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Morphological and Molecular Identification of Mycobiota Associated with *Juniperus monosperma* (Engelm.) Sarg. Blight in Mexico

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Authors' contributions

This work was carried out in collaboration among all authors. Author MEGC designed the study. Author ASA wrote the protocol. Author ECDA performed the analysis of results. Authors SFVM and JLAV managed the analyses of the study as the literature searches. All authors approved the final manuscript.

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ABSTRACT

The aim of the investigation was to identify the agents associated with blight in *Juniperus monosperma* at Lirios region, Arteaga, Coahuila. Botanical material was collected at Lirios, Arteaga, and taken to the laboratory. Pathogens were isolated in ADP culture medium and identifying by morphological criteria and molecular using primers ITS1 and ITS4. DNA extraction by the Dellaporta method, the visualization of obtained DNA was performed by electrophoresis on a 2% (p/v) agarose gel. DNA quantification was performed on a NanoDrop 1000 spectrophotometer, and DNA amplification was carried out in the Veriti thermocycler. Obtained sequences were aligned and compared with those available in the GenBank database of the National Center for Biotechnology Information (NCBI), using the BLAST algorithm (Basic Local Aligment Search Tool) to find conserved sequences. Pathogens were inoculated in stems of *J. monosperma*. The agents associated with blight were *Alternaria* sp., *Aspergillus* sp. and *Rosellinia* sp. and the sequence obtained compared with BLAST coincided only with *Alternaria* sp. and *Aspergillus* sp. with the

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access codes KP027305.1 and MG551283.1, respectively. According to data taken in field, *Alternaria* sp. behaved as the highest prevalence species and severity associated with blight in *J. monosperma*, and *Rosellinia* sp. in laboratory conditions.

Keywords: Incidence; inoculation; pathogen; severity.

1. INTRODUCTION

Juniperus is the second most diverse genus of conifers in the world, with approximately 100 species, with 67 species of this genus and 28 different varieties distributed worldwide [1]. *J. monosperma* (Engelm.) Sarg. is considered of low value for short-term rehabilitation programs, but of great value in program of long term rehabilitation and can be affected by biotic and abiotic factors, and can be affected by biotic and abiotic factors. Among the biotic factors, different wood-degrading fungi of living trees are considered one of the main pathogens of tree species [2]. Endophytic fungi comprise a large, but little explored portion of fungal diversity [3], [4-6]. Communities of wood-degrading organisms varies depending on geographic location. As a result of their activity, factors such as pH, organic matter, soil microflora and microfauna can be altered [7], for instance, climate change, has been suggested to increase forest damage in northern latitudes [8]. Twig and branch dieback is a common sight in many juniper plantings (Juniper blight) in Kentucky, caused by *Phomopsis juniperovora* Hahn and Kabatina juniper (Schneider y von Arx) Morelet, attacking several species of *Juniperus*, including red cedar, common juniper and creeping juniper [9-12]. The genus *Sphaeropsis* spp. cause regressive death and cancer in trees' branches and roots (mainly in *Pinus* spp.) [13, 14]. *Diplodia sapinea* (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko, was reported for the first time in 2013 in Sweden [15] causing shoot dieback, canker, blue stain, and root disease on pines [16, 17]. Due to the above mentioned, the aim of the investigation was to identify the agents associated with blight in *J. monosperma* at Lirios region, Arteaga, Coahuila.

2. MATERIALS AND METHODS

2.1 Sampling

The samplings was performed in 2018-2019 at Sierra de Arteaga, Experimental Agricultural Crop, Los Lirios, Arteaga, Coahuila, Mexico, latitude 25°23"30°N, longitude 100°37"W (Fig. 1).

Fig. 1. Arteaga, Coahuila, Mexico

2.2 Tree Selection for Sample Collection

Two randomized samplings were conducted. The studied area was divided in quadrants: North, (N), South (S), East (E) and West (W). Ten trees were selected and evaluated considering the presence and severity of blight (%, proportion). In addition, height and orientation of each tree was measured. Bark, branches and other tree samples were collected (Fig. 2) and placed in sealed bags to be taken to the Phytopathology laboratory of the Universidad Autonoma Agraria Antonio Narro.

2.3 Isolation, Purification and Identification of Fungi

Diseased tissue samples were cut in 0.5 cm pieces, then disinfected with 3% sodium hypochlorite solution for 2 min and washed with sterile distilled water for 2 min. Severed pieces were placed in Petri plates with Agar Potato Dextrose (APD) culture medium and kept at 27°C for 192 h. Developed fungal strains were isolated from the previous stage by successive reseeding in ADP culture medium and kept at 20°C for 120 h. Morphological identification of pathogens was performed on microscope inspection considering morphological keys features [18]. Molecular analyses were performed at the Instituto Potosino de Investigacion Cientifica y Tecnologica, analyzing the ITS1 and ITS4 sequences using the pair of primers of the ITS regions to identifiy the isolates. DNA extraction was performed by the Dellaporta method [19], visualization of obtained DNA was performed by electrophoresis on a 2% (p/v) agarose gel. DNA quantification was performed in a NanoDrop 1000 spectrophotometer, and DNA amplification was carried out in the Veriti thermocycler. Samples were sequenced with labeled dideoxynucleotides method in the 3130 Genetic Analyzer sequencer. Obtained sequences were aligned and compared with those available in the GenBank database of the National Center for Biotechnology Information (NCBI), using the BLAST algorithm (Basic Local Aligment Search Tool) to find conserved sequences.

2.4 Strain Increase

An explant 0.5 cm of the mycelium of each fungi (*Alternaria* sp., *Rosellinia* sp. and *Aspergillus* sp.) was taken and placed in 15 Petri plates with ADP culture medium kept at 25°C for 192 h.

2.5 Fungal Inoculation in *J. monosperma* Stems

Spore suspensions of each fungal isolates (*Alternaria* sp., *Rosellinia* sp. and *Aspergillus* sp.) were prepared. Firstly, mycelial growth of each strain was scraped and mixed in 50 mL spray bottles of sterile distilled water, adjusting to 10^8 spores mL^{-1}, (McFarland standards). Finally, stems of *J. monosperma* were atomized in plastic trays with each solution with a negative control (only with sterile distilled water), kept to 25°C for 240 h.

Fig. 2. Tree and seed of *J. monosperma* at Sierra de Arteaga, Experimental Agricultural Crop, Los Lirios, Arteaga, Coahuila, Mexico

3. RESULTS AND DISCUSSION

Pathogens identified were *Alternaria* sp., *Aspergillus* sp. and *Rosellinia* sp. (Fig. 3) and sequences obtained compared with BLAST coincided with *Alternaria* sp. and *Aspergillus* sp., with accession codes KP027305.1 and MG551283.1, respectively (Table 1). Analyses performed on genomic DNA from *Alternaria alternata* isolates obtained from various host plants showed a high level of genetic variation conducive to the colonization of different plant species [20].

Table 2 shows incidence and severity of pathogen infection associated with blight in *J. monosperma* in field conditions. The incidence was 100% and severity was 0 to 100%, the fungi with the highest severity was *Alternaria* sp. with 100% damage and the pathogens with less damage in the field were *Rosellinia* sp. and *Aspergillus* sp. with damage of 10 and 20%, respectively. The fungi of the genus *Alternaria*, due to their prevalence and genetic variation, constitute a great threat in the cultivation of plants [21]

Inoculation of three tested pathogens generated wilt and death observed in inoculated tissue, the etiological pathogen that caused the most damage was *Rosellinia* sp., and those that presented less damage were *Aspergillus* sp. and *Alternaria* sp. Stem inoculation of *J. monosperma* in all treatments showed tissue damage (yellowing and tissue death), due to the tissue being ideal host for used the strains (Fig. 4). *Alternaria* sp. causes diseases in various crop plants being present on all continents [22, 23] and ornamental plants, trees and fruit shrubs [24-27]. Factors that increase the development of the pathogen are: presence of old trees with their roots in process of decomposition, frequency of rains, observing an almost insignificant incidence where the frequency of rains is low [28]. and Arteaga, Coahuila with only 300 - 700 mm of precipitation [29]. In plantations of cocoa *Theobroma cacao* L, the occurrence of *Rosellinia pepo* Patouillard and *Rosellinia bunodes* (Berkeley & Broome) Saccardo was observed in plants with eight months of age, where the removal of organic material and cultural remains was not carried out during the eradication of dead plants [30]. *Aspergillus* spp. are among the most common molds isolated from soils and litters; they occur in desert, forest, wetland, and cultivated soils around the world [31].

Table 1. Pathogens identified in tree of *J. monosperma*

Pathogen	Access Key	Top Score	% of identity
Alternaria sp.	KP027305.1	2664	94.06
Aspergillus sp.	MG551283.1	1250	92.67

Table 2. Data of incidence, severity of blight and pathogens presents in tree *J. monosperma*

Incidence (%)				Severity (%)				*Pathogen*
NA	**O**	**A**	**I %**	**N**	**S**	**E**	**O**	
1	SE	1.8	5	10	0	25	100	*Aspergillus* sp. *Alternaria* sp.
2	SE	2.1	1	15	20	10	20	*Alternaria* sp.
3	SE	1.4	35	100	10	10	5	*Alternaria* sp. *Aspergillus* sp.
4	SE	1.8	20	0	1	5	40	*Rosellinia* sp. *Aspergillus* sp.
5	SE	2	15	5	10	20	50	*Alternaria* sp.
6	N	5	30	30	5	1	1	*Alternaria* sp.
7	N	2	5	5	20	5	10	*Alternaria* sp.
8	N	6	10	10	15	5	20	*Alternaria* sp.
9	N	5	10	5	50	5	5	*Alternaria* sp.
10	O	8	15	0	10	100	5	*Alternaria* sp.

N A= Tree number, O= Orientation, A= Height, I= Incidence, N= North, S= South, E= East, O = West, SE= Southwest.

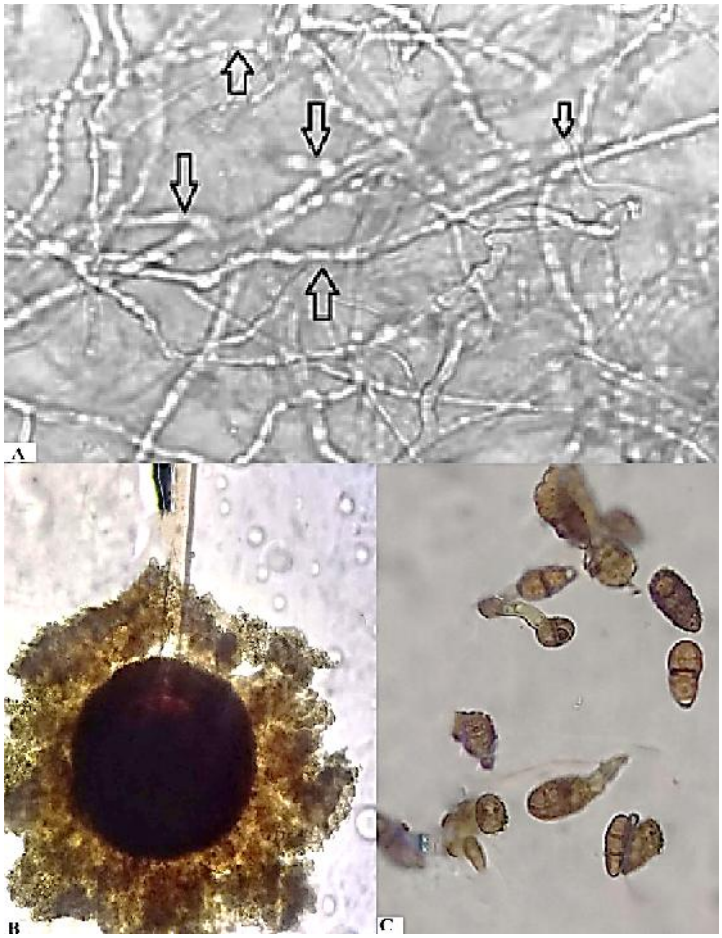

Fig. 3. Pathogens of *J. monosperma*. A=Morphological characteristics of *Rosellinia* sp. Typical pear-shaped swelling in the septa union of mycelia, B=*Aspergillus* sp., C=*Alternaria* sp.

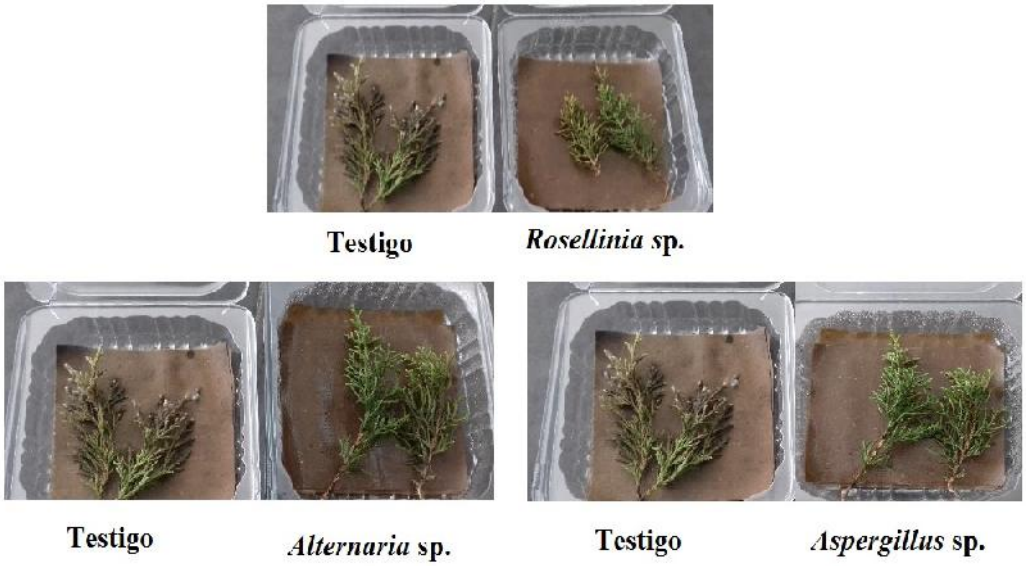

Fig. 4. Inoculation of pathogens in stems of *J. monosperma*

4. CONCLUSIONS

A total of three fungal pathogens: *Aspergillus* sp., *Alternaria* sp. and *Rosellinia* sp. were identified associated with blight of *J. monosperma* at Sierra de Arteaga Experimental Agricultural in Los Lirios area in Arteaga, Coahuila, Mexico.

According to data taken in field, *Alternaria* sp. behaved as the highest prevalence species and severity associated with blight in *J. monosperma*, whereas under laboratory conditions it was *Rosellinia* sp.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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