Revista Mexicana de Ciencias Forestales Vol. 12 (66)
Julio – Agosto (2021)

DOI: https://doi.org/10.29298/rmcf.v12i66.806

Article

Schizophyllum commune Fr. asociado a *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. en México

Schizophyllum commune Fr. associated to *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. in Mexico

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Resumen

Hevea brasiliensis es la especie de mayor importancia económica del género *Hevea*, de la cual se obtiene 99 % de la producción mundial de hule natural. En México, durante 2018 se registró una producción total de 75 922 toneladas que se obtuvieron de 28 172 ha plantadas en cinco estados: Chiapas, Oaxaca, Tabasco, Veracruz y Puebla. De ellos, Veracruz ocupa el primer lugar, con 66 % de la producción. Las enfermedades en el árbol del hule afectan la óptima producción de látex en todo el mundo. En 2018, se detectó la incidencia en vivero de una pudrición en tocones de hule injertados con el clon IAN-873 en el municipio Martínez de la Torre; por lo anterior, el objetivo de esta investigación fue identificar el agente asociado a la pudrición de tocones en dicho lugar. Se cultivaron muestras de tejido leñoso con pudrición y micelio en medio Extracto de Malta Agar (MEA) y se incubaron a 28 ± 1 °C por 72 h; se desarrollaron colonias algodonosas, con micelio color blanco-crema. Con base en la morfología del cultivo en el medio MEA y el análisis filogenético cono la secuencia de la región ITS, obtenida mediante la amplificación con los *primers* ITS5/ITS4 del aislamiento, se identificó a *Schizophyllum commune* como el hongo asociado a la pudrición de los tocones de *H. brasiliensis,* lo que corresponde al primer registro para México.

Palabras clave: Filogenia, hule, identificación, tocones, pudrición, *Schizophyllum commune* Fr.

Abstract

Hevea brasiliensis is the most economically important species of the *Hevea* genus, from which 99 % of the world's natural rubber production is obtained. During 2018, total production was 75 922 tons obtained from 28 172 ha planted in five states of Mexico: *Chiapas, Oaxaca, Tabasco, Veracruz* and *Puebla. Veracruz* is in first place with 66 % production. The diseases in the rubber tree affect the optimal production of latex worldwide. In 2018, the incidence in nursery of a rot in rubber stumps grafted with the clone IAN-873 in *Martínez de la Torre* municipality was detected; therefore, the objective of this investigation was to identify the agent associated to stump rot in this place. Samples of woody tissue with rot and mycelium were cultured in Malta Agar medium (MA) and incubated at 28 ± 1 °C for 72 h from which colonies with white-cream, cottony mycelium developed. Based on the culture morphology in the MA medium and the phylogenetic analysis by amplification of the ITS region with the ITS5 and ITS4 universal primers from the isolation, *Schizophyllum commune* was identified as the fungus associated to *H. brasiliensis* stump rot, thus becoming the first report in Mexico.

Key words: Phylogeny, rubber, identification, stumps, rot, *Schizophyllum commune* Fr.

Fecha de recepción/Reception date: 20 de junio de 2020
Fecha de aceptación/Acceptance date: 16 de diciembre de 2020

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Introduction

The *Hevea* genus belongs to the Euphorbiaceae family and includes the species *H. brasiliensis* (Willd. Ex A. Juss.) Müll. Arg., *H. spruceana* (Benth.) Müll. Arg., *H. benthamiana* Müll. Arg., *H. guianensis* Aubl., *H. pauciflora* (Spruce ex Benth.) Müll. Arg. and *H. rigidifolia* (Spruce ex Benth.) Müll. Arg. Among these, the most important is *H. brasiliensis* from which 99 % of the world's natural rubber production is obtained (Picón *et al.,* 1997).

In Mexico, rubber cultivation and latex production is an important economic activity; During 2018, a total production of 75 922 tons of latex was recorded in the states of *Veracruz, Chiapas, Oaxaca, Tabasco* and *Puebla.* Among these*, Veracruz* is the main producer with 48.7 % of the planted area and 50 229 tons concentrated in the areas of *Uxpanapa, Las Choapas, Hidalgotitlán* and *Tezonapa*, which as a whole, represents 66 % of the total national production (SIAP, 2018).

Diseases in the rubber tree are an important limitation that annually cause considerable losses in terms of latex production (Jaimes and Rojas, 2011). Most of these diseases are of fungal etiology that can affect different phenological stages and types of tree tissue. Worldwide, the main diseases in *H. brasiliensis* include "South American Leaf Disease" (SALB) caused by *Microcyclus ulei* (Henn.) Arx; anthracnose by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Castro, 2011); and downward death or stem rot by *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Izquierdo, 2008; Grupo Técnico Procaucho, 2012), among others.

Rot diseases in *H. barasiliensis* that have been identified include mold rot by *Ceratocystis fimbriata* Ellis & Halst.; in roots the white rot (Rigidoporus sp.), brown rot (*Fomes noxius* Corner), black rot (*Xylaria thwaitesii* Berk. & Cooke) and red rot (*Ganoderma pseudoferreum* (Wakef.) Overeem & B. A, Steinm.) (Rodríguez, 1993; Izquierdo, 2008).

In Mexico, there is little research that addresses the study of rubber diseases in the main producing areas. During 2018, in a nursery in *Martínez de la Torre* municipality, Ver., an incidence between 10 and 15 % of a rotting disease was detected,

presumably of fungal etiology, with a proliferation of grayish-white mycelium in the woody tissue affecting stumps grafted with the rubber clone IAN-873. Therefore, the objective of this study was to identify morphologically and genetically the fungus associated with the rot of rubber stumps in the place.

Materials and Methods

Mycelium isolation

Vegetable tissue samples were collected from rubber tree stumps grafted with the IAN-873 clone with rot symptoms in a nursery in *Martínez de la Torre* (20°05'57.88" N, 97°04'52.3" W), *Veracruz,* Mexico . The samples were placed on brown paper and inside sealed plastic bags for analysis in the Phytobacteriology Laboratory of the *Colegio de Postgraduados, Montecillo* Campus.

The mycelium isolation was carried out from fungal structures associated with the tissue with the symptoms of rot disease. From each sample, pieces of the sporome aroind 0.5 cm^2 were cut and disinfested with sodium hypochlorite (NaOH) at 1.5 % for 1 min, followed by three washes with sterile distilled water. The disinfected pieces were seeded in Malt Agar Extract (MEA) culture medium and incubated at 28 ± 1 °C for 72 h. Mycelium was obtained in axenic culture and preserved in slants in MEA culture medium and sterile mineral oil for subsequent studies.

Morphological description

Partially developed basidiomas were collected from stumps of rubber trees with rot symptoms for microscopic measurement of fungal structures, as well as those grown in the MEA medium; the identification was based on the taxonomic keys elaborated by Olivo and Herrera (1994) for *Schizophyllum* species.

Genetic characterization

For the genetic characterization, a colony of mycelium from an axenic culture of eight days of growth in MEA medium of the fungus isolated and identified in this study with the code MZVMT_01 was used. DNA extraction was carried out by the modified AP method (Sambrook and Russell, 2001). The ITS region was amplified with the universal primers *ITS5* (5'-GGAAGTAAAAGTCGTAACAAGG-3 ') and *ITS4* (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990), which are located in the conserved regions of the *18S* genes and 28S, respectively. The PCR reactions were carried out in a volume of 25 µL, with 2 µL of DNA at 50 ng μL^{-1} and 1 µL of each of the primers at a concentration of 10 µM; PCR buffer 0.5x final concentration; $MgCl_2$ at 1.25 mm; dNTP's at 0.2 mm and 0.1 U μL^{-1} of Taq DNA Polymerase (Promega). The amplification was carried out in a T100 thermal cycler (Biorad) under the following conditions: initial denaturation temperature at 94 °C for 3 minutes, 35 cycles of 94 °C for 30 seconds, alignment of 58 °C for 30 seconds and an extension of 72 °C for 1 min; final extension at 72 ºC for 7 minutes and conservation at 4 °C. The amplified fragments were visualized by 1 % agarose gel electrophoresis, with 0.4 µL of ethidium bromide for 50 minutes at 90 V. Gels were visualized in an Infinitity-ST5 Vilber Lourmat photodocumenter.

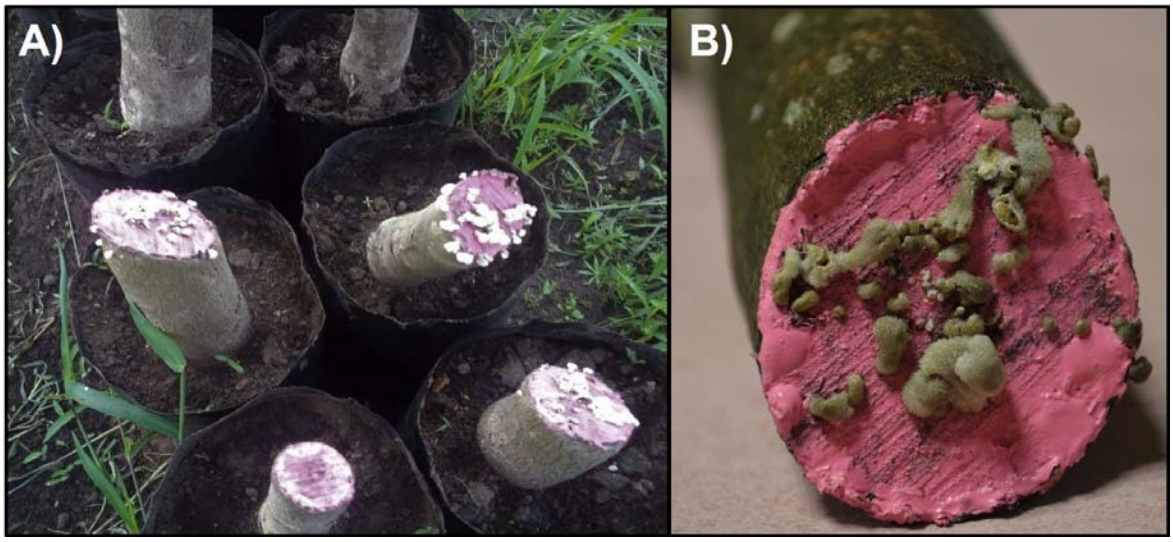
The PCR product was purified with the WIZARD® SV Gel kit and the PCR Clean-Up System, according to the manufacturer's specifications (Promega Corporation, 1999). The purified PCR products were sequenced at Macrogen Korea in Seoul, Republic of Korea.

The DNA sequences were edited and assembled to create the consensus sequence of the amplified regions with the BioEdit Sequence Alignment Editor version 7.0.5.3 program. The assemblies were used to perform a similarity search using BLAST (Basic Local Alignment Search Tool) in the nucleotide database of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/Blast). For the phylogenetic analyzes, the consensus sequences were aligned with the clustal method with the CLUSTAL OMEGA 1.2.2 program (Sievers and Higgins, 2014) and the search for the best nucleotide substitution model for each of the species was carried out with the program ModelTest-NG (Darriba *et al*., 2019). Phylogenetic reconstruction was performed with Bayesian inference using Markov Chains Monte Carlo

(MCMC), implemented in the BEAST v1.10.4 program (Suchard *et al*., 2018) with different generations until the chains stabilized. For the best tree annotation, 25 % of the trees produced were discarded and the subsequnt probability was determined with the remaining trees.

Results and Discussion

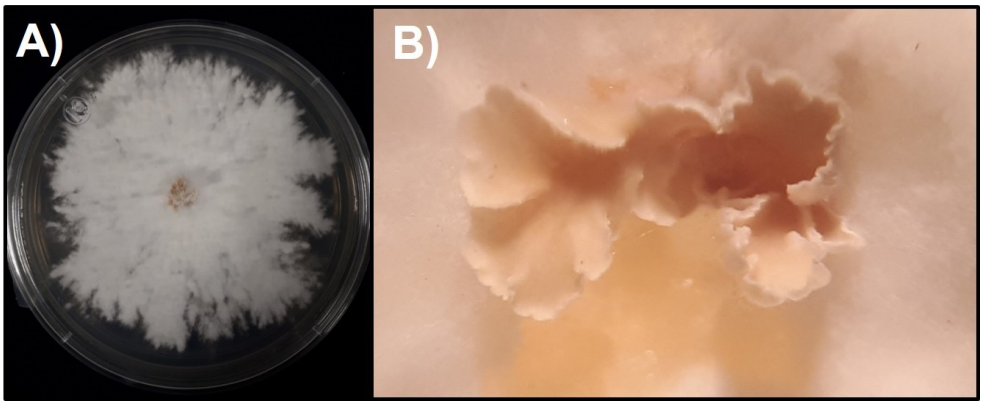
Of the rot symptoms in stumps of *H. brasiliensis* from the *Martínez de la Torre* nursery, small partially developed basidiomas were observed, which did not exceed one centimeter in their widest area, fan-shaped, of a grayish-white color (Figure 1).



A) Diseased stumps; B) Fruiting bodies in the tissue.

Figure 1. Rot symptoms and fungal structures in stumps of the rubber tree in *Martínez de la Torre*.

From the basidiome fragments that were inoculated in the MEA medium, pure strains of the fungus were isolated that formed creamy-white, cottony colonies and sporomas 10 days after sowing (Figure 2).



A) White-creamy, cottony colony in MEA culture medium; B) Formation of fruiting bodies 10 days after sowing.

Figure 2. Morphological characteristics of the fungal isolation of *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. stumps.

Morphological description

The sporomas observed in the stumps of *H. brasiliensis* with rot were grouped, small in size, between 0.5-2 cm wide, without stipe with an irregular shell shape, with white to grayish mycelium. The sporoma was gymnocarpal with the sporiferous part limited only to the lower part of the lid and the hymenophore limited to the lower part. The culture of the sporomas in the MEA medium developed colonies of white to cream color, cottony, with branched and irregular growth, of smooth texture and rough surface. Microscopic analysis of the sporomes identified the fungus associated with stump rot of *H. brasiliensis* as *Schizophyllum commune* Fries (1815). From the last taxonomic scrutiny, it is classified (Kirk, 2020) as:

Kingdom: Fungi

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

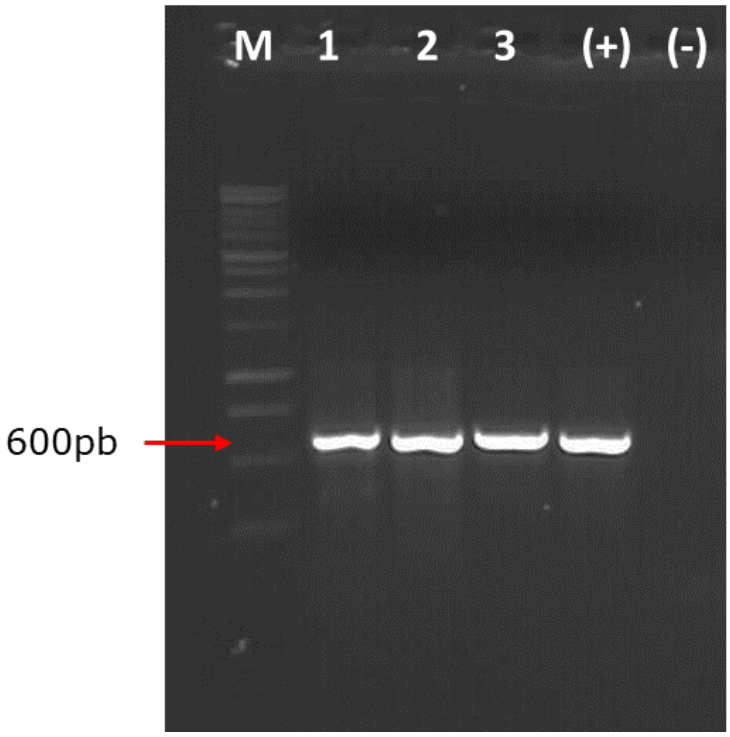
Family: Schizophyllaceae

Genus: *Schizophyllum*

Species: *S. commune*

Genetic identification

Genetic analysis confirmed the morphological identification of the fungus associated with *H. brasiliensis* stump rot. The PCR products with the ITS4 and ITS5 rDNA primers amplified an approximate 600 bp fragment of the MZVMT_01 strain morphologically identified as *S. commune* (Figure 3). Buzina *et al.* (2001) obtained amplifications with these same primers in the 660 bp range for *S. commune* isolates (Figure 3).



M = 1kb molecular marker, lane 1 to 3 samples of *Schizophyllum commune*; (+) = Positive control; (-) = Negative control (Nuclease-free water).

Figure 3. Amplification of the PCR product of *Schizophyllum commune* Fr. associated with stump rot of *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.

The search for similarity analysis by BLAST identified *Schizophyllum commune* with a maximum identity of 99.47 % with sequences deposited in the NCBI (accession number MN781967 and EU853847).

The phylogenetic tree of *Schizophyllum commune* was generated from 2 000 000 generations. Phylogenetic reconstruction showed that the MZVMT_01 isolate clustered in the same clade with the *S. commune* sequence MN781967 (Figure 4), which corresponds to a strain in a study of wood decay fungi in Northwest Arkansas forests (Alshammari and Stephenson, 2018).

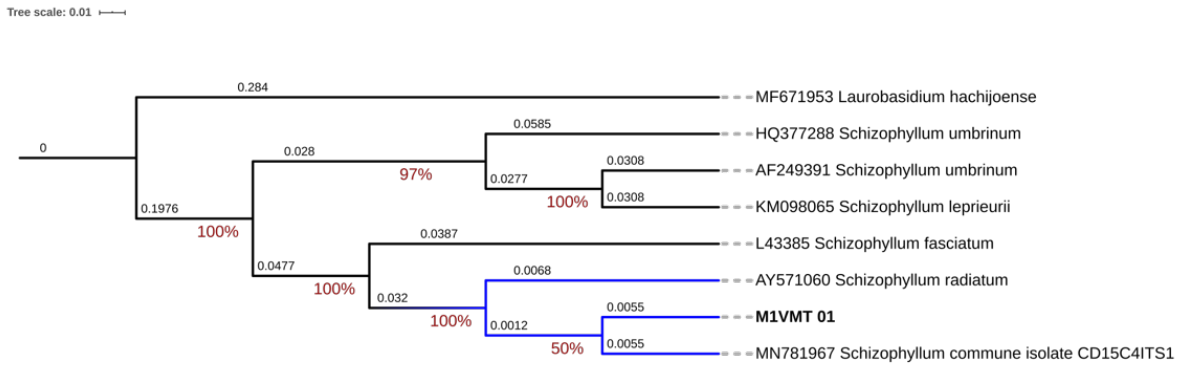


Figure 4. Phylogenetic consensus tree based on Bayesian inference illustrating the relationship of *Schizophyllum commune* Fr. associated with *Hevea brasiliensis* (Willd. Ex A. Juss.) Müll. Arg. stump rot in Mexico.

S. commune is a cosmopolitan fungus with wide distribution on all continents and is associated with the colonization of woody tissue and wood rot. It was previously considered a weak pathogen and was considered more of a saprophytic microorganism related to the decomposition of branches, dead wood and stored wood. However, it is now recognized as an important emerging pathogen in plants (Schmidt and Liese, 1980; Ohm *et al.,* 2010; Takemoto *et al.,* 2010) and humans (Chowdhary *et al.,* 2013; Saha *et al.,* 2013; Michel *et al.,* 2015).

S. commune is included in the group of pathogens that cause the "white rot" disease, among which Schwarze *et al.* (2000) have stated that it harbors the greatest variety of degradation mechanisms; it is considered an omnivorous pathogen that invades living tissue and is highly aggressive among basidiomycetes (Takemoto *et al.,* 2010).

The host range includes at least 150 genera of timber, coniferous, fruit, and ornamental trees; in Asia more than 260 species in 150 plant genera are identified as natural hosts and only in Japan 32 species in 22 woody plant genera (De Jong *et al*., 2006 cited by Ohm *et al.,* 2010; Takemoto *et al.,* 2010). Interaction with other pathogens has been demonstrated in young apple trees (*Malus* sp.) and propagation material that causes white root rot (Havenga *et al.,* 2019).

Genome sequencing of *S. commune* revealed unique characteristics and increased numbers of genes among other basidiomycetes that code for the production of enzymes that degrade pectin, cellulose, hemicellulose and a unique mechanism to degrade lignin (Ohm *et al.,* 2010). Other studies determined the high production of xylanase, laccase, cellulase, esterases and peroxidase (Schmidt and Liese *et al.,* 1980; Špániková *et al.,* 2007; Hirai *et al.,* 2008). Likewise, among 75 basidiomycetes analyzed, *S. commune* showed greater pectinolytic activity due to the production of polygalacturonases (Xavier *et al.,* 2004). Therefore, it is postulated that *S. commune* is capable of degrading practically all the components of the cell wall in woody tissue cells (Ohm *et al.,* 2010).

Studies on the colonization of S. commune showed that it invades the woody tissue through the lumen vessels, tracheids, woody vessels, fibers and xylem where cellulose, hemicellulose or pectin are used as carbon sources for its development and subsequent use of lignin and polysaccharides contained in woody tissue (Ohm *et al.,* 2010; Padhiar and Albert, 2011; 2012).

The worldwide distribution of *S. commune* indicates that it is one of the fungi that adapts to a wide variety of conditions, since it inhabits both temperate and tropical climates. It is stated that the degree of severity of white rot depends on the host species, environmental conditions and growth rate of the fungus; temperature, high relative humidity, global warming and pH have been associated among the most important environmental conditions that influence the aggressiveness of this pathogen (Schmidt and Leise, 1980; Takemoto *et al.,* 2010). This adaptation to diverse climate and host conditions suggests that genetic variability may exist; this was demonstrated among populations of *S. commune* that formed different lineages

with a close relationship with the geographic origin in North America and Europe; and a more recently expanding lineage in the Caribbean (James *et al.,* 1999; 2001). The above could suggest that it is also possible that there are differences in virulence and aggressiveness between populations as a phytopathogen; however, there are still no studies documenting these differences as a plant pathogen.

S. commune was recorded in Mexico, in the humid tropics with annual rainfall levels exceeding 2,500 mm (Carreño-Ruíz *et al*., 2019). In the state of *Tabasco*, *Ficus benjamina* L., which also produces latex, has been recognized in fallen wood in *Centro, Huimanguillo, Tacotalpa, Teapa, Tenosique* and *Macuspana* municipalities (Olivo and Herrera, 1994; Carreño-Ruíz *et al.,* 2019). These climatic conditions related to the presence of *S. commune* exist in Mexico in several rubber-producing localities in the states of *Chiapas, Oaxaca, Tabasco, Veracruz* and *Puebla*, with hot humid climates with abundant rains in summer or all year round and ranges of annual precipitation between 1 900 and 4 500 mm per year (Inegi, 2009).

The dissemination of *S. commune* occurs mainly due to the abundant dispersal of basidiospores by air, which colonize the woody tissue, mainly in young trees; injuries to tissue due to cold, frostbite and sunburn are the main routes of entry and infection (Takemoto *et al.,* 2010). Likewise, pruning, improper pruning, poor nursery management and fertilization practices promote a high incidence of *S. commune* (Snieðkienë and Juronis, 2001); the wide dispersal ability and prevalence of *S. commune* up to four years have been documented by Badalyan *et al.* (2002).

In *Hevea brasiliensis*, the basidiomycete *Rigidoporus microporus* (Sw.) Overeem (1924) has been identified as a causal agent of "white rot" in roots (Oghenekaro, 2016), but not *S. commune*. In the interaction of *Hevea brasiliensis* with *S. commune*, the little information available is limited to the study of the association of this basidiomycete as part of the saprophytic community in the decomposition of fallen branches and stored wood (Hong, 1982; Seephueak *et al.,* 2011a; 2011b), but not as a parasite and pathogen in plants. The high genetic uniformity in the rubber crop, the nursery management and the climatic conditions could be important factors in the establishment, dispersal and aggressiveness of *S. commune* in *Hevea brasiliensis* in Mexico.

Conclusions

Schizophyllum commune was identified in the rot symptoms in the woody tissue that was affecting stumps grafted with the IAN-873 rubber clone in *Martínez de la Torre, Veracruz*. The climatic conditions and annual precipitation in the main rubber-producing states in Mexico are located in the environmental range for the optimal development of *Schizophyllum commune*, so future studies deserve to deepen on the distribution, virulence and severity of this fungus in the different clones used in other rubber producing states, as well as the establishment of efficient control strategies.

This is the first report *of Schizophyllum commune* associated with stumps of *Hevea brasiliensis* with rot symptoms in Mexico; no international investigations were found that refer to the pathogenicity of *Schizophyllum commune* in *Hevea brasiliensis*.

Conflict of interests

The authors declare n conflict of interests.

Contribution by author

Iris Marley Pérez Gálvez: laboratory analysis and writing of the manuscript; Victoria Ayala Escobar: morphological identification; Elías Ortiz Cervantes: field trips and sampling; Adriana Rosalía Gijón Hernández: support in molecular analysis; Sergio Aranda Ocampo: research follow-up, review and correction of the manuscript.

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