity of *Diplodia seriata* causing branch canker on *Pinus pinea* in Tunisia. Plant Pathology & Quarantine 12(1), 124–132, Doi 10.5943/ppq/12/1/10

Abstract

Several species of Botryosphaeriaceae family are among the most aggressive pathogens associated with botryosphaeria dieback of agricultural and forestry trees. Particularly, *Diplodia* spp. having a cosmopolitan distribution, are well-known as virulent of woody plant hosts including *Pinus* spp. In recent years, symptoms of canopy wilt, branch dieback, necrosis, and trunk cankers have been noticed on *Pinus pinea* trees in Tunisian forests. However, this has been less well-documented in North Africa and especially in Tunisia. The aim of this study is to characterize the causal agent of *P. pinea* dieback in northeastern Tunisian forest. A collection of thirty-eight isolates obtained from symptomatic branches of *P. pinea* trees was identified as *Diplodia seriata* by means of morphological characteristics, and phylogenetic analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. A pathogenicity test was conducted on 3-years-old *P. pinea* seedlings, confirmed the virulence of the fungus. To the best of our knowledge, this is the first record of *D. seriata* associated with branch canker on *P. pinea* in Tunisia.

Keywords – botryosphaeria dieback – forestry trees – phylogenetic analysis – virulence

Introduction

Forest decline has occurred in the Mediterranean region having a several impact on a wide variety of plant species, including conifers (Tsopelas et al. 2004, Landmann & Dreyer 2006, Peñuelas et al. 2007, Di Filippo et al. 2010). Tree dieback proliferate due to the combined effect of warming and drought (Allen et al. 2010) followed by a decrease in its defense ability and the establishment of favorable conditions for the infection by biotic pathogens (De Sousa et al. 2008). Thus, fungal pathogens are able to cause severe damage and also to persist in latency inside the

Submitted 11 August 2022, Accepted 26 September, Published 27 October 2022
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living tissue of their hosts (Luque et al. 2000, Franceschini et al. 2005). Moreover, fungus is emerging as one of the most significant threats to the survival and function of Mediterranean forests. In particular, the most widespread and virulent species belonging to the Botryosphaeriaceae family.

Botryosphaeriaceae species are among the most important pathogenic fungi because of their ability to cause diseases on a wide range of valuable plant species (Hilario et al. 2020). Several investigations have shown that these species are directly involved in the aetiology of disease symptoms in Mediterranean basin (Deidda et al. 2012, Larignon et al. 2001, Manzanos et al. 2017). Furthermore, various species of *Diplodia* have been reported to cause canker, leaf spot and fruit rot on mulberry worldwide (Arzanlou & Dokhanchi 2013). Reports of the virulence of *Diplodia* spp. vary depending upon the crop, varieties and hosts involved and it is often regarded as a stress-related pathogen taking advantage of weak or stressed plants. Recently, *Diplodia scrobiculata*, has been recorded as the cause of branch canker on *Pinus halepensis* in Tunisia (Hlaiem et al. 2019). In common with other members of the Botryosphaeriaceae, *D. seriata* is capable of living endophytically inside plants (Crous et al. 2006, Slippers & Wingfield 2007) and latent infections of fruits can result in storage rots. This pathogen is dispersed through both pycnidia and ascospores with conidia regarded as the most important inoculum source for short-distance spread.

During field survey in the northeastern Tunisian forest, a generalized withering and serious dieback problems affecting the stone pine trees (*Pinus pinea* L.) have been detected associated with divers symptoms, namely wood necrosis, branch canker, innumerable pycnidia on infected tissues, stem blight with a poor growth and sudden death of the whole tree. The stone pine trees are susceptible to fungal pathogens, which may create a major ecological problem. It is among the most widely used species in the reforestation program after Aleppo pine (Sghaier et al. 2006). Actually, *Pinus pinea* L. eventually be one of the most valuable species in Tunisian reforestation programs, not merely for wood production, but as well for its nuts, widely used in a lot of traditional dishes (Boutheina et al. 2013).

Nevertheless, insufficient information is available about fungal pathogens associated with forest decline and mainly on *Pinus pinea* in North Africa and particularly in Tunisia. This crucial information is required to develop strategies for disease management. Given the great economic and ecologic values of *P. pinea* trees in the Mediterranean basin and specifically in Tunisia, suitable control strategies have become of primary importance in order to overcome the increasing spread of serious forest decline phenomena. Thus, the present study aims to characterize the causal agent associated with branch canker and dieback symptoms on *P. pinea* trees and to prove its virulence.

Materials & Methods

Sampling and fungal isolation

In total, forty-three stone pine trees were examined in Northeastern Tunisia (36.30'.406" N; 10.38'.780"E). Samples were collected from infected branches of declining *Pinus pinea* trees. From each symptomatic tree, 10 branches showing necrosis were selected and carried to the laboratory for fungal isolation. Fragments (2 × 2 mm) were taken from the margins between the necrotic and healthy tissues of the branches. Samples were then surface-disinfected in 70% ethanol for 2 min and rinsed three times in sterilized water before being placed in Petri dishes containing potato dextrose agar (PDA) containing streptomycin sulphate (0.05 g/l), and incubated in darkness at 25°C for 3 days (Franceschini et al. 2005). Cultures were purified by the hyphal tip technique and incubated under the same conditions described above. Aiming to raise sporulation, sterile pine needles were placed over actively growing cultures and incubated under white light at 25°C.

Morphological and molecular characterization

The obtained isolates were primarily identified based on 7-day-old cultures morphology and conidial features characteristics according to Phillips et al. (2007).

Based on morphological characterization, one representative isolate (TN.3) was selected for molecular characterization. Total DNA from fresh mycelium grown on PDA was extracted using an innuPREP Plant DNA Kit (Analytik Jena AG, Germany) according to the manufacturer's instructions. The ITS rDNA region was amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) and performed as Crous et al. (2013) methods. PCR products were sent for Sanger sequencing (Laboratory of the Interdepartmental Center for Biotechnology Services of Agricultural, Chemical and Industrial Interest-CIBIACI, Italy). New sequence was edited with FinchTV v1.4.0 (Geospiza, Inc., Seattle, Washington, USA; (http://www.geospiza.com/ finchtv) and compared with those deposited in GenBank through BLAST searches (http://www. ncbi. nih.gov/).

Phylogenetic analysis

The DNA sequence alignment was performed using MUSCLE 3.8 (https://www.ebi.ac.uk/ Tools/msa/muscle/). Newly ITS sequence was deposited in the National Center for Biotechnology Information (NCBI) (https:// www.ncbi.nlm.nih.gov/) under an accession number. The ITS sequence obtained in this study was supplemented with further sequences of *Diplodia* species retrieved from GenBank based on BLAST searches and the literature Phillips et al. (2013). Sequence of the isolate TN.3 was aligned with ClustalX v. 1.83 (Thompson et al. 1997). Phylogenetic tree was generated using the Neighbour-Joining (NJ) method with MEGA v. 6 (Tamura et al. 2013).

Pathogenicity test

Pathogenicity assay were carried out on 3-year-old *P. pinea* seedlings grown in plastic pots (N = 5), were inoculated with the putative pathogen at the nursery in natural conditions using Linaldeddu et al. (2008) methods: briefly, a mycelial plug (3–4 mm^2) of the isolate, taken from the margin of an actively growing colony on PDA, was put in a shallow wound made with a sterile scalpel on the stem foot of each seedling. PDA plugs were placed into similar wounds on control seedlings of pine specie (N = 5). The inoculation points were covered with Parafilm for 7 days to maintain moisture.

Results

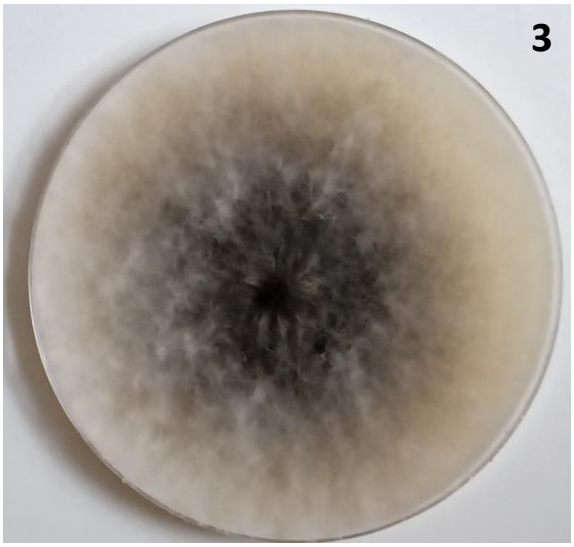
Disease symptoms

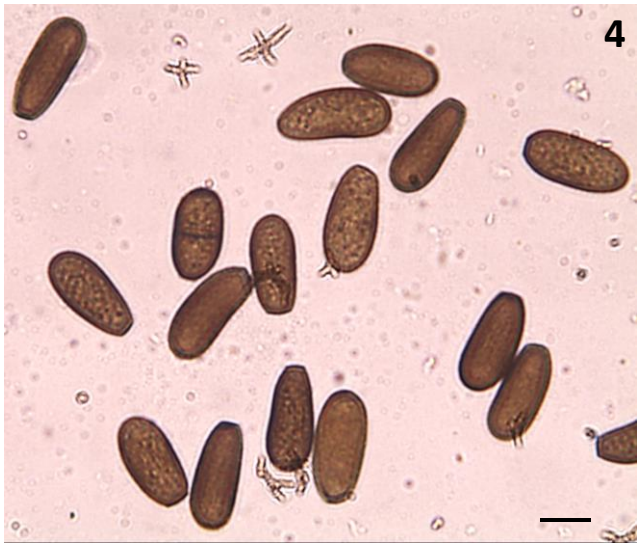
Our investigation revealed that 67% of the examined *Pinus pinea* trees showed branch canker and dieback. The utmost frequently noticeable symptoms include defoliation, yellowish brown needles, canopy wilt, shoot blight, numerous pycnidia on the surface of infected branches, necrotic lesions and vascular discoloration in wood and trunk cankers often associated with gummy exudates. On the branches, damage started as small depressed sunken spots expand to cover larger areas on infected tissues and resulted in branch death (Figs 1–2).

Morphological characterization

A total of thirty-eight isolates were obtained from symptomatic branches of *P. pinea* trees. Cultures of isolates were initially white with fast-growing mycelium, fluffy and highly dense. Then, became grey to dark-grey and finally turned to black. All isolates produced black unilocular and thick-walled pycnidia, semi-immersed on sterilized pine needles plated on PDA within one week of subculturing. Conidiophores reduced to hyaline conidiogenous cells, cylindrical, thin-walled and smooth, and produced a single conidium at the tip. The conidia were brown and oval, broad at the apex and truncated or rounded at the base, and its wall was rough. Immature conidia were aseptate and occasionally produced one septa as they mature, measuring 25.2 ± 0.43 x 9.6 ± 0.17 μm (Figs 3–4). These morphological characteristics were accordant with the description of *Diplodia seriata* (teleomorph: *Botryosphaeria obtusa*) (Phillips et al. 2007).

Figs 1–2 – Disease symptoms on *Pinus pinea* trees. 1 symptomatic trees with canopy wilt. 2 brown necrosis and numerous pycnidia on infected branches.





Figs 3–4 – Cultural and morphological characteristics of *Diplodia seriata*. 3 colony incubated on PDA at 25°C for 5 days. 4 dark brown conidia, Scale Bars = 10 µm.

Molecular diagnosis and phylogenetic analyses

Morphological identification of the Botryosphaeriaceae isolate was confirmed by analysis of the internal transcribed spacer regions (ITS). DNA fragments of approximately 580 bp (ITSr DNA) was amplified in PCR reactions. BLAST searches in GenBank revealed that the isolate TN.3 showed 99% identity with *Diplodia seriata* De Not. including the ex-epitype strain CBS585.50 (Phillips et al. 2012). The obtained sequence was deposited in GenBank and are available under the accession number MT711987. Phylogenetic tree was inferred using the sequence data of ITS region from *D. seriata* (TN.3) isolate and other known *Diplodia* species collected from various other plant species. Phylogenetic relationships among *Diplodia* isolates were assessed using the Neighbour-Joining tree from the DNA sequences of the ITS region were constructed with MEGA 6.0 software (Tamura et al. 2013). Bootstrap values (1000 replications) are provided to indicate support levels for tree nodes (Fig. 5).

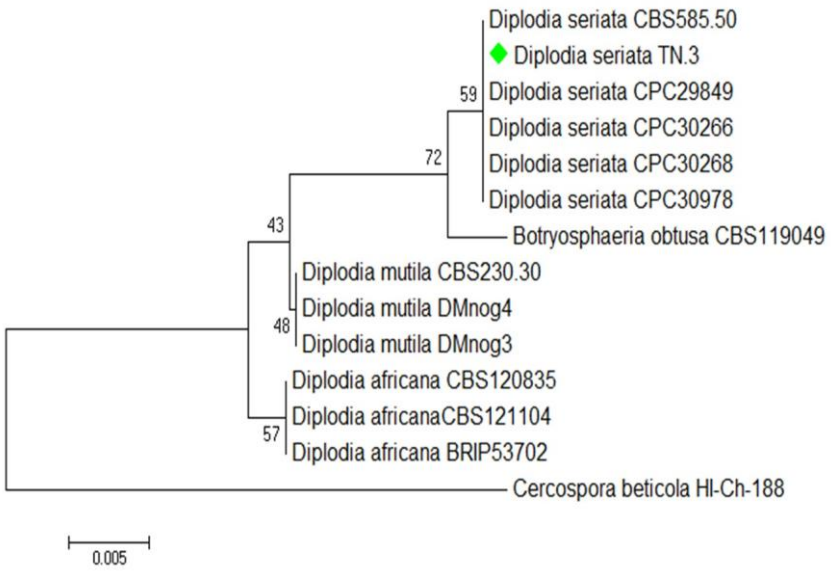
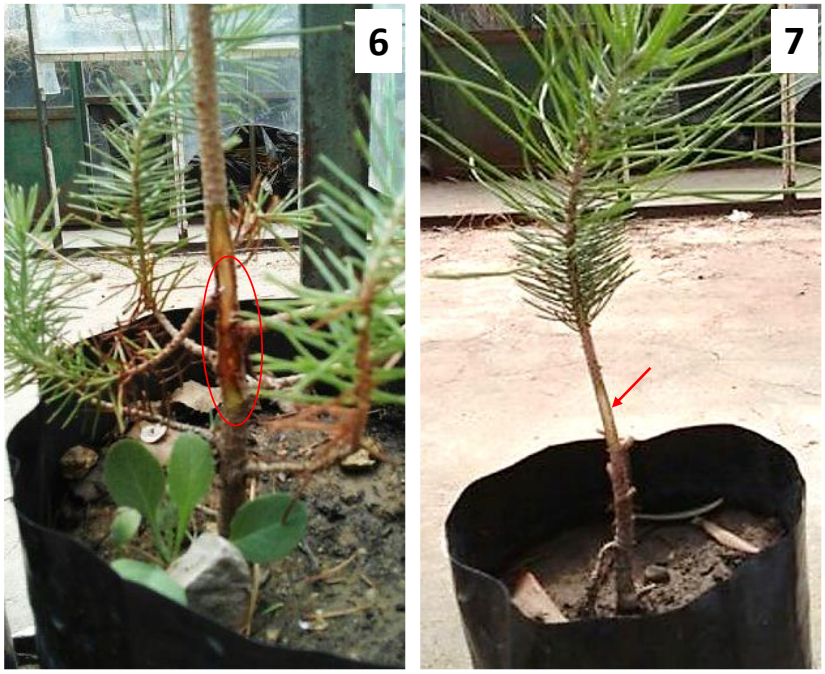


Fig. 5 – A neighbor-joining phylogenetic tree obtained from the ITS regions and 5.8S rDNA sequence data. Bootstrap support values from 1000 replicates are indicated on the nodes. The tree was rooted to *Cercospora beticola*. The scale bar indicates 0.005 substitutions per site.

Pathogenicity assay

Pathogenicity tests achieved on healthy seedlings of *Pinus pinea* yielded to the same symptoms as detected in the forest in natural conditions: blight needles fall, tip blight, brown discolorations on bark and wood tissues, and excessive resin flows from infected twigs. The lower canopy turns brown and eventually leading to death. Thirty post-inoculation days, visible necroses extending from inoculation points were noticed, confirming the virulence of *Diplodia seriata* isolate TN.3 (Fig. 6). Stem lesions measured 5.6 ±0.35 cm. Controls did not develop any disease symptom (Fig. 7). The fungus was successfully re-isolated from necrotic bark and the margin of symptomatic wood tissues, thus fulfilling Koch's postulate.



Figs 6–7 – Pathogenicity test. 6 Stem necrotic brown lesion caused by *Diplodia seriata* on *Pinus pinea* seedling, one-month post-inoculation. 7 asymptomatic control seedling.

Discussion

During our surveys, disease symptoms including crown wilt, yellowish brown needles, shoot dieback, branch cankers, and vascular discoloration were observed on *P. pinea* trees. Similar symptoms to those described herein have been previously reported by Slippers et al. (2004) on Eucalyptus trees in Italy, Van Niekerk et al. (2006) on grapevine in South Africa, Urbez-Torres (2011) on grapevine in California and Phillips et al. (2012) on apple trees in Portugal, and found to be associated with *Diplodia* spp. This genus is a cosmopolitan and plurivorous pathogen occurring on woody hosts (Punithalingam & Waller 1973, Phillips et al. 2007, Slippers et al. 2007). In this current study, the representative isolate TN.3 characterized both on the basis of the morphological features (Phillips et al. 2007, 2012) and molecular analysis confirmed its identity as *Diplodia seriata* clustered with the other *D. seriata* isolates from other host plant species.

Moreover, the pathogenicity assay confirmed the virulence of *D. seriata* on *P. pinea* showing disease symptoms and necrotic lesions around the inoculation point. Diverse works assessing pathogenicity of *Diplodia* species causing canker and dieback, wounded trunks or branches have been inoculated by mycelial plugs of pathogen colony on PDA plates (Taylor et al. 2005, van Niekerk et al. 2004). As well, this method of inoculation was chosen due to wounds that are considered as primary infection sites of several Botryosphaeriaceae family (Bestre et al. 2007) including *D. seriata*. Furthermore, *Diplodia* spp. are usual recognized as wound pathogens, infection is through wounds, natural openings, or direct penetration of the host tissue (Van Niekerk et al. 2006). Similar findings regarding the virulence of *D. seriata* have been reported by Urbez-Torres & Gubler (2009) and Luque et al. (2009). Additionally, studies of Auger et al. (2004), van Niekerk et al. (2006) have confirmed the pathogenicity of *D. seriata* by artificial inoculations on grapevine. Phillips et al. (2012) showed that *D. corticola*, *D. pinea*, *D. mutila* and *D. seriata* are well-known as pathogens of woody plants. In Iran, *D. seriata* was recorded as the causal agent of twig dieback and canker disease on the mulberry (Arzanlou & Dokhanchi 2013) and on cherry laurel plants in Italy (Quaglia et al. 2014). Moreover, this fungus has been reported as pathogen associated with grapevine dieback in Spain (Armengol et al. 2001), California (Urbez-Torres & Gubler 2009), and Lebanon (Zebib 2011). Additionally, *D. seriata* has been occurred on olive drupes in Spain (Moral et al. 2008) and Croatia (Kaliterna et al. 2012). Likewise, Xie et al. (2010) reported this fungal specie as pathogen associated with leaf dieback of *Osmanthus* sp. in China. At a recent time, *D. seriata* has been recognized also as pathogen causing branch canker of *Quercus coccifera* in Tunisia (Hlaiem et al. 2020). In fact, this fungus causes canker, dieback, fruit rot and leaf spot diseases on economically important forest and horticultural species (Farr & Rossman 2020). To the best of our knowledge this paper reports *Diplodia seriata* as a new pathogen involved in *Pinus pinea* trees dieback in the North Africa and specially in Tunisia.

Conclusion

This study exhibited that *Diplodia seriata* is associated with branch canker and *Pinus pinea* decline in Tunisia. Thereby, further in-depth studies are necessary for the purpose of more elucidate the etiology of *P. pinea* dieback and to better understand the mechanisms of *D. seriata* and to define its contribution in this disease. Hence, it is necessary to enhanced this coniferous evergreen tree sustainability by apply silviculture, reforestation, restoration and providing control strategies.

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