



***Heterotruncatella spartii* causal agent of dieback disease on *Pinus pinea* in Tunisia**

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Abstract

Disease is a syndrome of complex origin due to dynamic interactions between the plant host and several abiotic and biotic causes. Fungal infections are the main cause of diseases in forest trees. Decline symptoms are visible at canopy, trunk and branch levels: Canopy transparency gradually increases; plants show necrosis and canker on twigs. Since 2012 symptoms of dieback were observed on *Pinus pinea*, located in the forest of “Henchir Kort” northeast of Tunisia in the region of Nabeul mixed with other species of shrubs. The causal agent obtained from symptomatic tissues was identified as *Heterotruncatella spartii* based on morphological features and genomic DNA sequences of the ITS region. The Koch’s postulates have been verified.

Keywords – Canker – Fungal infections – ITS region – Syndrome

Introduction

The stone pine, *Pinus pinea* L., is a tree species found around the Mediterranean basin. It has been successfully introduced in Tunisia at the beginning of the 20th century along the Mediterranean coast line to consolidate the littoral dunes of Bizerte in the north and along the north east coast in the region of Cap Bon (Hasnaoui 2000). Today *Pinus pinea* covers an area of 21,165 ha (El Khorchani 2010) and becomes one of the most valuable species in Tunisian reforestation programs, not only for wood production, but also because it is much appreciated for its nuts, widely used in a lot of traditional dishes, such as cakes.

In Tunisia, the forest cover is in continuous decline (Ben Jamaa et al. 2007). Furthermore, harmful attacks of bark beetles and the occurrence of their association with plant pathogenic fungi in various pine forests have been reported (Ben Jamaa et al. 2007). There are a very few reports about pine diseases induced by fungal pathogens. Some of the species in the *Truncatella* genus are endophytic, colonizing plant tissues without causing visible symptoms, while others are known pathogens on a wide array of plants.

The aim of this study was to isolate and identify the fungal species associated with the diseased *Pinus pinea* presumed to be a species of *Truncatella*.

Materials & Methods

Collection of sample sand isolation

In October 2016, typical symptoms of the disease was collected from stems of *Pinus pinea* and transferred to the laboratory, fragments taken from the margin of infected tissues were placed in Petri dishes containing potato dextrose agar (PDA) added with streptomycin sulfate (0.05 g/l) antibiotic for isolate identification according to the technique used by Franceschini et al. (2005) and incubated in the dark at 25°C for 3 days. 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.

Morphological and molecular identification

The causal agent was identified based on its cultural traits, conidial morphological characteristics and molecular analysis.

Colony morphology including colour, shape, and growth rate was determined after 7 days of incubation on PDA at 25 °C in darkness. Microscopic characters were studied according to the technique explained by Arzanlou et al. (2007). Dimension of microscopic structures were calculated based on 50 measurements for conidia morphology (shape, colour, and number of appendages), size (length and width of conidia). Regarding the molecular identification, fungal DNA was directly extracted from mycelia growing on plates, using a commercial Kit Macherey-Nagel- 07/2014, Rev.09. PCR reactions were carried out with ITS1 and ITS4 primers (White et al. 1990) to amplify the ITS region of the ribosomal RNA as described by Alves et al. (2004) using the following conditions: 95°C for 3 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; final extension at 72°C for 10 min. Products from PCR reactions 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, Transgen Biotech. Amplified products were sequenced and the sequences obtained were blasted in GenBank.

Pathogenicity test

To fulfill Koch's postulates, 3-year-old *P. pinea* seedlings grown in plastic pots (five plants), were inoculated with the fungal isolate at the nursery in natural conditions using the procedures described by Linaldeddu et al. (2008): A mycelial plug (3–4 mm²) of the strain, taken from the margin of an actively growing colony on PDA, was put in a shallow wound made with a scalpel on the stem foot of each seedling. Sterile PDA plugs were placed into similar wounds on five control seedlings of pine specie. The inoculation points were wrapped in Parafilm for one week to retain moisture.

Results

Morphological and cultural characteristics

Taxonomy

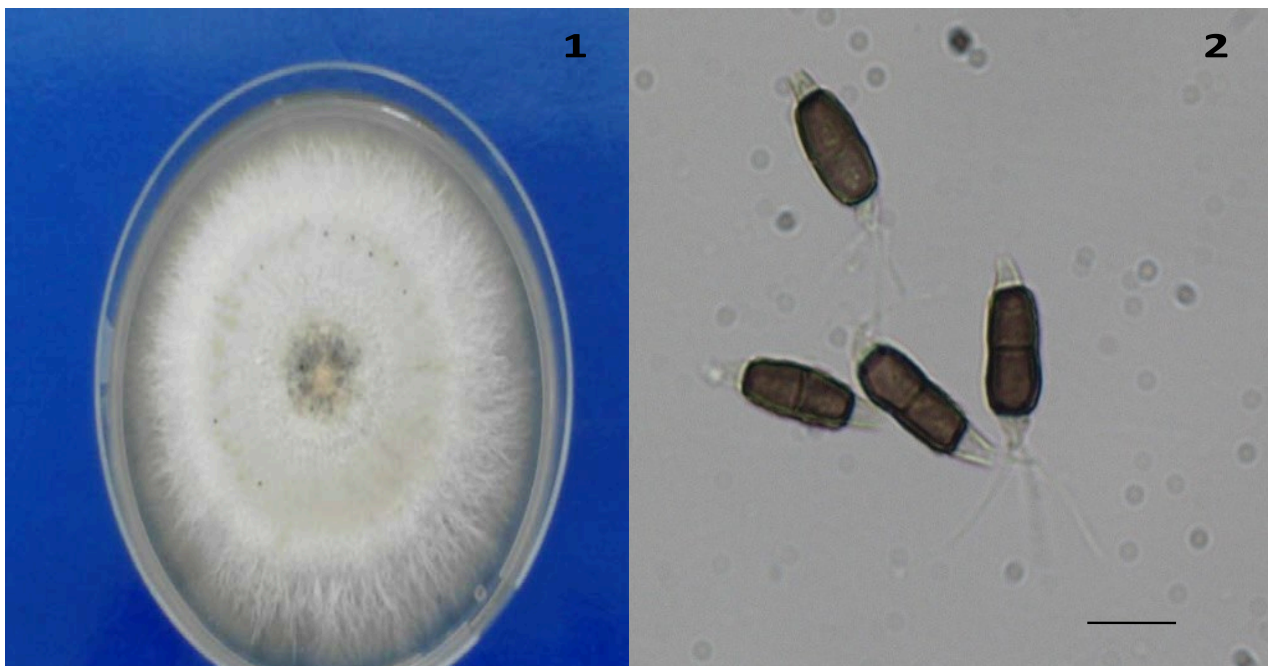
Heterotruncatella spartii (Senan., Camporesi & K.D. Hyde) F. Liu, L. Cai & Crous, in Liu, Bonthond, Groenewald, Cai & Crous, Stud. Mycol. 92: 343 (2018) [2019]

MycoBank MB828362.

Basionym: *Truncatella spartii* Senan. et al., Fungal Diversity 73: 91. 2015. (Holotype of *Truncatella spartii* MFLU 15-0721, ex-type culture MFLUCC 15-0537)

Fungal isolate named TN 8 was obtained from *P. pinea* twigs cankers. The culture characteristics on PDA were flat off-white at first that reached a diameter of 45–55 mm before 7 days in the dark at 25°C and later turned to pale luteous. Fungal colonies developed superficial black acervuli mainly in the center of the PDA plates after 10 days. Conidia were 3-septate (4 cells) and were fusoid to ellipsoid, straight to slightly curved measured 23.5µm (20.5-26.0) × 6 µm (4.7-

8.9). There were 2 median cells dark brown. Both apical and basal cells were hyaline, the apical cell with 3 or 5 appendages (mostly 3) and the basal cell with a truncate base. Morphological and cultural characteristics as well as type of conidia observed were very similar and typical of the genus *Truncatella* (Figs 1–2).



Figs 1–2 – *Heterotruncatella* colony cultured after 12 days on Potato-Dextrose-Agar at 25°C (1); Conidia with apical and basal appendages (2). Scale Bars = 10 µm.

Phylogenetic analysis

Phylogeny inferred using the sequence resulting from the PCR amplification of the nuclear rDNA operon using the primers ITS1 and ITS4 from the isolate obtained in this study TN 8 with other known isolates of *H. spartii* from GenBank clustered our isolate with 96 percent bootstrap value (Fig. 3). The fungus revealed a high degree of similarity (99%) with the ITS region of *H. spartii* CBS 143894 (MH554134) (Liu et al. 2019) was identified as *Heterotruncatella spartii* and the representative sequence was deposited in GenBank (MK418461).

Pathogenicity test

Pathogenicity tests performed on healthy seedlings of *Pinus pinea* led to the same symptoms as observed in field conditions: blight needles fall. Necrosis at branches, cankers at the level of the trunks and excessive resin flows from infected twigs and branches. The lower canopy turns brown and eventually dies.

After 4–6 weeks of inoculation all seedlings inoculated with *H. Spartii* showed symptoms of tip blight and brown discolorations of the bark and wood tissues in the stem and eventually died. Stem lesions measured 5.3 ± 0.5 cm (Fig. 4). Controls did not develop any disease symptom (Fig. 5). The fungus was successfully re-isolated from necrotic bark and the margin of symptomatic wood tissues, thus fulfilling Koch's postulate.

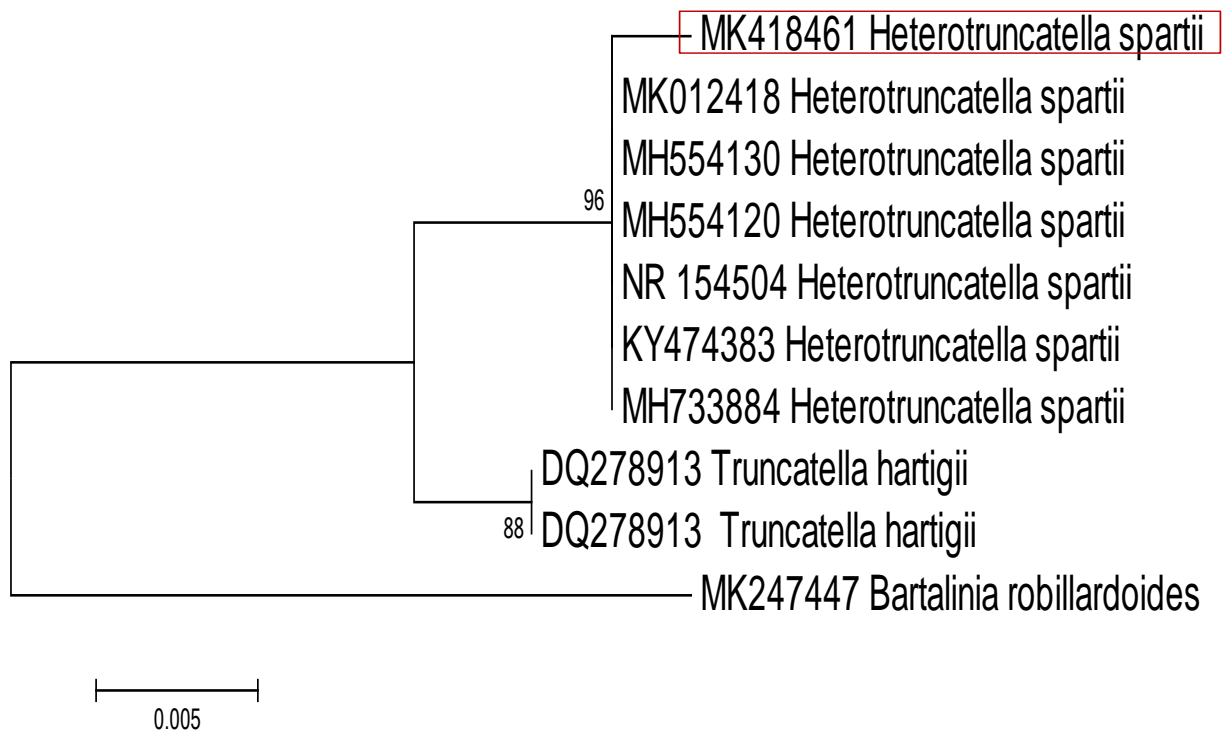


Fig. 3 – A neighbor-joining phylogenetic trees obtained from the ITS regions and 5.8S rDNA sequence data. Bootstrap support values from 1000 replicates are indicated on the nodes. The tree was rooted to *Bartalinia robillardoides*. The scale bar indicates 0.005 substitutions per site.



Figs 4–5 – Pathogenicity test. 4 Stem necrotic brown lesion caused by *Heterotruncatella spartii* on *Pinus pinea* seedling, 4 weeks after inoculation. 5 asymptomatic control seedling.

Discussion

The causal agent was identified as a member of the genus *Heterotruncatella* based on morphological criteria of conidia. The morphology of our isolates was in full agreement with the description for *Heterotruncatella* (Senanayake et al. 2015). The fungus produces colonies similar to those of *Pestalotiopsis* and produces similar conidiomata (Pitt & Hocking 1997). *Truncatella* was easily identified based on its four-celled, fusiform conidia (Maharachchikumbura et al. 2016). The morphological criteria used for the delineation of pestalotioid fungi are insufficient and overlap among different genera (Lee et al. 2006, Barber et al. 2011). With the service of DNA sequence data, taxonomy of pestalotioid fungi has undergone drastic revision (Jeewon et al. 2002, 2003, 2004, Kang et al. 1998, 1999, Lee et al. 2006) and now the boundaries of the genera are more clear (Lee et al. 2006, Tanaka et al. 2011). Phylogenetic analysis carried out in this study further confirmed the identity of our isolates TN 8 as *Heterotruncatella spartii*.

Neopestalotiopsis, *Pestalotiopsis* and *Truncatella* belong to the order Xylariales and are generally known as pestalotioid fungi (Maharachchikumbura et al. 2014). The genus *Truncatella* was introduced by Steyaert (1949) to accommodate species having three-septate conidia.

The results of pathogenicity tests revealed *H. spartii* to be pathogenic on *Pinus pinea*. The genus *Truncatella* represents a well known plant pathogen, encompassing some 23 species (Crous et al. 2004). These taxa are significant phytopathogens causing postharvest fruit rot and trunk diseases in grapevines in many countries (Arzanlou et al. 2013, Jayawardene et al. 2015). *Neopestalotiopsis*, *Pestalotiopsis* and *Truncatella* species are associated with grapevine trunk diseases in France (Maharachchikumbura et al. 2016).

To the best of our knowledge this paper reports *H. spartii* as a new pathogen on pine and first record for the genus *Heterotruncatella* in Tunisia.

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