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SHORT NOTES

FIRST REPORT OF THE LIGNIVOROUS FUNGUS *PLEUROSTOMA RICHARDSIAE IN CEDRUS ATLANTICA* M. IN MOROCCO

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ABSTRACT

Our study is interested in isolating fungi from the wood parts of cedar trunks withered and identifying these lignivorous fungi. A sample was isolated from the cedar grove of Tazekka National Park located south of the city of Taza in Morocco. The culture and purification of the isolated fungus were done on a water agar medium and then on a PDA medium. After the purification of the fungus, a morphological study by electron microscope allowed us to identify the pathogen *Pleurostoma richardsiae*. A molecular characterization confirmed these results with a coverage percentage of 97% and an identity of 99%. To our knowledge, this is the first report of *P. richardsiae* in decayed cedar wood.

KEYWORDS: *Cedrus atlantica* M., cedar wood. *Pleurostoma richardisae*, red ring rot, M'jej, cubic brown rot, Saboune.

INTRODUCTION

The Atlas cedar (*Cedrus atlantica* M) is a natural forest wealth in Morocco. The wood of this plant is renowned for its quality. However, it is exposed, like all other trees, to the attacks of several pests and fungi such as *Trametes pini* causing the red rot (M'jej), and *Ungulina officinalis* causing the cubic rot (Saboune). Indeed, these two diseases are the cause of cedar wood decay.

The cedar is a very sought-after plant worldwide since this tree can adapt to different biotopes (Güney et al. 2020), as well as for the excellent quality of its wood, which is ranked among the best woods in the world. The genus *Cedrus* includes four species: the Atlas cedar (*Cedrus atlantica* Manetti), the Lebanon cedar (*Cedrus libani* Richard), the Cyprus cedar (*Cedrus brevifolia* Henry), and the Himalayan cedar (*Cedrus deodara* Don) (Forgione et al. 2019). Cedar, like all plant resources, is rich in bioactive molecules, such as himachalenes, atlontones and in general the terpenes (Saab et al. 2018), these molecules are known for their crucial antimicrobial power as well as for their cytotoxic effect (Belkacem et al. 2021, Elias et al. 2019). The cedar wood, in addition to its good quality, which makes it very requested by the sawyers, and its richness in secondary metabolites, which are the base of several medicines, is rich in molecules exploitable in the perfumery such as the 4-acetyl-1-methylcyclohexene and the vestitenone (Uehara et al. 2017).

Cedar groves in the world suffer from the attacks of several pests. These attack the seed, roots, stem, trunk, twigs, and fruit. *Cedrus atlantica* is also exposed to these pests that cause decay and decomposition of the wood (Abourouh and Morelet 1999). This decomposition of the wood is mainly due to two diseases known to Moroccan sawyers: Saboune and M'jej: *(1)* Cubic brown rot (Saboune) caused primarily by *Fomitopsis pinicola* or *Ungulina officinalis*, it is characterized by a brown, cubic rot of the heartwood and sapwood of dead trees and often on the wood of stumps, logs, or live trees. The first signs of infection are the appearance of a pale yellow to brownish discoloration of the wood. In the advanced stage of the attack, the wood takes on a brown and cubic appearance and crumbles over time into cubic pieces. This brown rot is typical; only the cellulose is degraded (High Commissioner of Water and Forests and The Fight Against Desertification 2009, Sarkhad et al. 2022). *(2)* Red ring rot (M'jej) caused primarily by *Trametes pini* or *Phellinus chrysoloma*. Fungi can cause heart rot in living trees, but their prominent role is to decompose the wood of trees killed by other pathogens. Infection usually begins via a wound already present on the tree. Since the fungi mostly colonize dead material, there can be many sites of infection. The first signs of infection are the appearance of a pale yellow to brownish discoloration of the wood, which often appears as a ring at the beginning of the attack. The wood decays over time (Wang et al. 2021). When the disease is well established, small spindle-shaped cells of white tissue, characteristic of the disease, become visible. The edges of infected sections show red fruiting bodies in broad bands. They are complex, lignified, and perennial, meaning they remain on the tree season after season. Over time, the fruiting bodies become gray and wrinkled, and the underlying wood becomes dry and light brown. The disease kills affected trees only in rare cases of very severe generalized infection. Damage is most severe on older or less vigorous trees. The attacked wood is seriously devalued and unusable (High Commissioner of Water and Forests and The Fight Against Desertification 2009).

Pleurostroma richardsiae, also known as *Phialophora richardsiae,* is a wood-rotting fungus and can also be involved in human infections (Ferreyra et al. 2020, Ghasemi-Sardareh and Mohammadi 2020, Levenstadt et al. 2012). This fungus causes essential damage in the aerial parts of the plants and especially the stem and the trunk (van Dyk et al. 2021a,b, Vijaykrishna et al. 2004). The systematic position of this fungus is given in Tab. 1.

The main objective of this study is the microscopic and molecular identification of the fungal pathogen isolated from infected cedar wood.

Tab. 1: Taxonomical classification of Pleurostoma richardsiae.

Kingdom	*Fungi*
Subkingdom	*Dikarya*
Division	*Ascomycota*
Subdivision	*Pezizomycotina*
Class	*Sordariomycetes*
Subclass	*Sordariomycetidae*
Ordrer	*Calosphaeriales*
Family	*Pleurostomataceae*
Genus	*Pleurostoma*
Species	*Pleurostoma richardsiae*

MATERIALS AND METHODS

Raw material

Samples in the form of small pieces of 1 to 2 cm of decomposed wood were taken from the trunks of cedar trees (*C. atlantica*) visually infected by the two diseases at the level of the Tazekka national park located south of the city of Taza in Morocco (Fig. 1). This fungus was isolated from a dead tree trunk infected with red ring rot (M'jej) at 34°05'05"N,4°10'35"W.

Fig. 1: Decayed cedar tree (Tazekka national park) from which a sample was taken: a) view into tree trunk from outside, b) view into tree trunk from inside.

Culture and purification

The pieces of wood of 1 to 2 cm were deposited after their collection in Petrie dishes containing only agar water (20 g of agar-agar per litre); the latter is a minimum medium to inhibit bacterial growth. After the mycelium's appearance, the fungal strains' purification was done by subculturing in malt-agar medium (30 g of malt + 20 g of agar-agar + 50 mg of the antibiotic

chloramphenicol per litre). The antibiotic is used for the inhibition of the bacterial growth. In 100 mL of a liquid medium containing 5 g of yeast extract + 5 g of malt extract + 5 g of sucrose + 50 mg of chloramphenicol per litre, the obtained strains were seeded to ensure an excellent mycelial growth. The mycelium obtained after one week was lyophilized in Eppendorf tubes for preservation and DNA extraction.

Morphological characterization

A fragment of the fungus was taken after purification and put under the microscope with one or two drops of methyl blue for observation and identification. Once the fungus structures were observed separately, their multiplication was performed for further studies.

DNA extraction and PCR

From 20 to 30 g of the mycelium, DNA was extracted by the Bioline ISOLATE II Plant DNA Kit, BIO-52070. A NanoDrop measured the purity of the obtained DNA. The ITS sequences of the rDNA genes were amplified by the two universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The reaction mixture volume of 25 µL consists of 1 µL of DNA, 2 µL of dNTP, 0.8 µL of Taq DNA polymerase, 2.5 µL of Buffer ×10, 1 µL of each ITS primer, and 16.7 µL of ultra-purified water. The amplification was under the following conditions: a denaturation phase at 95°C for 2 min, a 35-fold cycle (denaturation phase at 95°C for 30 s, hybridization phase at 57°C for 30 s, and elongation phase at 72°C for 30 s), with a final extension at 72°C for 1 min. After amplification, a 1% agarose gel electrophoresis was performed to check the quality of the PCR product obtained.

PCR product purification

The purification kit (ExoSAP-IT™ PCR Product Cleanup Reagent) was used to purify the PCR product by adding to 5 uL of DNA (PCR product) 1uL of EXO-SAP-IT, the thermocycler program is 37°C/15 min to activate the enzymes, and 80°C/15 min to inactivate these enzymes.

Sequence reaction

BigDye™ Terminator v3.1 Cycle Sequencing Kit is used in the sequence reaction by adding to 1 uL of purified PCR product the following reagents: 2 uL of BIG DYE Seq 3.1, 2 uL of 5X Buffer, 1 uL of Primer (10µM), and 4 uL of ultrapure H2O. The conditions for the sequence reaction were as follows: 96°C/ 1 min, 25-fold cycle (96°C/10S, 50°C/5 s, 60°C/4 min). The purification of the sequence reaction was done following the manual of the Xterminator SAM solution kit.

Sequencing and bioinformatics analysis

Sequencing was done using an Applied Biosystems 3130xl Genetic Analyzer Sanger sequencer. The DNA Baser Assembler software did the assembly of the results. The ITS sequences obtained were blasted with the NCBI nucleotide library collection.

RESULTS AND DISCUSSIONS

Colonies multiply on PDA medium after 3 to 5 days of incubation at 22-25°C in the dark. They appear with a round aspect, powdery to woolly or in clumps, a gray-brown coloration in the center, and a light brown aspect at the end. Under the electron microscope, the mycelium appears septate, two types of conidia are generated: the first, brown, thick-walled conidia, which are spherical to subspherical, formed on dark brown, thin, tapered phialides with flared collars. The second, hyaline conidia that are allantoid or cylindrical, formed on discrete, peg-like phialides on thin-walled hyphae (Fig. 2).

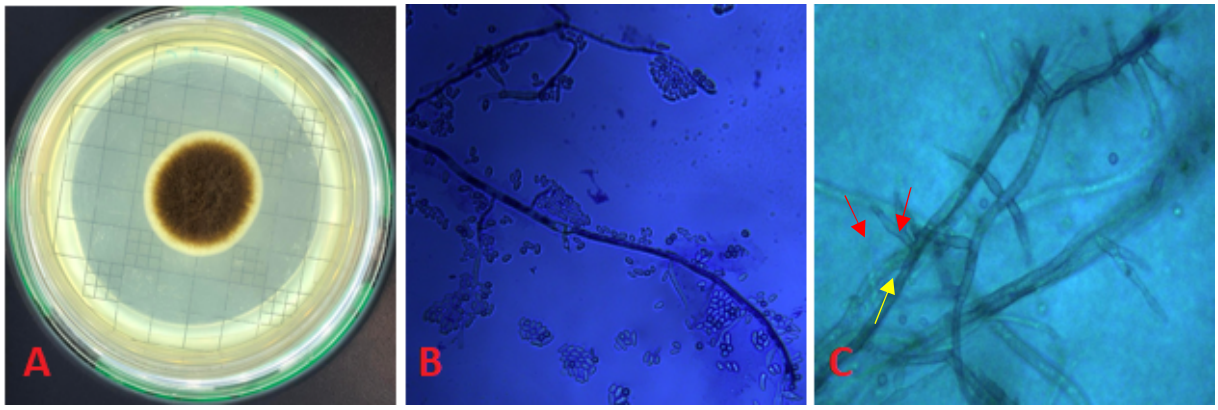

Fig. 2: Pleurostoma richardsiae: a) colony on PDA medium after 5 days of incubation at 22-25°C, b) mycelium of the fungus with different forms of phialides and conidia, c) two phialides are visible in the center of the picture (red arrows) with distinct septa visible at their base (yellow arrows). Microscopic observation ×40.

The blast of the DNA sequence obtained after the assembly of the sequencing results allowed us to identify *Pleurostoma richardsiae*, with a coverage percentage of 97% and a similarity of 99%.

Pleurostoma richardsiae is known for its pathogenicity and the degradation of the trunk wood of infected trees (Raimondo et al. 2019, Sohrabi et al. 2020). This fungus causes severe damage to olive trees, causing leaf browning and leaf drop, wilting of apical shoots, dieback of twigs and branches, and brown streaks under the bark of the trunk, branches, and twigs. It was recognized as the most aggressive and first reported as a pathogen of olive (Carlucci et al. 2013). It has also been isolated from olive trees in California and identified as a pathogen that causes branch and trunk cankers (Lawrence et al. 2021). In Spain and Croatia, *P. richardsiae* was first reported as an agent of trunk and twig wood decay in olive trees (Calvo-Peña et al. 2021, Ivic et al. 2018). Also, in Spain and Turkey, *P. richardsiae* has been reported as an aggressive causal agent of grapevine trunk decline (Özben et al. 2017, Varela et al. 2016). In cedar groves, the fungi *Trametes pini*, *Ungulina officinalis*, *Phellinus chrysoloma*, *Porodaedalea pini*, and *Coniophora puteana* have been isolated as parasitic fungi of cedar, and other agents have not yet been identified (Aberchane et al. 2003, Zaremski et al. 2007).

CONCLUSIONS

Pleurostoma richardsiae is a lignivorous fungus that causes wood dieback on trunks and twigs. Several studies have reported it as an aggressive and pathogenic agent of olive trees in Spain and California, as well as an agent of vine decline in Turki and Spain. Our study identified this fungus in a sample isolated from a dead and depleted cedar trunk, which shows symptoms of severe infection by a disease known as M'jej in Morocco. This disease causes the decay of the cedar wood, which leads to losses of 40% of the wood. To our knowledge, this is the first report of *P. richardsiae* in Cedarwood.

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