ARTICLE / INVESTIGACIÓN

Molecular characterization of endophytic fungi from pine (*Pinus oocarpa*) in Honduras

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Abstract: Pine is one of the important coniferous genera in America. In Honduras, Pinus oocarpa is the most critical conifer species since it plays an essential role for the country's economy and represents a symbol of national identity. Endophytic fungi are defined as microorganisms that reside in the internal tissues of plants without causing any obvious immediate negative effects and are an integral part of their associated microbial communities. This study aimed to isolate and characterize the endophytic fungi present in the pines of Honduras through the amplification and sequencing of the ribosomal RNA gene's internal transcribed spacer (ITS). A total of 7 pines from the department of El Paraíso in eastern Honduras were analyzed. A total of 14 fungi grouped into 6 genera and 7 species were isolated: *Fusarium lateritium, F. pseudocircinatum, Pestalotiopsis pini, P. microspora, Xylaria grammica, Trichoderma atroviride*, y *Nigrospora oryzae*. To our knowledge, this is the first report of endophytic fungal species in pines in Honduras. Although some endophytic fungi may be mutualistic or saprophytic, the present study shows the presence of several genera of endophytic fungi that have been reported as pine pathogens. The presence of these fungi in the pines of Honduras represents a potential threat to the health of the forest. Further research is needed to increase knowledge about the importance of these fungi and the potential impact they could have on pine forests in Honduras.

Key words: Endophytic fungi, *Pinus oocarpa*, Honduras.

Introduction

Pinaceae is the most important of the eight families of conifers. Within this family are grouped 11 genera distributed in boreal, arid, subalpine, temperate and tropical forest ecosystems[1,2]. The *Pinus genus* is one of the most important within this family and includes more than 100 species distributed mainly in the forests of the countries of the northern hemisphere in Europe, Asia, North Africa, North America, and Central America. "Pines", as they are commonly called, have high ecological importance since they are the predominant tree species in many of the countries of these geographical regions[3]. Also, pine trees represent a fundamental pillar in the economies of several countries, and its by-products contribute significantly to the Gross Domestic Product (GDP), mainly in low- and middle-income countries that export the wood as raw material[4,5].

Endophytic fungi are microorganisms that reside in the internal tissue of plants without causing any apparent immediate adverse effects and that, together with other microorganisms, are an integral part of the microbial community of the plant[6,7]. The association between fungi and plants represents a universal and cryptic phenomenon in nature6. Most of these microorganisms are transmitted horizontally through the production of conidia or spores that spread to adjacent uninfected plants using physical or mechanical agents[6]. Most of the endophytic fungi belong to the Ascomycota phylum; however, some genera belonging to the Glomeromycota, Basidiomycota and Zygomycota phyla have also been reported[6,8]. Endophytic fungi are associated with plants as saprophytes, mutualists, or parasites[7,9,10], although these fungi are generally found in host tissue as mutualists[9]. Factors such as stress, physical changes and the level of maturation of the plants are some of the causes that could be involved in the development of infections by this microorganisms[7].

Endophytic fungi have been recognized as possible disease-causing agents in conifers, mainly in pines[11-13]. Honduras has an estimated forest cover of 6,314,815 hectares, of which 1,951,978 hectares (30.91%) are pine forests[14] including seven species and five varieties: *P. oocarpa var. oocarpa and var. trifoliata, P. maximinoi, P. caribaea var. hondurensis, Pinus ayacahuite var. ayacahuite, P. hartwegii, P. pseudostrobus var. pseudostrobus, and P. tecunumanii*[15]. In Honduras, pine is the most critical conifer species, and *Pinus oocarpa* has been selected as a national tree for its relevant economic role and scenic importance. Given the pathogenic potential that some genera of endophytic fungi could represent and due to the economic and cultural importance of pine trees in Honduras, this study aimed to characterize endophytic fungi isolated from pine using molecular methods.

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Materials and methods

A total of 7 pine trees (*Pinus oocarpa Schiede ex Schltdl*) from the department of El Paraíso in Honduras were analysed. The trees showed a change in the color of the needles from their apex to the base, exudation of resin at the base of the fascicles, and vascular damage (Figure 1). Endophytic fungi were isolated using the superficial sterilization technique, previously described by Stone *et al.*[7]. Briefly, 4 samples of 1 cm of pine needles were randomly selected from each tree. The outside of the needles was sterilized with 4% sodium hypochlorite solution for 3 min, then rinsed three times with sterile distilled water. Once disinfected, the needles were cultured independently on PDA (Potato Dextrose Agar) and 10% tartaric acid. The Petri dishes were incubated at 25ºC and observed daily for 7 days to detect the appearance of colonies (Figure 1), then all the isolates were purified using the hyphal tip or single spore methods. DNA extraction was performed by inoculating the mycelium of each strain in 3 ml of GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone)[16]. All cultures were incubated for two days at 26ºC. Subsequently, the mycelium mats were recovered and subjected to a protocol based on organic solvents. Briefly, the mycelium was resuspended in 500 µl of lysis buffer composed of 10 mM Tris, pH 8; 1 mM EDTA, pH 8; and 100 mM NaCl. This suspension was heated to 100ºC for 1 min in a water bath and then shaken three times in a Disruptor Gene® system (Scientific Industries) with 0.1 mm glass beads for 2 min with rest intervals of 1 min. 400 µL of phenol-chloroform-isoamyl alcohol was added (25:24:1), vortex mixed and centrifuged at 13,000 rpm for 15 min. The supernatant was recovered, and a volume of chloroform-isoamyl alcohol (24:1) was added, mixed with vortex, and centrifuged at 13,000 rpm for 5 min. The aqueous phase was transferred to a new vial and precipitated with a volume of absolute cold ethanol and 1/10 of the volume with sodium acetate (3M, pH 5.2), mixed by inversion and centrifuged at 13,000 rpm. The supernatant was removed, and the sediment was subsequently washed with 70% ethanol. The dried pellet was resuspended in 50 µL of nuclease-free water. The DNA concentration was calculated with a NanoDrop® spectrophotometer (Thermo Fisher Scientific Inc.), diluted to a final concentration of 40 ng/µL and stored to −20ºC until further use.

Molecular identification of all isolates was performed by amplification and sequencing of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA gene. The ribosomal ITS region is recognized as a universal marker for the identification of fungi[17]. Polymorphisms in both length and sequence, the availability of databases to compare sequences, universal primers and a high rate of successful amplification among all fungal lineages are some of the reasons why this marker has been recognized as a good outlet for the identification of endophytic fungi[8,9,17,18].

PCR amplifications were carried out under the following conditions in a 50 µL volume: 25 µL of PCR Master Mix (Promega Corp. Madison, WI, USA), 1 µL of 10 µM ITS1 and ITS4 primers 5'-TCCGTAGGTGAACCTGCGG-3'/5'-TCCTCCGCTTATTGATATGC3', and 1 µL of DNA (40 ng/ µL). Reactions were carried out with an initial denaturation step at 95ºC for 5 min, 37 cycles of 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s, with a final extension at 72ºC for 10 min. Amplicons were visualized in 1.5% agarose gel electrophoresis with ethidium bromide. The amplified products were sequenced on both flanks with their respective primers according to Psomagen company protocols (https://lims.psomagen.com/). The quality of the sequences was analyzed in the software with the Geneious®9.1.7 software and queried against international databases contained in NCBI to confirm the identity of the sequences. The sequences were compared with sequences deposited in GenBank, registering the result with the highest percentage of similarity as the most likely identification of each isolate. The sequences were deposited in GenBank, and accession numbers were assigned for 12 isolates.

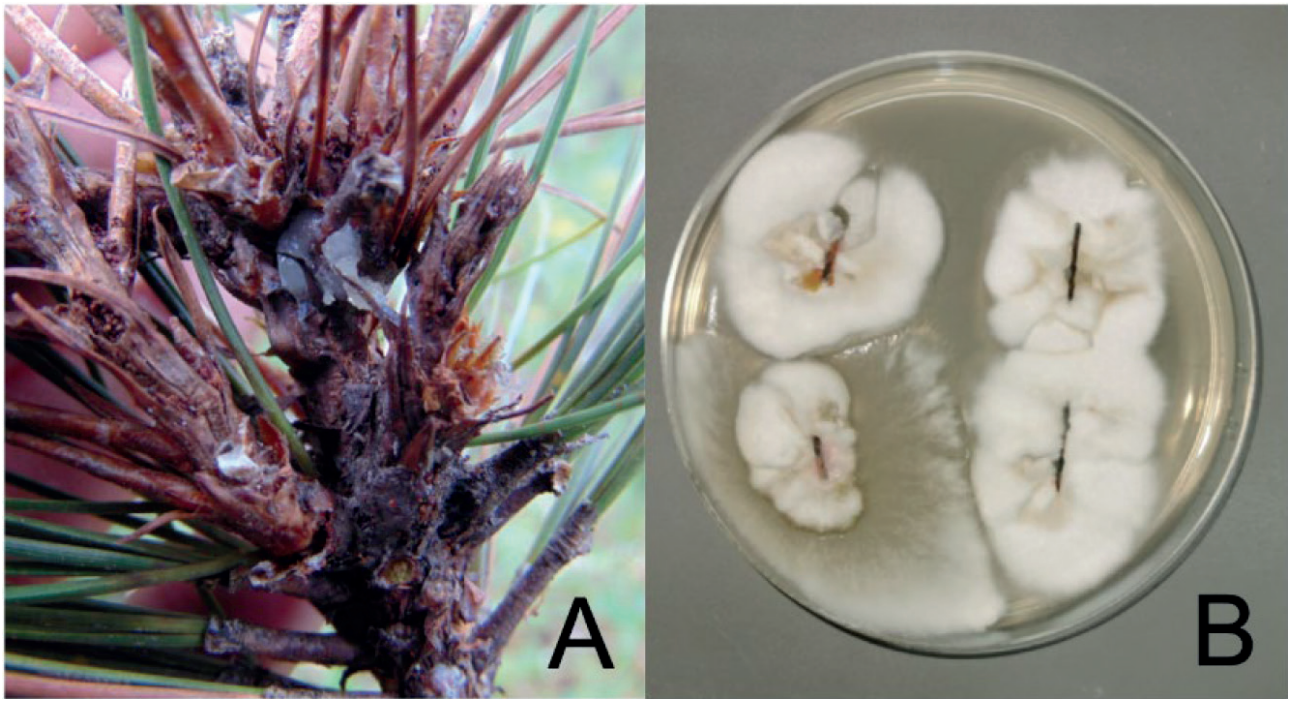

Figure 1. (A) Pine needle lesions and resin exudation at the base of the fascicles; (B) Endophytic fungal growth from pine needles.

Results and discussion

A total of 14 monoaxenic isolates were obtained from the 7 trees analyzed. All samples were successfully amplified and sequenced. The size of the amplicons ranged from 500 to 700 bp. The sequencing results yielded 6 genera and 7 different species. The endophytic fungi identified were F*usarium lateritium, F. pseudocircinatum, Pestalotiopsis pini, P. microspora, Xylaria grammica, Trichoderma atroviride*, and *Nigrospora oryzae*. The most frequently identified species was *P. pini*, while *Trichoderma atroviride* and *Xylaria grammica* were the least frequent (Table 1). All the fungal isolates in this study belong to the Ascomycota phylum, which agrees with what has been reported in the literature regarding the classification of endophytic fungi in plants[6,8,19]. However, the isolates were grouped under the Sordariomycetes class, and this finding contrasts with what was previously reported in Germany and Spain, where the Dothideomycetes class was the most frequent in *Pinus silvestris*[19, 20]. Something similar happened with the endophytes described in *Pinus nigra* subsp.

On the other hand, endophytic fungi of the Arthoniomycetes class were the most frequent in *Pinus silvestris* from Sweden[24], and the Leotiomycetes class was the most commonly found in the United States and South Korea in *Pinus taeda, P. leiophylla* and *P. densiflora* respectively[25-27]. The Sordariomycetes class was the second in frequency in Spain, South Korea and the USA[19, 26, 27], while in a study carried out in Germany, fungi of the class Sordariomycetes were found in 31% of all isolates[20]. Moreover, according to what was reported by *Bullington et al.* in the USA and *Taudiere et al.* in Croatia this class was the least frequent in both studies[21,22]. The high diversity of microorganisms that pine species can harbor in different geographic regions is evident, and multiple factors could influence the prevalence and distribution of fungal communities in coniferous species. Further studies are needed to clarify the main determinants of the prevalence of various classes of fungi in this group of trees.

Of 14 isolates, 6 (42.9%) belonged to two species of the genus *Pestaloptiopsis*: *P. pini*, (n = 4) and *P. microspora* (n = 2). Fungi grouped in this genus are generally considered secondary pathogens that can be responsible for a wide variety of plant diseases including cankers, dieback, leaf spots, needle blight, tip blight, grey blight, and severe chlorosis, among others[13]. The presence of *Pestalotiopsis spp.* in pines has been reported in Iraq, Spain, Portugal, China, USA, Slovakia and some other European countries[13, 28-32], but as far as we know this is the first report of two species of the genus *Pestalotiopsis* in Honduras.

Similarly, *Fusarium lateritium* (n = 2) and *F. pseudocircinatum* (n = 1) were identified in this study. *Fusarium spp.* it is among the main genera of endophytic fungi. This genus includes important plant pathogens that affect both forest and agricultural species. Although the presence of *F. lateritium* and *F. pseudocircinatum* has been reported in pine trees[33], there is little information in the literature on these two species that cause pine infection. Romon *et al.* suggested that *F. lateritium* was a potential agent for the biological control of infections by phytopathogens, mainly *F. circinatum*[34]. It is important to highlight that, although the ITS regions are considered a reliable universal marker for identifying *Fusarium*, the combination of at least two loci could be more informative and conclusive for the discrimination between species, which is a limitation in this study.

On the other hand, two isolates of the genus *Nigrospora*, one *Xylaria grammica* and one *Trichoderma atroviride* were identified. These fungi have also been identified as endophytic microorganisms of pine trees in the western part of the Himalayas, Spain, and China[19,35]. To our knowledge, this is the first report of these three genera of endophytic fungi isolated from *Pinus oocarpa* in Honduras.

Conclusions

This is the first report of endophytic pine fungi isolated in Honduras. The presence of these fungi in the pines of Honduras represents a potential threat to the health of the pine forests. The information of these fungi in the country is limited and more studies are needed to increase our knowledge about the presence of these microorganisms and the potential impact they could have on the forest.

Author Contributions

B.O., L.E., and G.F., conceptualized the study; K.M., Y.Y., Y.S., S.G., K.A., performed the mycological diagnosis; B.O., K.A., performed molecular experiments; B.O., K.A., Y.S., S.G., L.E., and G.F., organized and cured the data; writing and original draft preparation, B.O., and G.F.; All the authors contributed with writing, review, and editing the manuscript; supervision, project administration, and funding acquisition, B.O., K.M., Y.Y., L.E., All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Any research article describing a study involving humans should contain this statement. Please add "Informed consent was obtained from all subjects involved in the study." OR "Patient consent was waived due to REASON (please provide a detailed justification)." OR "Not applicable." for studies not involving humans. You might also choose to exclude this statement if the study did not involve humans.

Written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Please state "Written informed consent has been obtained from the patient(s) to publish this paper" if applicable.

Data Availability Statement

In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "Bionatura Research Data Policies" at https://www.revistabionatura.com/policies.html. You might choose to exclude this statement if the study did not report any data.

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Code	Identification	Blast results (Accession number)	Query Cover	Taxonomic class	Order	GenBank accession number
P1	*Fusarium lateritium*	*Fusarium lateritium* (MF380827.1)	96%	*Sordariomycetes*	*Hypocreales*	MZ711187
P2	*Fusarium lateritium*	*Fusarium lateritium* (KC787693.1)	95%	*Sordariomycetes*	*Hypocreales*	OK605019
P3	*Pestalotiopsis pini*	*Pestalotiopsis pini* (MT374681.1)	94%	*Sordariomycetes*	*Xylariales*	OK303421
P4	*Xylaria grammica*	*Xylaria grammica* (JQ673413.1)	97%	*Sordariomycetes*	*Xylariales*	OK303435
P5	*Pestalotiopsis microspora*	*Pestalotiopsis microspora* (MW081271.1)	97%	*Sordariomycetes*	*Xylariales*	OK303422
P6	*Pestalotiopsis microspora*	*Pestalotiopsis microspora* (MW081271.1)	95%	*Sordariomycetes*	*Xylariales*	OK303423
P7	*Fusarium pseudocircinatum*	*Fusarium pseudocircinatum* (KX385054.1)	96%	*Sordariomycetes*	*Hypocreales*	MZ711228
P8	*Trichoderma atroviride*	*Trichoderma atroviride* (MW532980.1)	*97%*	*Sordariomycetes*	*Hypocreales*	OK303424
P9	*Pestalotiopsis pini*	*Pestalotiopsis pini* (MT374681.1)	96%	*Sordariomycetes*	*Xylariales*	OK303436
P10	*Pestalotiopsis pini*	*Pestalotiopsis pini* (MT374689.1)	95%	*Sordariomycetes*	*Xylariales*	OK303425
P11	*Nigrospora oryzae*	*Nigrospora oryzae* (HQ262527.1)	97%	*Sordariomycetes*	*Trichosphaeriales*	OK303426
P12	*Nigrospora spp*	*Nigrospora spp* (MG711604.1)	85%	*Sordariomycetes*	*Trichosphaeriales*	-
P13	*Pestalotiopsis pini*	*Pestalotiopsis olivacea* (MT374682.1)	*96%*	*Sordariomycetes*	*Xylariales*	OK303427
P14	*Nigrospora oryzae*	*Nigrospora oryzae* (KF998977.1)	*96%*	*Sordariomycetes*	*Trichosphaeriales*	OK303428

Table 1. Identified species of endophytic fungi and accession numbers assigned by GenBank.

Forestal, Áreas Protegidas y Vida Silvestre de Honduras (ICF).

Conflicts of Interest

The authors declare no conflict of interest.

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