CENTRO DE CIENCIAS BÁSICAS

DEPARTAMENTO DE MICROBIOLOGÍA

TESIS

Epidemiologia y Confirmación de la Presencia de *Taphrina Caerulescens* Infectando *Quercus Spp*. en La Sierra Fría de Aguascalientes, México.

PRESENTA:

M. en C. Gregg Evans

PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS BIOLÓGICAS

AREA: BIOTECNOLOGÍA

TUTORES

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Aguascalientes, Ags., 11 diciembre 2018

Autorizaciones

UNIVERSIDAD AUTONOMA
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GREGG EVANS
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Estimado alumno:

Por medio de este conducto me permito comunicar a Usted que habiendo recibido los votos aprobatorios de los revisores de su trabajo de tesis y/o caso práctico titulado: **"EPIDEMIOLOGIA Y CONFIRMACION DE LA PRESENCIA DE *TAPHRINA CAERULESCENS* INFECTADO *QUERCUS* SPP. EN LA SIERRA FRIA DE AGUASCALIENTES, MEXICO"**, hago de su conocimiento que puede imprimir dicho documento y continuar con los trámites para la presentación de su examen de grado.

Sin otro particular me permito saludarle muy afectuosamente.

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Por este conducto informo a usted que la contribución con clave: 18-115, intitulada: **ISOLATION AND IDENTIFICATION OF *Taphrina caerulescens* IN *Quercus eduardii* IN AGUASCALIENTES, MEXICO,** cuyos autores son: Gregg **Evans**, Onesimo **Moreno Rico**, José De Jesús **Luna Ruíz**, Joaquín **Sosa Ramírez**, Celeste Elizabeth **Moreno-Manzano**, se encuentra aceptada para publicación.

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Acrónimos

µL: micrólitro

µm: micrómetro

18S: Small subunit Ribosomal RNA (Subunidad pequeña ARN ribosomal)

28S: Large subunit Ribosomal RNA (Subunidad grande ARN ribosomal)

ADN (DNA): Ácido desoxirribonucleico

Ags: Aguascalientes

BLASTN: Basic Local Alignment Search Toot Nucleotide

C: Centígrado

cm: Centímetro

Conabio: Comisión Nacional para el Conocimiento y Uso de la Biodiversidad

CTAB: Cetrimonium bromide (Cetrimonium bromide)

d: día

EI: epidermis inferior

ELMA: Extracto de levadura y malta Agar

EMA: Extracto de malta agar

ES: Epidermis superior

et al.: et alii (y otros)

ET: Estomas

Fig.: Figura

gr: Gramos

Hr: Hora(s)

HR: Humedad Relativa

Inifap: Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias

IDT: Integrated DNA Technologies®

Km: Kilometro

L: Litro (s)

MEB: Microscopio electrónico de barrido

MI: Micelio

min: Minuto

mL: Mililitro

mm: Milímetro

Mx: México

NCBI: National Center for Biotechnology Information

pb: Pares de bases

PCR: Polymerase Chain Reaction (Reacción en cadena de la polimerasa)

PDA: Potato dextrose agar (Agar dextrosa de patata)

PE: Parénquima en empalizada

PJ: Parénquima esponjoso

PVP: Polivinilpirrolidona (Polyvinylpyrrolidone)

spp.: especies

TC: Tricoma

RESUMEN EN ESPAÑOL

Los bosques de encino son recursos forestales importantes para el hombre debido a los beneficios ecológicos que le brinda. En los bosques templados de la Sierra Fría, Aguascalientes, México, se observaron síntomas de la enfermedad causada por *Taphrina caerulescens* en las hojas de *Quercus* spp. Este fue el primer reporte del patógeno en México. *T. caerulescens* afecta exclusivamente a las plantas del género *Quercus*, sin embargo, no hay mucha información disponible sobre la enfermedad, ya que se ha estudiado poco. La identificación y el aislamiento de este hongo es difícil debido a su naturaleza dimórfico y su crecimiento extremadamente lento en los medios de crecimiento artificiales. Los objetivos del estudio fueron: 1) Aislar e identificar (mediante síntomas, estudios morfométricos, bioquímicos y técnicas moleculares) al patógeno *T. caerulescens*, y 2) Describir los cambios histopatológicos que ocurren en las hojas de *Q. eduardii* infectadas con *T. caerulescens*. Para cumplir con el primer objetivo se realizaron una serie de pruebas que incluyeron: análisis de síntomas, análisis morfológicos, pruebas bioquímicas, análisis moleculares y pruebas de patogenicidad. Como resultado de estas pruebas, se confirmó que el hongo causante de la enfermedad era *T. caerulescens*. Para satisfacer el segundo objetivo, se procesaron secciones delgadas de hojas, de *Q. eduardii*, infectadas y no infectadas para su estudio con microscopía óptica y electrónica. Los resultados mostraron que, una vez infectada la hoja, el hongo causa hipertrofia, hiperplasia y destrucción de sus células huéspedes.

PALABRAS CLAVES: Histopatología, Identificación, *Quercus, Taphrina caerulescens*,

ABSTRACT

Oak forests are important forest resources due to the ecological benefits that they provide. In the temperate forests of the Sierra Fria, Aguascalientes, Mexico, disease symptoms caused by *Taphrina caerulescens* were observed affecting leaves of *Quercus spp.* This was the first report of the pathogen in Mexico. *T. caerulescens* exclusively affects plants of the *Quercus* genus, however, not much information is available on the disease as it has been poorly studied. The identification and isolation of this fungus is difficult due to its dimorphic nature and extremely slow growth habit in artificial growth medium. The objectives of the presented studies were: 1) first to isolate and identify (through symptoms, morphometric studies, biochemical and molecular analysis) the pathogen *T. caerulescens*, and 2) Describe the histopathological changes that occur in *Q. eduardii* leaves infected with *T. caerulescens.* To satisfy the first objective a series of tests were carried out these included: analysis of symptoms, morphological analyses, biochemical tests, molecular analysis and pathogenicity tests. As a result of these tests, the fungus was confirmed to be *T. caerulescens.* To satisfy the second objective thin sections of infected and non-infected leaves were processed for optic and electronic microscopy. The results showed that once infected the fungus causes hypertrophy, hyperplasia and destruction of its host cells.

Keywords: Histopathology, Identification, *Quercus, Taphrina caerulescens*

INTRODUCCION GENERAL

La Sierra Fría

La Sierra Fría se encuentra entre las coordenadas Latitud N: 21° 52' 45'' a 23° 31' 17'' y Longitud W: 102° 22' 44'' a 102° 50' 53'' y pertenece a los estados de Aguascalientes y Zacatecas. Tiene una superficie total de 1,419 km². La Sierra fría del estado de Aguascalientes es un área natural protegida que comprende una importante diversidad biológica y es de mucha importancia en razón a que el 90% de los bosques templados del estado se encuentra allí. Esta Sierra tiene un rango altitudinal de 2,200 a 3,050 metros y la precipitación anual oscila entre los 600 y los 700 mm (SEDESO, 1993; Sosa-Ramírez *et al.*, 2014).

La zona tiene una gran diversidad de especies de fauna, plantas y microorganismos. En total se tienen registradas 591 especies de plantas terrestres pertenecientes a 325 géneros y a 87 familias; 37 especies de plantas acuáticas y subacuáticas, que pertenecen a 28 géneros y a 21 familias. Además de plantas tiene 95 especies de hongos, (Sosa-Ramírez *et al*., 2014). La vegetación actual, principal de la Sierra Fría de Aguascalientes está constituida por bosques templados secos, dominados por pino, táscate y encino (García *et al*., 1995).

Importancia de los encinos

Los encinos (*Fagaceae, Quercus* spp.) son los árboles y arbustos predominantes en gran parte de las sierras templadas Latinoamericanas. Se encuentran también distribuidos en gran parte de Europa y Norteamérica. Una razón de su exitosa colonización es que es una planta altamente adaptable a diferentes tipos de ambientes. Puede crecer en casi cualquier suelo, en varios climas y en diferentes altitudes. Se regeneran mediante la clonación o las semillas de algunas especies, pueden llegar a ser árboles muy grandes, mientras que otros son arbustos. Invaden campos abandonados; crecen en tierras de pastoreo y a lo largo de las vías de ferrocarriles. Cuando se encuentran creciendo en los bosques crecen en poblaciones grandes o dispersos entre otros árboles. Algunas especies son encinos de hoja perenne con sistema

radicular profundo mientras que las especies caducifolias tienen raíces poco profundas (Rackham, 2006).

Los encinos y los ecosistemas asociados a él son el hogar de un número diverso de especies de plantas. Muchas de estas especies no se producen en áreas donde no hay asociación con las especies de encino. Estos hábitats también soportan cientos de especies de aves, anfibios y mamíferos. También, interactúan con cientos de especies de hongos e insectos. La asociación de encinos con tantas especies se debe a una compleja red de factores que contribuyen a que el encino sirva como hábitat para muchas especies de organismos. El largo ciclo de vida y las diferentes etapas de decaimiento y descomposición de los árboles de encinos son probablemente razones importantes para la alta biodiversidad. El encino es un hábitat muy estable. Un encino moribundo o en descomposición no desaparece ni experimenta cambios dramáticos durante un par de cientos de años. En muchos hábitats se ha observado que cuando los bosques de encinos desaparecen, muchas especies de insectos, hongos y líquenes también lo hacen. Un ejemplo es el pájaro *Dendrocopos medius* que desapareció, en 1982, en Suecia, aunque todavía existe en algunos sitios con viejos encinos alrededor de Europa. En el siglo XVIII fue un pájaro común en el sur de Suecia, hasta que la protección de los encinos desapareció en 1832 y los grandes bosques de los encinos empezaron a fragmentarse. Entre los insectos que se asocian con el encino, *Lucanus cervus* fue muy común en los bosques de Europa, sobre todo cuando los bosques de encinos eran muy abundantes en los pastos y prados, hasta principios del siglo XIX, pero a medida que la cantidad de encinos ha disminuido, también lo han hecho los hábitats para *L. cervus*, por lo que ya no es tan común hoy en día. Otro insecto, altamente asociado con el encino de Europa, es el *Osmoderma eremita* (Rackham, 2006).

Los encinos también son importantes para la flora silvestre. Generan mucha materia orgánica, en ocasiones son de las pocas especies que se desarrollan en sitios con suelos delgados y con poca materia orgánica. Las ramas actúan como substrato para el desarrollo de variadas epifitas, como orquídeas (Orchidaceae), bromeliáceas (Bromeliaceae), y muerdagos (Viscaceae). Además, muchas especies de insectos y otros invertebrados habitan el follaje, frutos, ramas y raíces de encinos. Entre los hongos, algunas especies dependen de los encinos, como las micorrizas, y otras llevan alguna relación simbiótica distinta con plantas de este género.

De acuerdo con De la Cerda (1999) y García *et al*. (1999), en Aguascalientes existen 17 especies de encinos. Sin embargo, en un estudio por Sosa-Ramírez *et al*., (2011), solo encontraron los siguientes 10 de las 17 especies mencionadas por De La Cerda: *Quercus*

potosina, Q. *eduardii, Q. sideroxyla, Q. rugosa, Q. resinosa, Q. chihuahuensis, Q. coccolobifolia, Q. laeta, Q. grisea* y *Q. microphylla*. Las especies de encino más comunes en toda la sierra Fría son *Quercus eduardii, Q. laeta* y *Q. potosina* (Siqueiros Delgado, 2008; Ramírez *et al.*, 2011). De las 10 especies identificadas en 2011, *Q. potosina* posee la distribución más amplia, habiéndose encontrado en 62%, Q. *sideroxyla* y *Q. eduardii* se encuentran en el segundo y tercer lugar, respectivamente (Ramírez et al., 2011). Sin embargo, en un estudio de 2016, realizado por Martínez *et. al*. (2017), se ha confirmado que en Aguascalientes se han encontrado y registrado 25 especies de *Quercus* y 21 de ellas se encuentran en la Sierra Fría de Ags.

Aprovechamiento del encino en México

En México, a pesar de las cualidades de la madera, los encinos son, en general, bastante explotado a escala local, pero muy poco a nivel industrial. Este hecho se debe principalmente a que la mayor parte de los bosques de *Quercus* de este país están formados por árboles de baja calidad y con troncos delgados. Además, los encinos son de crecimiento relativamente lento y los que alcanzan tamaños mayores tampoco se utilizan mucho, entre otras razones por la inaccesibilidad del terreno, porque no se conocen bien las características de su madera o porque se ignoran las técnicas para su debido secado (Rzedowski, 1981).

En México la madera de encino se emplea para construcciones, muebles, postes y muchos otros usos, pero más que nada como combustible, bien sea directamente, o bien transformada en carbón, cuyo uso tiene profundo arraigo y tradición en el pueblo. Grandes extensiones de encinares mexicanos se han consumido debido a la explotación desmedida para la obtención de carbón vegetal, sobre todo en el siglo pasado y aunque parece que en las últimas décadas la demanda ha disminuido debido al uso más frecuente de otros combustibles, en algunas regiones la devastación sigue en auge (Zavala, 1990). A nivel nacional, la madera de encino ocupa el segundo lugar de aprovechamiento y los usos a los que se destina principalmente son: celulosa 54%, escuadría 30%, leña 7% y carbón 6% (La Paz, 2000).

Principales fitopatógenos de *Quercus* spp. en México

México siendo uno de los centros de diversificación del genero *Quercus*, también es un centro de la mayoría de los hongos que los afecta. Dentro de los hongos más importantes a nivel nacional se puede señalar los siguientes: *Ceratocystis fagacearum* patógeno que causa marchitamiento de los encinos, el cual debilita el sistema conductor del agua. *Phytophthora cinnamomi* es otro fitopatógeno importante en la república, causa la muerte regresiva en muchas especies de encino, además, es uno de los patógenos más importante en el aguacate en Michoacán. En el 2001, este hongo produjo una extensa muerte de bosques en los estados de Colima, Jalisco y Guerrero (Cibrián-Tovar *et al.,* 2007). *Hypoxylon atropunctatum* se considera como un patógeno débil, se encuentran solamente en árboles estresados, sin embargo, Moreno *et al*. (2010), reportaron este hongo atacando arboles sanos en la Sierra Fría de Aguascalientes, en Nayarit también hay reportes de este hongo. *Ganoderma lucidum,* ha sido señalado por Romero (1999) como causante de marchitez de encinos. Vázquez *et al*. (2004), observaron que *Nectria galligena* e *Hypoxylon thouarsianum* actúan en conjunto para causar marchitez de encinos en Guanajuato. Alvarado *et al*. (2007), encontraron que *Hypoxylon atropunctatum, Pythium*, *P. cinnamomi*, *H. atropunctatum*, *Ganoderma curtisii* y *Armillaria* spp. causaron muerte de encinos en varios estados de la república mexicana.

Principales fitopatógenos de *Quercus* spp. en Aguascalientes, México

En el estado de Aguascalientes, Moreno *et al.* (2010), Moreno (2015) y Sosa Ramírez *et al.* (2011), realizaron estudios en la Sierra Fría de Aguascalientes para determinar las enfermedades en encinos e identificaron como principales causantes de cancrosis a *Phellinus robustus* (Karst.) Bourd. et Galz., *Phellinus gilvus* (Schw.) Pat. y *Phellinus everhartii* (Ell. y Gall.) A. Ames. Ellos encontraron que *Ganoderma lucidum* (W. Curt. ex Fr.) Karst se observó presente tanto en arboles jóvenes como en árboles maduros, asimismo, *Hypoxylon thouarsianum* (Lèveillè) Lloyd y *Biscogniauxia atropunctata* (Schwein), Fr.) pouzar., fueron los más frecuentemente encontrados en el estudio y fueron observados en los troncos de muchos de los árboles de *Quercus* afectando arboles vivos y muertos. Por otra parte, Luévano-Rodríguez *et al*. (2016), identificaron hongos Ophiostomatoides causando manchado de la

madera de *Q. potosina* a partir de las galerías causadas por el insecto *Megapurpuricenus magnificus*. Ellos identificaron a *Ophiostoma pluriannulatum*, a su anomorfo *Sporothrix*, a *Pesotum* y a *Ceratocystis* como los causantes del manchado de la madera.

Recientemente, Moreno *et al.* (2015), reportaron por vez primera en México, la presencia de una enfermedad foliar que causa deformaciones, a manera de ampollas, en las hojas de *Q. potosina, Q. resinosa* y *Q. eduardii*, en la sierra Fría de Ags. Ellos realizaron estudios morfométricos preliminares que aunados a los síntomas que presentaban las hojas pudieron identificaron el fitopatógeno como *Taphrina caerulescens*.

Taphrina caerulescens

Taphrina caerulescens es un hongo patógeno Ascomiceto que causa ampollas y deformaciones de la hoja de más de 50 especies de encinos, tales como: (*Q. acutissima* (Carruth), *Q. agrijolia* (Nee) *Q. alba* (L.), *Q. borealis* (Michx), *Q. cerris* (L), *Q. cinerea* (Michx), *Q. coccijera* (L), *Q. coccinea* (Muench), *Q. crispula* (Blume), *Q. dentata* (Thunb), *Q. douglasii* (Hook), *Q. ellipsoidalis* (Hill) , *Q. jruticosa* (Brot) , *Q. gambelii* (Nutt), *Q. geminala* (Small), *Q. georgiana* (Curtis), *Q. ilicifolia* (Wangh), *Q. imbricaria* (Michx), *Q. kelloggii* (Newb), *Q. laurifolia* (Michx), *Q. lobata* (Nee), *Q. macrocarpa* (Michx), *Q. marilandica* (Muench), *Q. maxima* (Ashe), *Q. mirbeckii* (Dur), *Q. mongolica* (Turcz), *Q. nigra* (L), *Q. palustris* (Moench) , *Q. phellos* (L), *Q. prinoides* (L), *Q. pubescens* (Willd), *Q. lanuginose, Q. rubra* (L), *Q. robur*. *Q. serrata* Thunb), *Q. sessiliflora* (Salisb), *Q. stellata* (Wangh), *Q. undulata* (Torr), *Q. utahensis* (Rydb), *Q. velutina* (Lam), *Q. virginiana* (Mill), *Q. eduardii* y *Q. potosina* (Mix, 1949; Moreno *et al*., 2015).

Al igual que todos los demás miembros de este género, *T. caerulescens* es un hongo dimórfico. En la mayoría de los casos los efectos de la enfermedad son principalmente cosméticos, sin embargo, se ha documentado, en estudios previos (Mix, 1949; Horst, 2008; Vann, 2011), que cuando la severidad de la enfermedad es alta, debilita las plantas dejándolas sin reservas para el invierno lo cual puede resultar en la muerte de las ramas jóvenes y de la propia planta.

Se han realizado pocos estudios sobre de *T. caerulescens* y, en general, se ha observado que las cepas de este patógeno aislado de una especie de encino no se puede utilizar para infecciones cruzadas a otras especies de *Quercus*, esto indica que hay un número de cepas

diferentes dentro de *T. caerulescens*. En un estudio realizado por Taylor y Birdwell (2000), se utilizaron cepas de *T. caerulescens* aisladas de *Quercus nigra* y de *Quercus falcata* para inocular *Quercus virginiana* en pruebas de patogenicidad y de infección cruzadas. Ellos encontraron que las cepas aisladas de *Quercus nigra* no fueron capaz de infectar a otras especies de encino y solo se desarrollaba en los arbolitos de *Q. nigra*, lo que indica la especificidad del huésped. También, se ha demostrado que las cepas de *T. caerulescens* difieren en sus perfiles metabólicos de nitrógeno y compuestos de carbono (Mix, 1949 y 1953).

OBJETIVO GENERAL

Confirmar la identidad de *Taphrina caerulescens* y analizar su epidemiologia en *Quercus eduardii* de la Sierra Fría de Aguascalientes.

Objetivos particulares:

1. Confirmar la identidad de *Taphrina caerulescens* mediante pruebas morfométricas, bioquímicas y moleculares.
2. Caracterizar los cambios anatómicos en células de hojas infectadas por *Taphrina caerulescens*. mediante cortes histológicos.
3. Analizar la epidemiologia de *T. caerulescens* en *Q. eduardii.*
4. Determinar la especie de *Quercus* más susceptible a *T. caerulescens* bajo condiciones controladas

HIPOTESIS

1. *Taphrina caerulescens* es el hongo que causa las ampollas de las hojas de *Quercus* spp. en la Sierra Fría
2. La incidencia y severidad de la enfermedad causada por *T. caerulescens* se incrementa conforme la humedad y temperatura son favorables a la dispersión e infección de este hongo
3. Bajo las condiciones de La Sierra Fría de Aguascalientes, *Q. eduardii* es más susceptible a *Taphrina* sp. que *Q. potosina, Q. resinosa* y *Q. sideroxyla*

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1. Chapter 1

Isolation and identification of *Taphrina caerulescens* in *Quercus eduardii* in Aguascalientes, Mexico

ABSTRACT

Taphrina caerulescens exclusively affects plants of the *Quercus* genus. The identification and isolation of this fungus is difficult due to its dimorphic nature and extremely slow growth habit in artificial growth media. The objective of this research was to isolate and identify the fungal pathogen *T. caerulescens*. Three methods were used to isolate the fungus, however, only the spore fall method was successful. In order to identify the fungus, a visual inspection of the host plants' infected leaves was carried out. The symptoms and signs of infection were noted. In addition to the typical blister formation on the infected leaves, an atypical symptom comprising of chlorotic spots covered with asci was also observed. Three biochemical tests were carried out to prove that the pathogen was in the genus *Taphrina*: They first tested the ability to utilize urea, the second, its capacity to produce extra cellular DNase and the third tested its reaction to Diazonium blue B (rapid blue salts). For species identification the following analyses were done: a) Carbon assimilation tests were also carried out using the following carbon sources: glucose, saccharose, D-xylose, raffinose, trehalose and sorbitol, b) Finally, molecular analysis tools were also utilized to extract and sequence the genomic DNA. The resulting sequences when analyzed with the NCBI Nucleotide blast tool, confirmed with 99 % similitude that the isolate was similar in nature to *Taphrina caerulescens* strain CBS 351.35 ascension number AF492081.1. Pathogenicity test were also carried out and resulted in the infection of 25 % of the inoculated plants

RESUMEN EN ESPAÑOL

Taphrina caerulescens afecta exclusivamente a las plantas del género *Quercus*. La identificación y el aislamiento de este hongo es difícil debido a su naturaleza dimórfico y su crecimiento extremadamente lento en los medios artificiales. El objetivo de esta investigación fue aislar e identificar el patógeno *T. caerulescens*. Se utilizaron tres métodos para aislar el hongo; sin embargo, solo el método de caída de esporas fue exitoso. Para identificar el hongo, se llevó a cabo una inspección visual de las hojas infectadas de las plantas hospederas. Se observaron los síntomas y signos de la infección. Además de los síntomas típicos de ampollas en las hojas infectadas, también se observó un síntoma atípico que comprende manchas cloróticas cubiertas con ascas. Se llevaron a cabo tres pruebas bioquímicas para demostrar que el patógeno era del género *Taphrina*: la primera, probó la capacidad de utilizar urea, la segunda, su capacidad de producir ADNasa extracelular y la tercera probó su reacción cuando está en contacto con el reactivo azul de diazonio B (sales de azul rápido). Para la identificación de la especie, se realizaron los siguientes análisis: a) pruebas de asimilación de carbono utilizando las siguientes fuentes de carbono: glucosa, sacarosa, D-xilosa, rafinosa, trehalosa y sorbitol y b) La secuencia del ADN genómico. Las secuencias resultantes, después de ser analizadas con la herramienta del nucleótido BLAST del NCBI, confirmaron, con una similitud del 99%, que el aislamiento era similar a la cepa CBS 351.35 de *Taphrina caerulescens* AF492081.1. También, se realizaron pruebas de patogenicidad y que resultaron en la infección del 25% de las plantas inoculadas.

1. 1. INTRODUCTION

Taphrina caerulescens belongs to the order Taphrinales. The first complete monograph of the genus was realized by Mix (1949) who divided the genus into 100 species. *Taphrina* spp. are dimorphic (Mix, 1949; Kramer, 1960). Dimorphic fungi are those fungi that exist either in yeast form or as mold (mycelial form) depending on environmental conditions, physiological conditions of the fungus or the genetic characteristics (Flannigan *et. al.*, 2001). When the fungus is within its host's tissue, it is present in its filamentous state, however, under adverse conditions, it is present in its yeast state, it is in this state that the fungus behaves like a saprophyte. In the past, *Taphrina* spp. were identified based upon geographical distribution, site and type of the infection and symptoms, localization of mycelium, morphology of the fungal structures and host range (Mix, 1949; Taylor and Birdwell, 2000; Rodríguez and Fonseca, 2003). Recently, molecular methods have been used to identify *Taphrina* spp. (Sjamsuridzal *et al.*, 1997; Rodríguez and Fonseca, 2003; Bacigálová *et al*., 2003; Tavares *et al*., 2004; Petrýdesová *et al*., 2013; Isheng *et al*.2014; Selbmann *et al.*, 2014). The most important species include: *T. deformans* which causes deformation of leaves, fruits and flowers and the defoliation of *Prunus persica* and *Prunus dulcis* (Mill.) D. A. Webb; *T. communis* which causes deformations in American plum, *Prunus americana*; *T. pruni* is found affecting European plum species; *T. wiesneri* (Rathay) Mix, syn. *T. cerasi* (Fuckel) Sadeb (Farr *et al.*, 1989), produces leaf blisters and witches broom in all cherry species; *T. armeniacae* (Georgescu y Badea) causes witches broom in apricot, *Prunus armeniaca*; *T. confusa* (Atk.) Giesen in *Prunus virginiana*; *T. farlowii* Sadeb causes blistering of the leaves of various shrubs and hedges, including *Prunus serotina*.

T. caerulescens causes leaf blister and deformation in approximately 50 species of oak (*Quercus*); *Q. acutissima* (Carruth), *Q. agrijolia* (Nee) *Q. alba* (L.), *Q. borealis* (Michx), *Q. cerris* (L), *Q. cinerea* (Michx), *Q. coccijera* (L), *Q. coccinea* (Muench), *Q. crispula* (Blume), *Q. dentata* (Thunb), *Q. douglasii* (Hook), *Q. ellipsoidalis* (Hill) , *Q. jruticosa* (Brot) , *Q. gambelii* (Nutt), *Q. geminala* (Small), *Q. georgiana* (Curtis), *Q. ilicifolia* (Wangh), *Q. imbricaria* (Michx), *Q. kelloggii* (Newb), *Q. laurifolia* (Michx), *Q. lobata* (Nee), *Q. macrocarpa* (Michx), *Q. marilandica* (Muench), *Q. maxima* (Ashe) , *Q. mirbeckii* (Dur), *Q. mongolica* (Turcz), *Q. nigra* (L), *Q. palustris* (Moench) , *Q. phellos* (L), *Q. prinoides* (L), *Q. pubescens* (Willd), *Q. lanuginose*, *Q. rubra* (L), *Q. robur*. Q. serrata Thunb), *Q. sessiliflora*

(Salisb), *Q. stellata* (Wangh), *Q. undulata* (Torr), *Q. utahensis* (Rydb), *Q. velutina* (Lam), *Q. virginiana* (Mill), *Q. eduardii* and *Q. potosina* (Mix, 1949; Moreno *et al*., 2015).

In 2015, the presence of the pathogen was confirmed in Mexico based upon the description of symptoms, signs and host range (Moreno *et al.*, 2015). Up until now the pathogen was thought to be affecting *Quercus* spp. solely in Asia, Europe and North America (USDA, 2017).

The purpose of this study was to isolate and identify the pathogen *T. caerulescens* and to confirm its presence in Aguascalientes, Mexico using available tools: visual symptoms, morphology, biochemical and molecular techniques.

1.2. MATERIALS AND METHODS

1.2.1. Sampling

Leaf samples of *Q. eduardii* with and without symptoms of the disease were collected in the Sierra Fria of Aguascalientes during the months of July to August of 2015 to 2017. The locations of sample collection were as follows: 22° 11' 3" N | 102° 36' 12" W; 22° 11' 19" N | 102° 36' 29" W; 22° 11' 31" N | 102° 36' 50" W; 22° 11' 51" N | 102° 37' 46" W; 22° 10' 52" N | 102° 38' 35" W and 22° 6' 11" N | 102° 41' 39" W. (Figure 1). The samples were placed in clear polyethylene bags containing a moist tissue and were placed in a cooler for no more than 24 hs. They were later processed in the Plant Pathology Laboratory of the Autonomous University of Aguascalientes in Aguascalientes, Mexico.

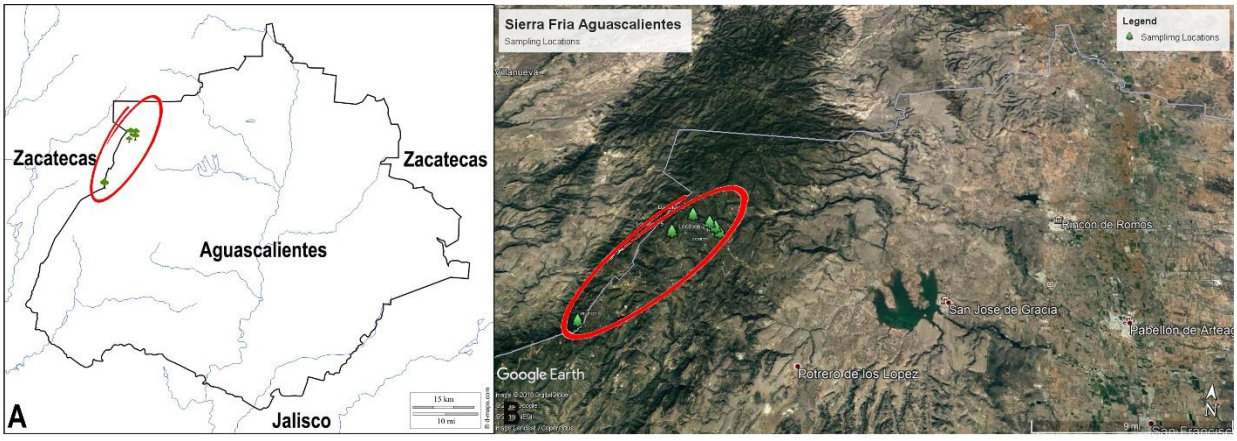

Figure 1 Map displaying locations in the Sierra Fria of Aguascalientes, Mexico, where samples were taken.

1.2.2. Isolation and identification of *T. caerulescens*

1.2.2.3. Description of Symptoms

The visual symptoms of infected leaves were described and compared to reports of Mix (1949), Taylor and Birdwell (2000) and Bacigálová (2010).

1.2.2.4. Isolation of *T. caerulescens*

Three methods were used to isolate the fungus. Method 1: The blastospores were obtained by the technique of leaf washing, a leaf sample (500 cm^2 of leaf) was shaken for 1 h with 500 ml of sterile distilled water containing one drop of Tween 80. One drop of the resulting suspension was then plated on PDA media and using sterile azar it was spread evenly over the surface (Quintana-Obregón *et al*., 2013).

Method 2: Sections of approximately 5 mm^2 were cut from freshly collected infected *Q. eduardii* leaves with symptoms of the disease. The leaf pieces were cut from the outer limit of the advancing lesions, they were disinfested in 1% sodium hypochlorite for 1 min, then washed with sterile distilled water and then placed in PDA and incubated at 20 C for 14 days (Agrios, 2005). Method 3: the blastospore fall method. Leaf tissue of *Q. eduardii* bearing asci of *T. caerulescens* was fastened to petri dish lids over PDA (Taylor and Birdwell, 2000; Bacigálová *et al*., 2003).

1.2.2.5. Maintenance of isolates

After the fungus was isolated from the host, the isolates were antiseptically purified and grown at 20 C for 2 weeks. The purified fungal colonies were maintained on Yeast Malt Extract Agar (YMEA) (malt extract 3.0 g, dextrose 10.0 g, yeast extract 3.0 g, peptone 5.0 g, agar 20.0 g, distilled water 1000.0 ml, final pH 6.2) and PDA (Yarrow, 1998). They were then stored at 4 C and sub cultured once a month.

Observations were made using a Leica DME, and Carls Zeiss microscopes and documented using the Nikon D3000 and D5100 DCLR cameras.

1.2.2.7. Biochemical differentiation of *Taphrina* from other yeast genus

In order to ensure that the isolates obtained from *Q. eduardii* leaves were *Taphrina* spp., they were subjected to a series of biochemical tests designed to differentiate *Taphrina* spp. from other yeast genus. The tests performed were the following: (1) Diazonium blue B (DBB) reaction (the DBB reagent was prepared by adding 15 ml of cold 0.25 M TRIS buffer (pH 7.0) to 15 mg of DBB technical grade; 95% pure; Sigma Chemical Co. The dissolved reagent was maintained in an ice bath and was used within 30 min). (2) Test for extracellular DNAse activity (tryptone 15 g, soya peptone 5 g, deoxyribonucleic acid (DNA) 2 g, sodium chloride 5 g, methyl green 0.05 g, agar 15 g, sterile distilled water 1 L, final pH 7.3±0.2) and (3) Tests for urease activity (urea 20 g, sodium chloride 5 g, monopotassium phosphate 2 g, peptone 1 g, dextrose 1 g, phenol red 0.012 g, agar 15 g, sterile distilled water 1 L, final pH 6.7 ± 0.2), all members of the genus *Taphrina*, should produce a negative DBB reaction, negative extracellular DNase activity and positive urease activity (Seeliger, 1956; Van der Walt and Hopsu-Havu, 1976; Sen and Komagata, 1979; Hagler and Ahearn, 1981; Nagao and Katumoto, 1998; Prillinger *et al.*,1990; Rodrigues and Fonseca, 2003).

1.2.2.8. Assimilation of Carbon Sources to Identify the Species of *Taphrina*

A modified version of carbon assimilation tests by Kali *et al* (2015) was used to confirm the organism's identity. A 10 ml suspension with a density of 3×10^8 / ml blastospores of *T. caerulescens* was prepared and stored at 4 C for 24 hs. This was done to starve and improve its utilization of the test sugars (Yarrow, 1998). For the control (negative control) a solution of sterile distilled water and phenol red indicator without the addition of test sugars was used. (In some of these experiments glucose is considered to be a positive control).

Sugar solutions of 2 % were prepared using each of the test sugars (glucose, saccharose, trehalosa, sorbitol, raffinose and D-xylose). Phenol red indicator (0.012 gm /L sterile distilled water) was added to each test sugar. All reagents were carefully mixed ensuring that the pH was between 6.8 and 7. The sugar solutions, as well as the negative control, were prepared under sterile conditions.

One hundred and fifty microliters (150 µl) of each sugar solution was pipetted into Eppendorf tubes. The samples were sealed and stored at 4 C for 24 hs. After a 24-hour time lapse, 100 µl of the yeast suspension were added to the sugar solutions and incubated at 25 C for 7 days. Tests were replicated 3 times (Yarrow, 1998).

1.2.2.9. DNA isolation from pure cultures of *Taphrina* sp.

The fungal DNA was isolated from pure culture colonies grown for 14 days at 24 C. One loopful of the cells grown on PDA was suspended in 1.5ml of sterile distilled water and was pelleted in a centrifuge at 20,000 × g for 5 min. The supernatant was eliminated and a modification of 'Bust and Grab' protocol (Harju and Peterson 2004) was used to obtain the fungal genomic DNA. The DNA quantity was estimated using the Nanodrop ND.- 1000 spectrophotometer (Thermo scientific) and the quality was determined after being mounted in 1 % agarose gels stained with redsafe dye and ran at 75 V for 48 min and viewed using the ChemiDoc XRS+System. Samples were run in duplicate in separate reactions.

1.2.2.10. DNA Isolation from *Quercus eduardii* leaves infected with *Taphrina* sp.

A modified CTAB and PVP extraction method was used to extract the genomic DNA from the leaves of *Q. eduardii* (Porebski *et al.,* 1997; Lian *et al.,* 2001; Shiran, *et al.,* 2004; Toader *et al.,* 2009; Azmat *et al.,* 2012). DNA yield and purity were determined by Nanodrop ND - 1000 spectrophotometer (Thermo scientific) and the quality was determined after being mounted in 1 % agarose gels stained with redsafe dye and ran at 75 V for 48 mins Samples were run in duplicate in separate reactions.

1.2.2.11. PCR Analyses

PCR reactions were conducted in 50-µl reaction volumes. Each reaction tube contained approximately 2 µl of 20-ng/µl DNA template. The master mix High-Fidelity 2X (New England Biolabs Ltd.) was used according to the manufacturer's instructions. Following White *et al.* (1990), the ribosomal internal transcribed spacer region was amplified and sequenced using ITS1 and ITS4 primers. The samples tested were the following: negative control (sterilized distilled water), pure fungal genomic DNA and genomic DNA extracted from leaves with blisters. The thermal cycling parameters were as follows: initial denaturation at 94 C for 3 min followed by 35 cycles consisting of denaturation at 94 C for 0.3 min, annealing at 65 C for 0.3 min, and extension at 72 C for 1 min. A final extension at 72 C for 5 min was done at the end of the amplification. The resulting PCR products were analysed through electrophoresis and then viewed using the ChemiDoc XRS+System. The products of the PCR reactions were submitted for sequencing to the McGill University and Génome Québec Innovation Centre.

1.2.2.12. Pathogenicity tests

Pathogenicity of *T. caerulescens* isolated from oak leaves of *Q. eduardii* was determined using cultured cells from a 2-week-old isolate, approximately 1.35 X 10^9 cells/mL were suspended in 0.01% Tween 80 and atomized onto the lower surfaces of leaves newly emerged from buds on 12 one-year-old greenhouse grown oak seedlings (Taylor and Birdwell, 2000). Inoculated seedlings were kept in a growth and maintained under lab conditions of 22

C and relative humidity of 100%. The inoculated plants were exposed to 12 h of light and 12 h of darkness. The plants were monitored weekly until the appearance of the disease symptoms.

1.3. RESULTS AND DISCUSSION

1.3.1. Description of Symptoms

The appearance of the disease symptoms is directly related the rainfall. The first symptoms of the disease on the leaves of *Q. eduardii* appear two to three weeks after the beginning of the season rains in Aguascalientes, Mexico. Most of the blisters appeared on the adaxial surface of the leaf (Figures 2A, 2B, 2E, 2F), however, blisters were also observed on the abaxial surface of *Q. eduardii.* These blisters reached a height of up to 0.5 cm. In some cases, the leaves did not form blisters but rather formed chlorotic lesions with irregular borders (Figures 2C, 2D). Both types of symptoms varied from a few millimetres to more than 4 cm in diameter. The lower side of the leaf corresponding to the blisters was light green in colour. In most of the leaves with blister symptoms, no asci were observed on the adaxial surface of the blister; however, asci were observed on the adaxial surface of leaves which had the atypical symptoms (chlorotic spots). The asci on the leaf surfaces with the atypical symptoms were initially white to cream in appearance (Figure 2D). By the end of the third week, they had turned grey or brown (Figure 2C). On some samples asci formed on both surfaces of the leaf. In most cases, however, more asci were observed on the adaxial leaf surface. In leaves heavily infected, chlorotic spots and or blisters joined together and covered large areas of the leaf surface.

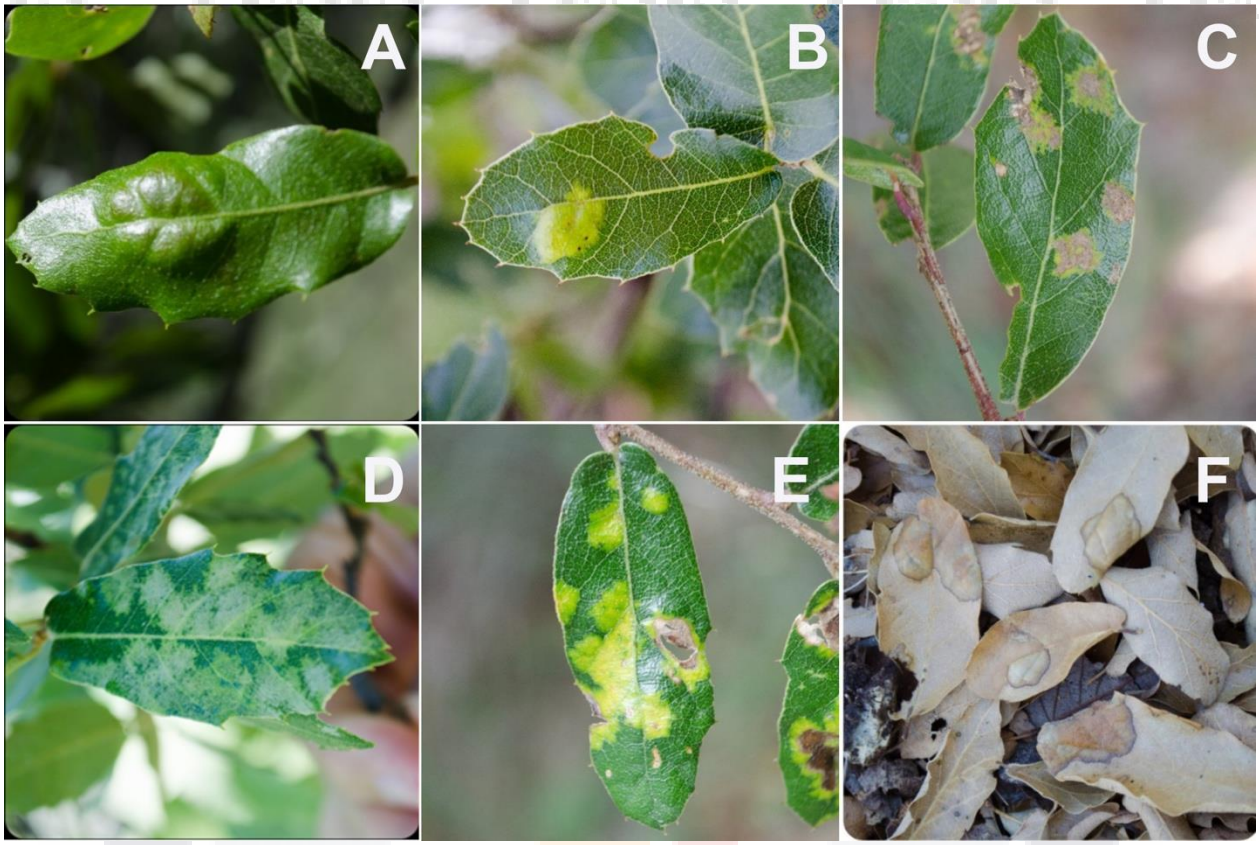

Figure 2: Symptoms of *T. caerulescens* on oak *Q. eduardii* leaves: A)., B). and E). Typical symptoms of leaf blister; C). and D). Atypical symptoms; F). Typical symptoms on dried leaves on forest floor.

1.3.2. Isolation and identification of *Taphrina* sp.

Of the three methods used to isolate pathogen only the blastospore fall method was successful. Spores were discharged onto the agar within 24 hs and isolated colonies (blastospores originating from single asci) that formed were transferred to fresh media and incubated at 20 C (Figure 3A). One isolate was purified and maintained form each sampling site. The mature colonies were light pink to cream in colour and with smooth convex surfaces with continuous borders (Figure 3B). This description fits the characteristics of *T. caerulescens* (Mix, 1949; Taylor and Birdwell, 2000; Bacigálová *et al.*, 2003; Selbmann *et al*., 2014).

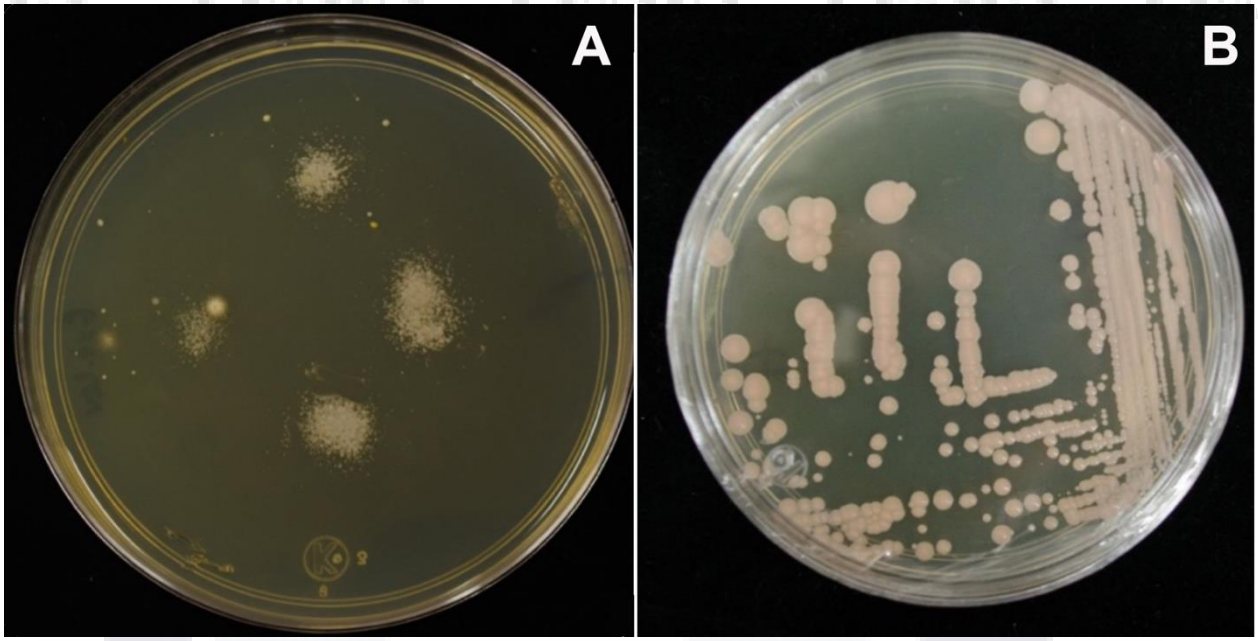

Figure 3: *T. caerulescens* pure cultures: A). Growth after 24 hours (Spore fall method); B). Fourteen-day old culture of *T caerulescens*

1.3.2.1. Description of asci

The asci were present on both the upper and lower epidermal surfaces, and varied in size and shape. They were cylindrical, claviform or a combination of the two. In addition, they were rounded at the apex and their base were flat to round (Figure 4) with some displaying root-like appendages; an important characteristic for differentiating *T. caerulescens* from other *Taphrina* spp. (Figures 4G, 4H, 4I). The asci were wedged between the epidermal cells and measured 45 - 75 µm x 10 - 22 µm with an average of 65 x 17 µm, (n = 30). Ascospores were not observed within the ascus, instead they were filled with hundreds of blastospores which measured 3 - 5.5 x 1.5 - 2.0 µm with an average of 4.2 x 1.8 µm (n = 30). The length was calculated with a standard error of 1.602 and the standard deviation was 8.777. The width was calculated with a standard error of 0.538 with a standard deviation of 2.947. These morphometric characteristics were within with the dimensions reported for *T. caerulescens* (Mix, 1949; Taylor and Birdwell, 2000).

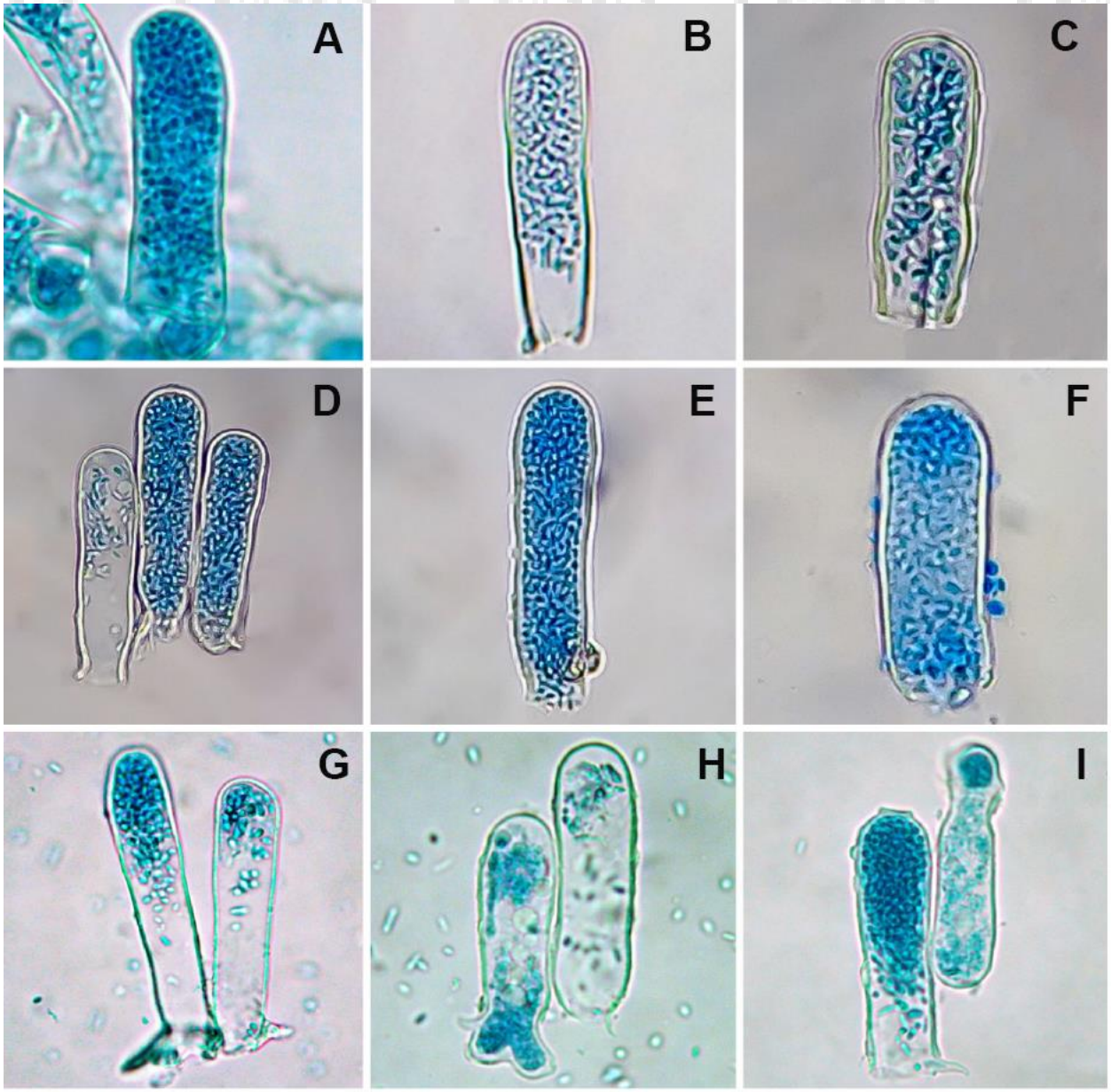

Figure 4: Variation in shapes of mature asci of *T. caerulescens* infecting *Q. eduardii*

1.3.2.2. Description of blastospores

Although the blastospores varied in size and shape, they were generally oval- shaped and reproduced by budding. On average, they measured 4.47 μm in length with a minimum and maximum range of 3 μm and 6 μm (n = 30). The standard error was 0.164 with a standard

deviation of 0.899. The average width was 2.63 μm with a minimum and maximum range of 2μm and 4μm. The standard error was 0.112 and the standard deviation was 0.614 (n = 30). These morphometric characteristics were similar to the dimensions previously reported for *T. caerulescens* (Mix, 1949; Taylor and Birdwell, 2000).

1.3.2.3. Biochemical analyses to differentiate *Taphrina* from other yeast genus

Of the six isolates of *Taphrina* obtained, 3 were used to perform these tests The reactions for Diazonium blue B (Figures 5C, 5D) and for DNAse (Figures 5E, 5F) activities were both negative, however, a positive reaction was observed for the urease activity (Figures 5A, 5B) where there was a colour change from orange to pink. These results were similar to those obtained by Hagler and Ahearn (1981), Prillinger *et al.*, (1990), Rodrigues and Fonseca (2003), to *Taphrina caerulescens*. All the isolates gave similar results.

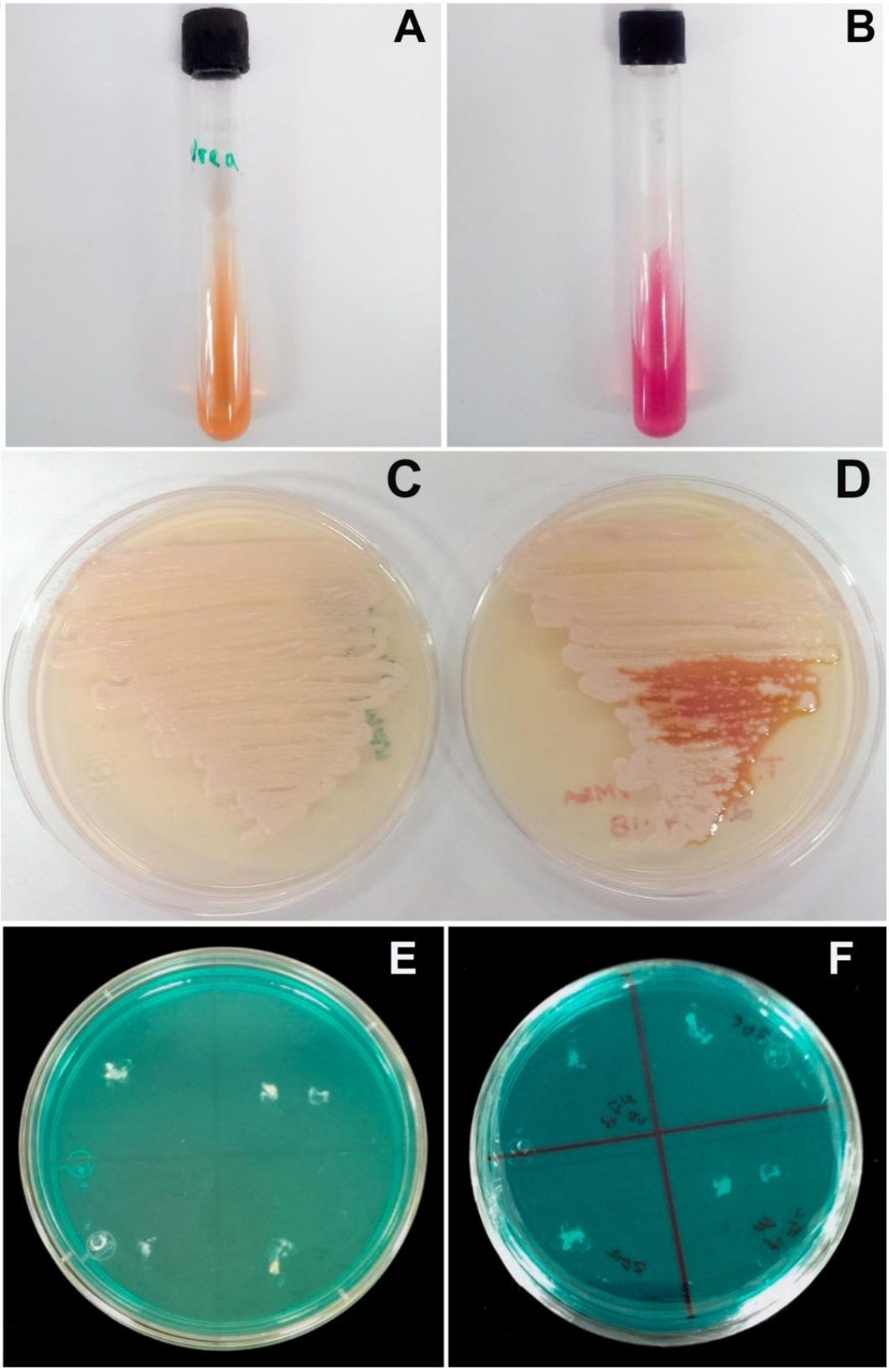

Figure 5: Differentiation tests: A). Test tube with urease media; B). Urease media 72 hs. after inoculation with *T. caerulescens* C). *T. caerulescens* in PDA zero hs after inoculation; D). *T. caerulescens* 24 hs after being inoculated with Diazonium blue B Salts; E).

1.3.2.4. Assimilation of carbon sources to identify the *Taphrina* sp.

Taphrina caerulescens was capable of assimilating glucose, trehalose, and D-xylose. This was evident as a colour change from orange to yellow was observed seven days after the solutions were inoculated with the yeast. There were no significant changes in the other samples: control, saccharose, raffinose and sorbitol (Figure 6). These provided further proof that the organism in question is indeed *T. caerulescens* as these results coincide with the results of previous studies (Seeliger, 1956; Van der Walt and Hopsu-Havu, 1976; Sen and Komagata, 1979; Hagler and Ahearn, 1981; Prillinger *et al.*,1990; Nagao and Katumoto, 1998; Rodríguez and Fonseca, 2003) All three isolates used gave similar results in all of the repetitions.

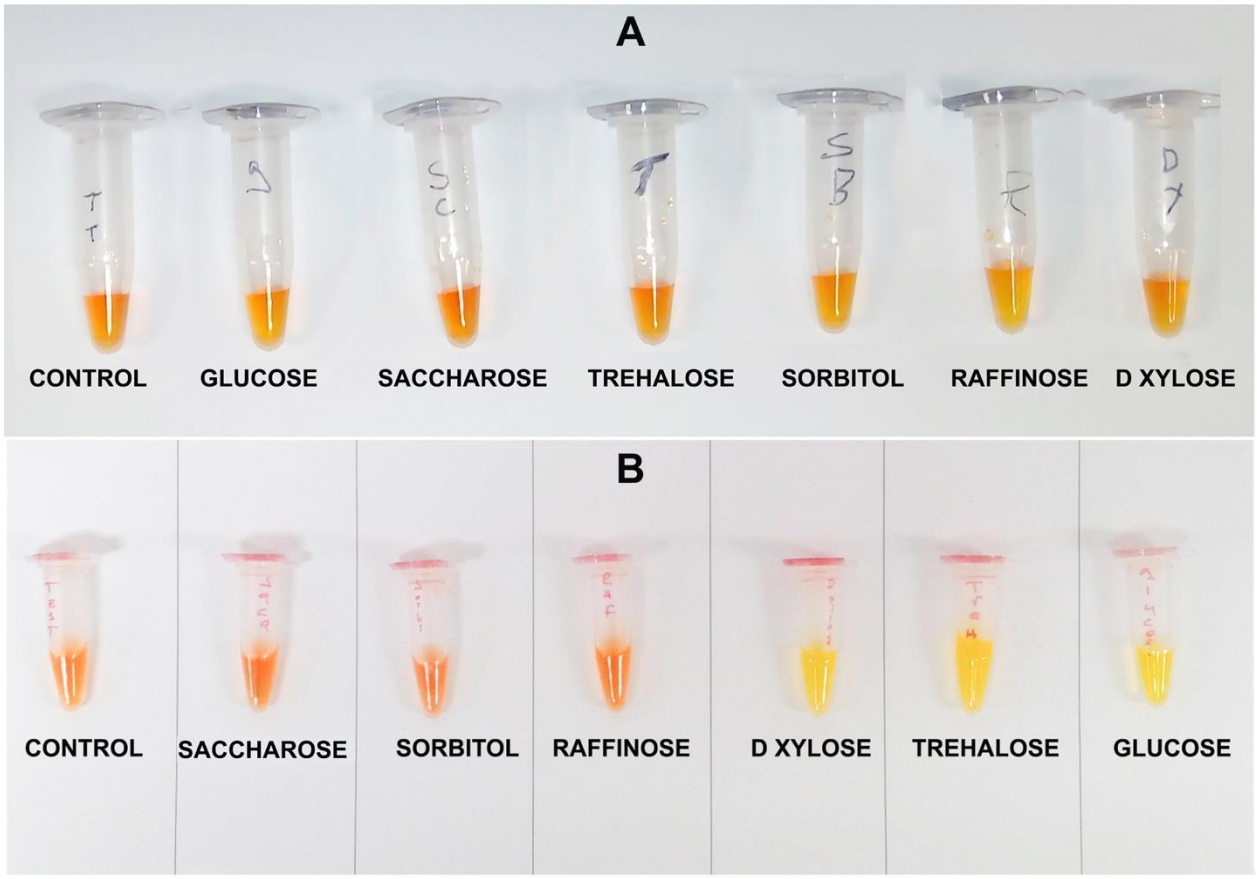

Figure 6: Results of biochemical test: A). Eppendorf tubes with 2 % sugar solution and phenol red indicator; B). Sugar solutions seven days after inoculation with *T. caerulescens*.

1.3.2.5. Molecular identification PCR reaction and sequencing.

Amplified DNA fragments were viewed using the ChemiDoc XRS+System after electrophoresis at 75 V on 1% agarose gel stained with redsafe (Figure 7). After being sequenced, they were analysed with the NCBI BLAST. The fragment sequence CGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTG AGTGTCATTAATCTCTCAACAGACCACTTGGTTCCAAACCGAGTCGGCCTGTCGA TGTTGGAGGCTGCGACGGGGTNNCGGCCCCTTCGCTCCTCTCAAATGA was closest to the *Taphrina caerulescens* strain CBS 351.35 ascension number AF492081.1 with 99 % similitude and a query cover of 100 %.

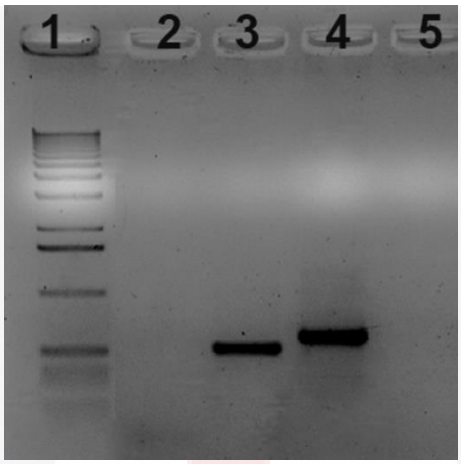

Figure 7:Gel of genomic DNA amplified with primer pair ITS 1 and ITS 4: Lane 1, 100bp ladder; Lane 2, negative control; Lane 3, Pure culture genomic DNA; Lane 4, genomic DNA from leaves with *T. caerulescens* symptoms

1.3.2.6. Pathogenicity.

The formation of blister symptoms was observed 35 days after inoculation in 25 % of the inoculated plants. Analysis of thin sections of the infected tissue by light microscopy revealed immature ascus on the upper epidermis, and hypertrophy and hyperplasia of the leaf's cells in the infected zone. This together with all the other experiments presented confirms that the specie of *Taphrina* on leaf of *Q. eduardii* in Aguascalientes, Mexico, is *T. caerulescens*.

1.4. CONCLUSION

Morphological, biochemical, molecular analysis combined with pathogenicity tests, confirms that the pathogen responsible for the leaf blisters of *Q. eduardii* in Sierra Fria of Aguascalientes, Mexico, is *T. caerulescens.* This is the first study which combines all of these techniques to confirm the identity of *T. caerulescens.*

1.5. ACKNOWLEDGEMENT

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2. Chapter 2

Modificación estructural en la hoja de *Quercus eduardii* (Trel.) causado por *Taphrina caerulescens* (Desm. & Mont.)

ABSTRACT

In the temperate forests of the Sierra Fria, Aguascalientes, Mexico, *Taphrina caerulescens* was observed affecting leaves of *Quercus eduardii*. This was the first report of the pathogen in Mexico and Central America. In addition to the typical symptom, the plant-pathogen interaction also results in the appearance of chlorotic lesions on the upper surface of some infected leaves. This was the first report of these atypical symptoms; these atypical do not result in the formation of blisters. The main objective of this study was to describe the histopathological changes that occur in *Q. eduardii* leaves infected with *T. caerulescens.* Of the 25 species of oak present in the Sierra Fria, the disease incidence and severity was highest in *Q. eduardii.* For histopathological studies, sections of healthy and infected leaf tissue were fixed, dehydrated, embedded in paraffin, cut with a microtome and stained with fast green and safranin. The differences in the healthy and infected leaf sections were observed with a Leica DME microscope and a Carl Zeiss microscope. Leaf samples were also processed for scanning electron microscopy. The following changes were observed in the affected blister tissue: there was hyperplasia and hypertrophy of cells of the upper and lower epidermis and of the palisade layer cells; there was destruction of the cells of the spongy mesophyll layer and there was destruction of the nucleus and other organelles of the palisade cells. The differences in the cell sizes between the infected and healthy leaf tissue were supported by morphometric and statistical analysis. No noticeable changes in the leaves which displayed atypical symptoms were observed.

RESUMEN EN ESPAÑOL

En los bosques templados de la Sierra Fría, Aguascalientes, México, se observó *Taphrina caerulescens* en las hojas de *Quercus eduardii*. Este fue el primer reporte del patógeno en México y Centroamérica. Además del síntoma típico, la interacción planta-patógeno también produjo lesiones cloróticas en la superficie superior de algunas hojas infectadas. Este fue el primer reporte de estos síntomas atípicos que no resultan en la formación de ampollas. El objetivo principal de este estudio fue describir los cambios histopatológicos que ocurren en las hojas de *Q. eduardii* infectadas con *T. caerulescens*. De las 25 especies de encinos presentes en la Sierra Fría, la incidencia y severidad de la enfermedad fue mayor en *Q. eduardii*. Para los estudios histopatológicos, secciones de tejido foliar sano e infectado se fijaron, deshidrataron, se impregnaron en parafina, se cortaron con un micrótomo y se tiñeron con verde rápido y safranina. Las diferencias en las secciones de hojas sanas e infectadas se observaron con un microscopio Leica DME y un microscopio Carl Zeiss. También, se procesaron muestras de hojas para microscopía electrónica de barrido. Se observaron los siguientes cambios en el tejido de la ampolla afectada: hubo hiperplasia e hipertrofia de las células de la epidermis superior e inferior y de las células de la capa de empalizada; hubo destrucción de las células de la capa del mesófilo esponjoso y destrucción del núcleo y otros orgánulos de las células en empalizada. Las diferencias en el tamaño de las células entre el tejido de la hoja infectada y el tejido sano fueron respaldadas por análisis morfométricos y estadísticos. No se observaron cambios notables en las hojas que mostraron síntomas atípicos.

2.1. INTRODUCTION

In Sierra Fria, state of Aguascalientes, oak trees (*Quercus* spp.) can be found in pure stands or mixed with pine trees (*Pinus* spp.), Tazcate (*Juniperus deppeana*), manzanita (*Arctostaphylos pungens*) and arbutus (*Arbutus* spp.), among others. All these trees are very important as they provide a wide range environmental services (CONABIO *et al*., 2008). Recently, Moreno-Rico *et al*., (2015), observed blisters and necrotic areas on leaves of *Quercus eduardii* (Trel.) in Sierra Fria. A study of symptoms in leaves along with morphometric studies of the organism affecting them, identified the fungus *Taphrina caerulescens* (Desm. & Mont.) (Fonseca and Rodrigues, 2010). *Taphrina* diseases are better known in Europe and North America (Ellis, 2000). Leaf blister disease of *Quercus* spp. caused by *T. caerulescens* (Desm. & Mont.) was thought to be endemic to North America. Hansen *et al*., (2007) reported all the known *Taphrina* spp. found in central and south America, however, *T. caerulescens* (Desm. & Mont) was not mentioned in this list.

The most studied species is *Taphrina deformans*. Ogawa *et al*., (1995) stated that *T. deformans* causes peach leaf curl. In peaches, the most noticeable symptoms are that leaves become severely distorted and reddish and eventually lead to premature aging and leaf abscission (Kern and Naef-Roth, 1975; Yamada *et al.,* 1990; Giosù *et al.,* 2000; Gauthier and Morgeson, 2015).

In *T. caerulescens* (Desm. & Mont.) symptoms appear as raised areas or blisters on the upper leaf surface, these blisters vary in size from a few millimetres to more than 1 cm; on the surface of these blisters the pathogen produces hundreds of naked asci which are filled with blastospores, a characteristic only observed in *T. caerulescens* (Desm. & Mont.). All other known *Taphrina* spp. produce 8 ascospores within their asci. These symptoms are initially light green in colour and eventually begin to turn brown resulting in premature death and or abscission of infected leaves, many of these changes are similar to modifications observed in peach and almond leaves infected with *T. deformans*. When the infection is severe the blisters unite and occupy and deform large areas of the leaf's surface (Mix, 1949; Taylor and Birdwell, 2000; Fonseca and Rodrigues, 2010; Kurtzman *et al.,* 2011 b).

These alterations in the structure of the leaf's surface caused by *T. deformans*, are due to changes in the cell's shape, size, number as well as the hormonal imbalances. The combined

effect of these modifications resulted in hyperplasia and hypertrophy and were described in detail by Adekunle *et al.,* (2005) and Tsavkelova *et al.*, (2006). In another study by Hansen *et al*. (2007), while studying effects of *T. entomospora* during infection of *Nothofagus*, they concluded that the pathogen follows a similar pattern, inducing hyperplasia and hypertrophy of the cells of the upper and lower epidermis, these changes also result in distortion of the host tissue. In available studies, although researchers agree that the pathogen induces pronounced changes in the host tissue, they have not reached a consensus about how the fungus infects and provokes these changes.

There is little research about the interaction *T. caerulescens* (Desm. & Mont.)-*Quercus* spp., for this reason the objective of this study was to describe the histopathological changes of *Q. eduardii* (Trel.) leaves infected with *T. caerulescens* (Desm. & Mont.).

2.2. MATERIALS AND METHODS

2.2.1. Study site and leaf sampling method

Oak leaves (two weeks after bud break) with and without blisters caused by *T. caerulescens* (Desm. & Mont.) were collected from mark trees every 15 days during the rainy period from June to August, 2016 and 2017. A total of 20 leaves were collected from the four cardinal points of two trees in each of the four sampling location of the Sierra Fria, Aguascalientes. The sample collection locations were as follows: 22° 11' 3" N | 102° 36' 12" W; 22° 11' 19" N | 102° 36' 29" W; 22° 11' 31" N | 102° 36' 50" W; 22° 11' 51" N | 102° 37' 46" W (Monte Grande); 22° 10' 52" N | 102° 38' 35" W (Laguna Seca), and 22° 6' 11" N | 102° 41' 39" W (La Angostura). The samples were placed in clear polyethylene bags containing a moist tissue and were placed in a cooler for no more than 24 hours. They were later processed in the plant pathology lab of the Autonomous University of Aguascalientes in Aguascalientes, Mexico.

2.2.2. Processing of leaves for histological studies for optical microscope analyses

For histological preparations, specimens were first fixed in formaldehyde alcohol acetic acid, 10 % :50 % :5 % + 35 % distilled water (F.A.A) for no less than 24 hours. The samples were then dehydrated in an ethyl alcohol series (10, 20, 30, 50, 70, 80, 90, 100 and 100%) for 4 hours in each concentration after which the samples were fixed in paraffin wax for 48 hours. Transverse sections of 8 and 10 µm were obtained using a rotary microtome. The paraffin wax was removed and the tissue was rehydrated in an ethyl alcohol series (100, 90, 80, 70%, 50, 30, 20 and 10%). The preparations were stained with safranin-fast green in order to study the effect of the parasite in its host (Johansen, 1940). All original measurements were made using an ocular micrometre ruler affixed to a compound microscope (Carl Zeiss include model and lenses (magnification) used). Measurements were made using Imagej (Version 1.51j8; Rasband, 2017) and descriptive analyses were realized. Observations were made using a Leica DME and Carl Zeiss compound light microscope and documented with a Nikon D3000 reflex mounted on the microscope. A Blackberry z10 cell phone was used in some instances because of the excellent quality of its images.

2.2.3. Scanning electron microscopic analyses

For electron microscopic analyses of the infected tissue, leaf sections of *Q. eduardii* (Trel.) with and without blisters caused by *T. caerulescens* (Desm. & Mont.) were fixed in 3 % glutaraldehyde pH 7.2 for 24 h. Samples were washed three times with a phosphate buffer for 1 min. Each sample was then dehydrated in series with ethanol solutions (30, 40, 50, 60, 70, 80, 90 and 100%) for 15 min in each of the solutions. The last three solutions were duplicated. Samples were critically dried with CO in a Sandri-2 780A® dryer (TOUSIMIS Research Corporation, Rockville, U.S.A.). They were placed on a sample holder using a double adhesive tape of copper (or charcoal) and coated with gold for 4 minutes in a JFC-1100® ionizer (JEOL LTD, Tokyo, Japan). The samples were observed using a scanning electron microscope JSM-35C® (JEOL LTD, Tokyo, Japan) in the Electronic Microscopy laboratory of the Department of Biology, Basic Sciences Centre of the Autonomous University of Aguascalientes (Dykstra and Reuss, 2003).

2.3. RESULTS AND DISCUSSION

2.3.1. Description of disease symptoms

Typical blister symptoms were presented as raised areas on the leaf upper epidermal surface, they were variable in shape and size and ranged from a few millimetres to more than 2 cm in diameter and in severe cases occupied the entire leaf surface. Blister were present on leaves during all stages of their development (Figure 8A, 8B, 8C). The blisters started out as pale green to yellow, as the leaf matured, the infected area became necrotic. A layer of asci generally formed on the upper leaf surface; however, asci also formed on the lower leaf surface.

Atypical non-blister symptoms were present as irregular, chlorotic spots on the leaf upper epidermal surface; they were variable in shape and size and ranged from a few millimetres to more than 2 cm in diameter. These spots overtime were covered by a whitish mass of asci (Figure 8D, 8E, 8F).

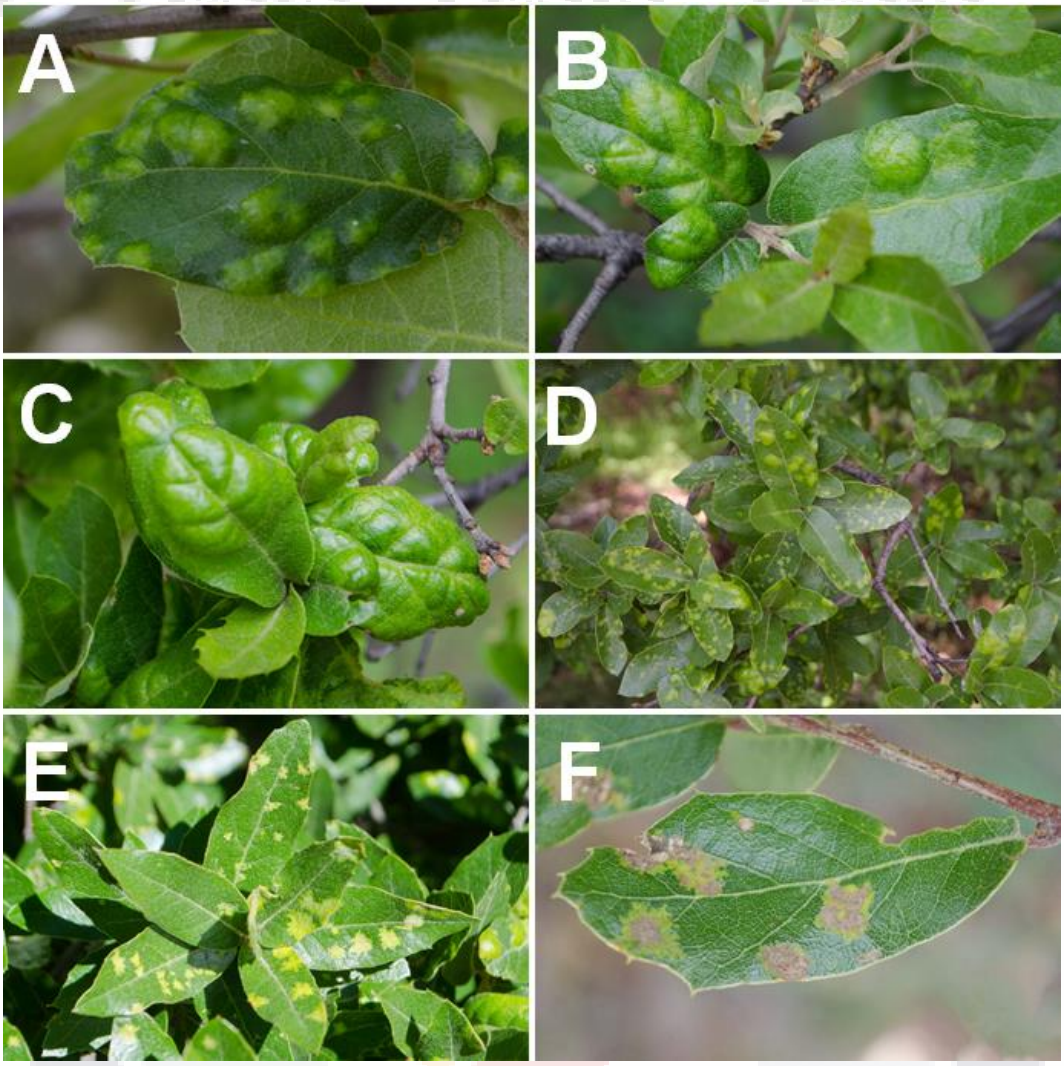

Figure 8: Symptoms caused by *T. caerulescens* (Desm. & Mont.) on oak leaves: A, B and C) Typical blister symptoms; D, E and F) Atypical symptoms.

2.3.2. Histology of leaf section with typical blister symptoms

On comparing the section of a healthy leaf (Figure 2A) with the infected tissue of the blister area there was a marked increase in the size and number of cells (Figure 2B, 2C), Asci were observed emerging from the cells of both the upper and lower epidermis (Figure 2D). Hypertrophy and hyperplasia were observed in the cells of the upper and lower epidermis (Figure 3A, 3B, 3C, 3D). These findings are similar to observations made by Syrop (1975) in her studies of the leaf curl disease of almond caused by *T. deformans.*

Palisade cells also increased in length and in number and there was less intercellular space between the cells, their cell walls were thin and nuclei and chloroplast suffered modifications (Figure 3E, 3F). Two or three layers of palisade cells were observed. The morphometric and statistical analysis of the control and of the infected leaves confirmed that the observed changes were significant (Table 1).

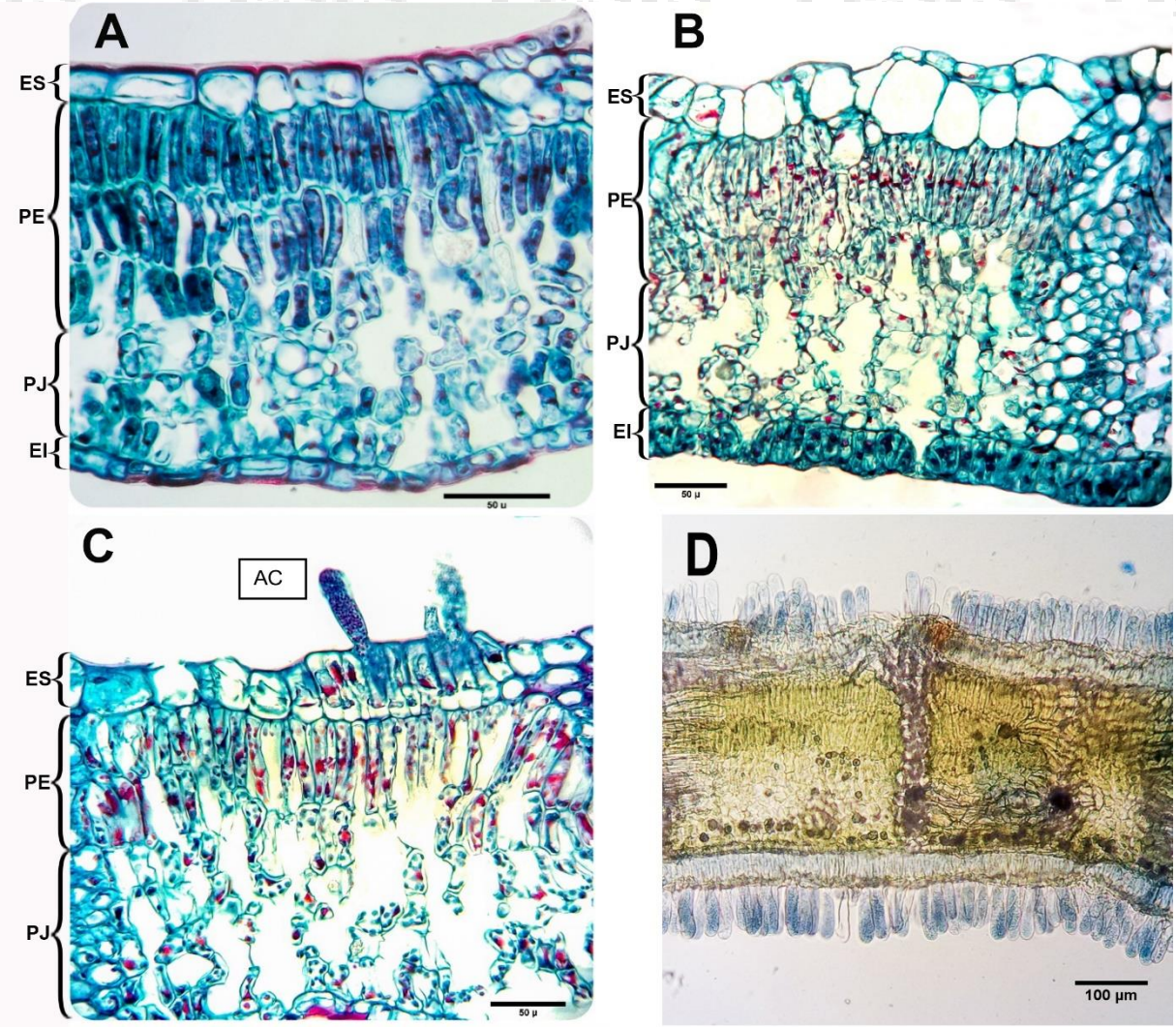

Figure 9: Health and infected sections of *Q. eduardii* (Trel.) leaves: A) Health section of Q. *eduardii* (Trel.) leaves; B, C and D) leaf sections infected with *T. caerulescens* (Desm. & Mont.) with asci on both upper and lower leaf Surface. (ES: superior epidermis; PE: palisade parenchyma; PJ: spongy parenchyma; EI: inferior epidermis).

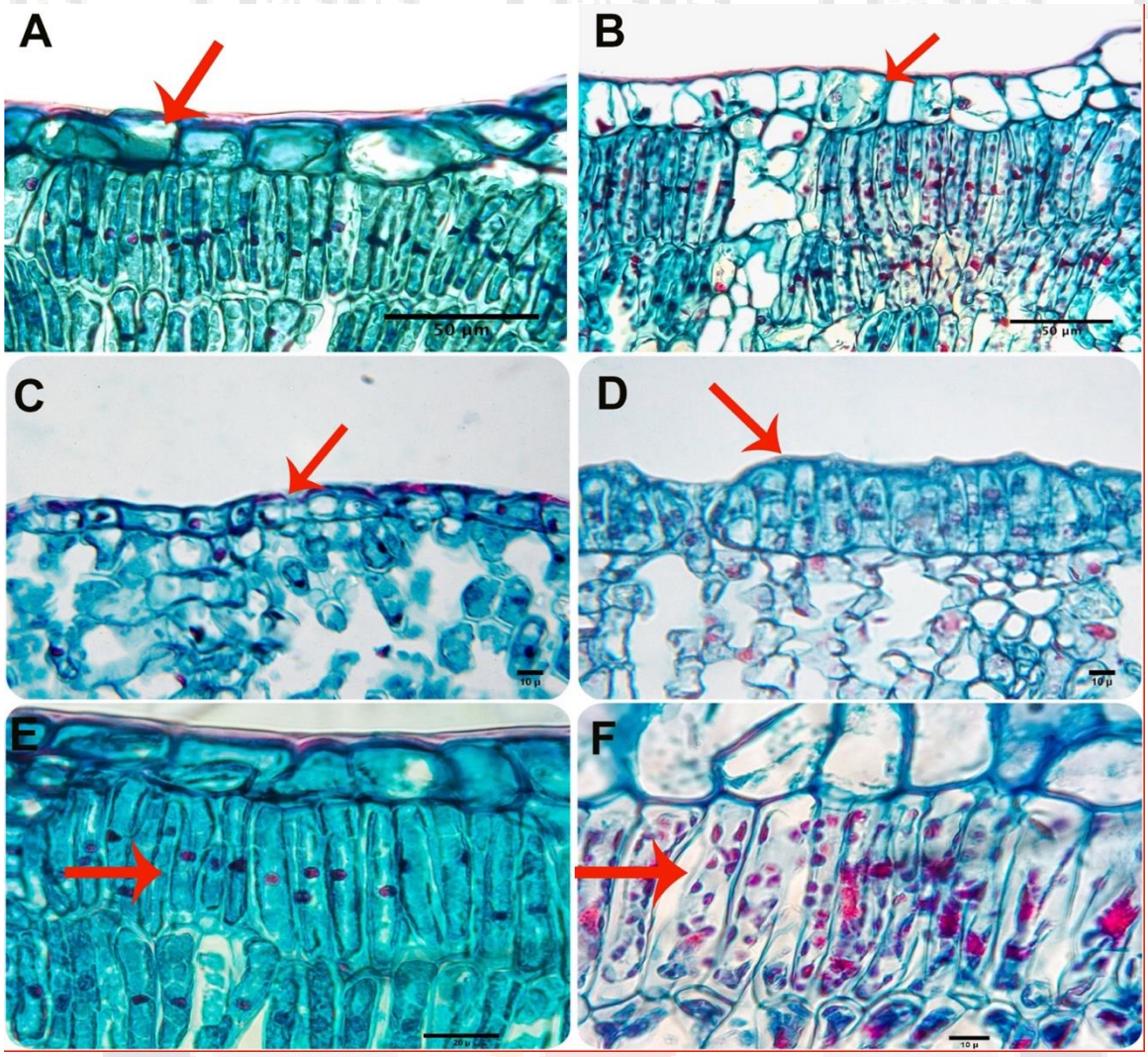

Figure 10: Comparison of transverse sections of healthy and infected leaf sections of *Q. eduardii* (Trel.) : A) uninfected cells of the upper epidermis; B) infected upper epidermis cells; C) healthy cells of the lower epidermis; D) infected cells of the lower epidermis; E) palisade cells from healthy leaf, with dense cytoplasm and intact organelles; F) palisade cells from infected leaf section, less dense cytoplasm with damaged organelles.

Table 1: Morphometric parameters of cells of *Q. eduardii* (Trel.) infected by *T. caerulescens* (Desm. & Mont.) with healthy cell measurements (measurements were made using 15 leaves).

Parameters	Status	Mean (µm) (N=30)	SD	Difference between means
Upper epidermal cells length	Healthy	28.58	4.71	7.09
	Infected	35.67	11.37	
Upper epidermal Cells width	Healthy	25.75	6.44	16.66
	Infected	42.41	13.91	
Lower epidermal cells length	Healthy	12.16	1.43	8.92
	Infected	21.08	3.39	
Lower epidermal cells width	Healthy	14.83	5.29	4.79
	Infected	19.62	4.52	
Palisade cells length	Healthy	43.41	6.55	11.34
	Infected	54.75	6.54	

SD: Standard Deviation

2.3.3. Statistical analysis

Analysis of variance showed that the effect of the disease on the different cells types of the infected tissue was significant.

Cells of the upper epidermis: ANOVA for the length of the cells, showed that changes were significant, $F(1, 58) = 406.28$, $p = .000$. The changes in the width were also significant $F(1, 58) = 165.09$, $p = .000$.

Cells of the lower epidermis: ANOVA for the length of the cells showed that change was significant, $F(1, 58) = 176.14$, $p = .000$. The changes in the width were also significant $F(1, 58) = 16.04$, $p = .000$.

Palisade cells: ANOVA showed that changes in the length of the palisade cells were significant, $F(1, 58) = 44.96$, $p = .000$.

2.3.4. Histology atypical symptoms

The cells of the epidermis and mesophyll layers displayed no signs of hyperplasia or hypertrophy; however, four parenchymal palisade layers were observed at times. Asci were located on the upper epidermis. Necrosis was observed in the cells of the upper epidermis and in the cells of the first layer of the palisade cells (Figure 4A, 4B), while cells of the spongy mesophyll appeared normal. In regions of the same leaf which did not have any symptoms, the cells were isodiametric in shape with 2 – 3 layers of palisade parenchyma cells and two layers of spongy parenchyma; cells of the epidermis were normal in size and shape, all of which fits the description of normal uninfected tissue as already described in Figure 2A. The presence of tannins was also observed in unaffected epidermal and sub epidermal cells close to the areas with atypical symptoms.

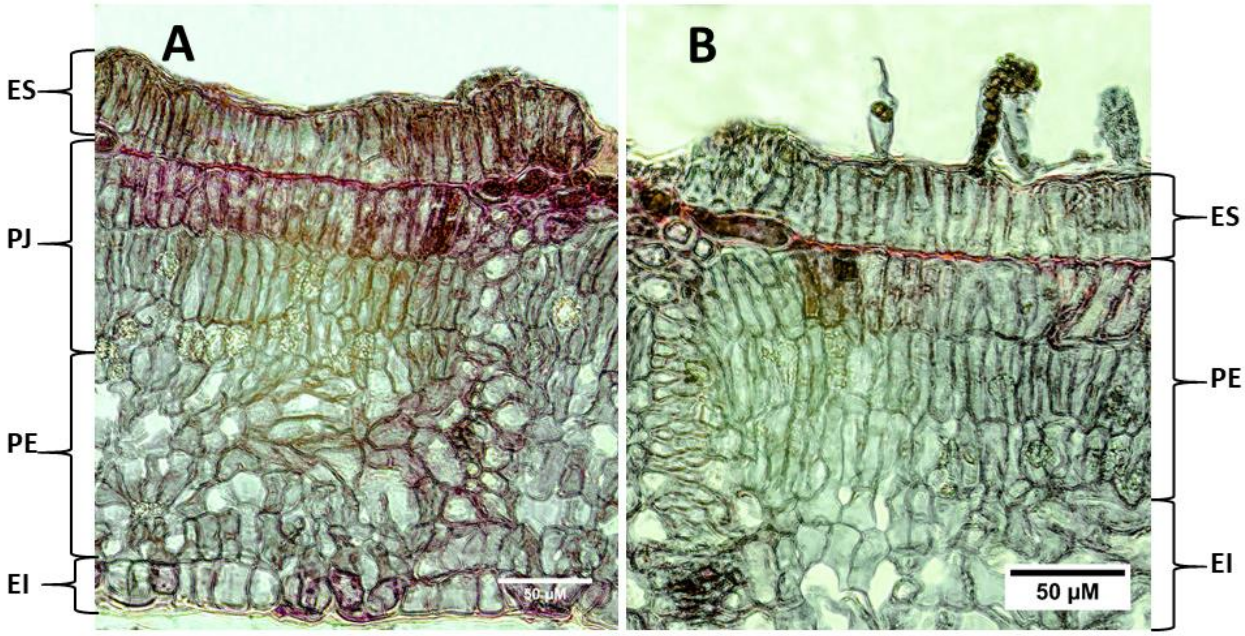

Figure 11: Atypical symptoms of *T. caerulescens* (Desm. & Mont.) affecting *Q. eduardii* (Trel.) leaf: A) longitudinal section of an infected zone of *Q. eduardii* (Trel.) showing necrosis of the upper epidermis and the first layer of the palisade parenchyma; B) *T. caerulescens* (Desm. & Mont.) ascus forming from ascogenous cells inserted between epidermal cells of *Q. eduardii* (Trel.).

2.3.5. Scanning electron microscopy analyses

These analyses were done using tissue from the typical blister symptoms. The mycelium of *T. caerulescens* (Desm. & Mont.) was observed infecting the leaves of *Q. eduardii* (Trel.) in the following ways: subcuticular, intercellular and also within the walls of the epidermis of its host (Figure 5A, 5B and 5C). These results were similar to what was published by Syrop (1975) in her studies of *T. deformans* in almond leaves. In this study the mycelium of *T. caerulescens* (Desm. & Mont.) was first observed colonizing the cells of the lower epidermis and then the entire internal leaf tissue (Figure 5A and 5B). Also in this study it was observed that the asci of *T. caerulescens* (Desm. & Mont) developed on both the adaxial and abaxial surfaces, this is in contrast to observations made by Syrop (1975) and Bassi *et al.* (1984), who only observed asci developing on the adaxial side of leaves (Figure 6). The mycelium of *T. caerulescens* (Desm. & Mont.) was extremely slender (Figure 5C and 5D) < 0.2 µm.

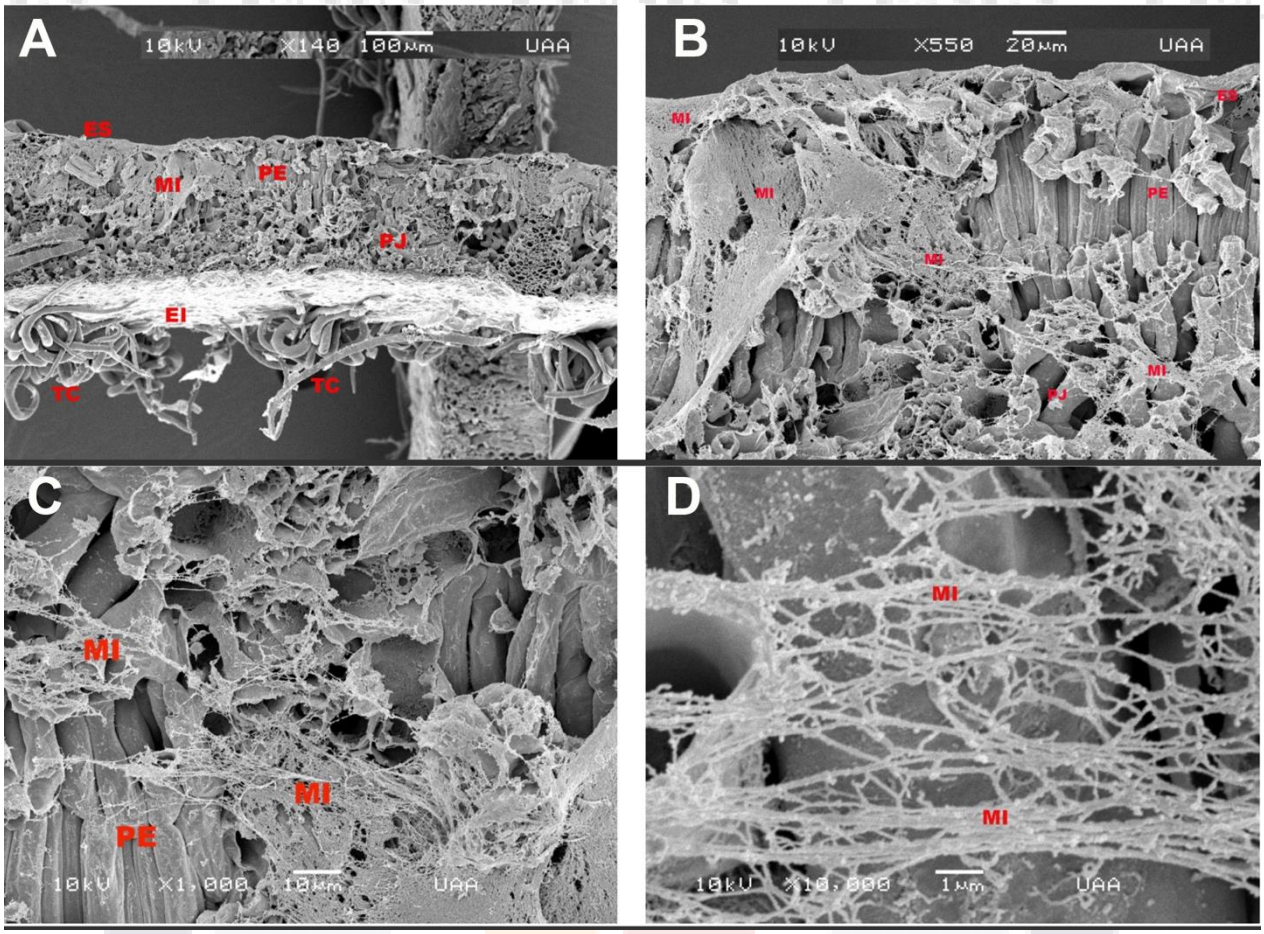

Figure 12: SEM of *T. caerulescens* (Desm. & Mont.) on *Q. eduardii* (Trel.): A) and B) Longitudinal section of *Q. eduardii* (Trel.) infected with *T. caerulescens* (Desm. & Mont.), showing the mycelium of *T. caerulescens* (Desm. & Mont.); C) and D) Mycelium of *T. caerulescens* (Desm. & Mont.), measuring < 0.2 µm, colonizing the entire leaf section of *Q. eduardii* (Trel.) in the infected zone. (ES = Superior Epidermis; EI = Inferior Epidermis; MI = mycelium; PE = Palisade Cells; PJ =. Spongy mesophyll; TC = Trichome).

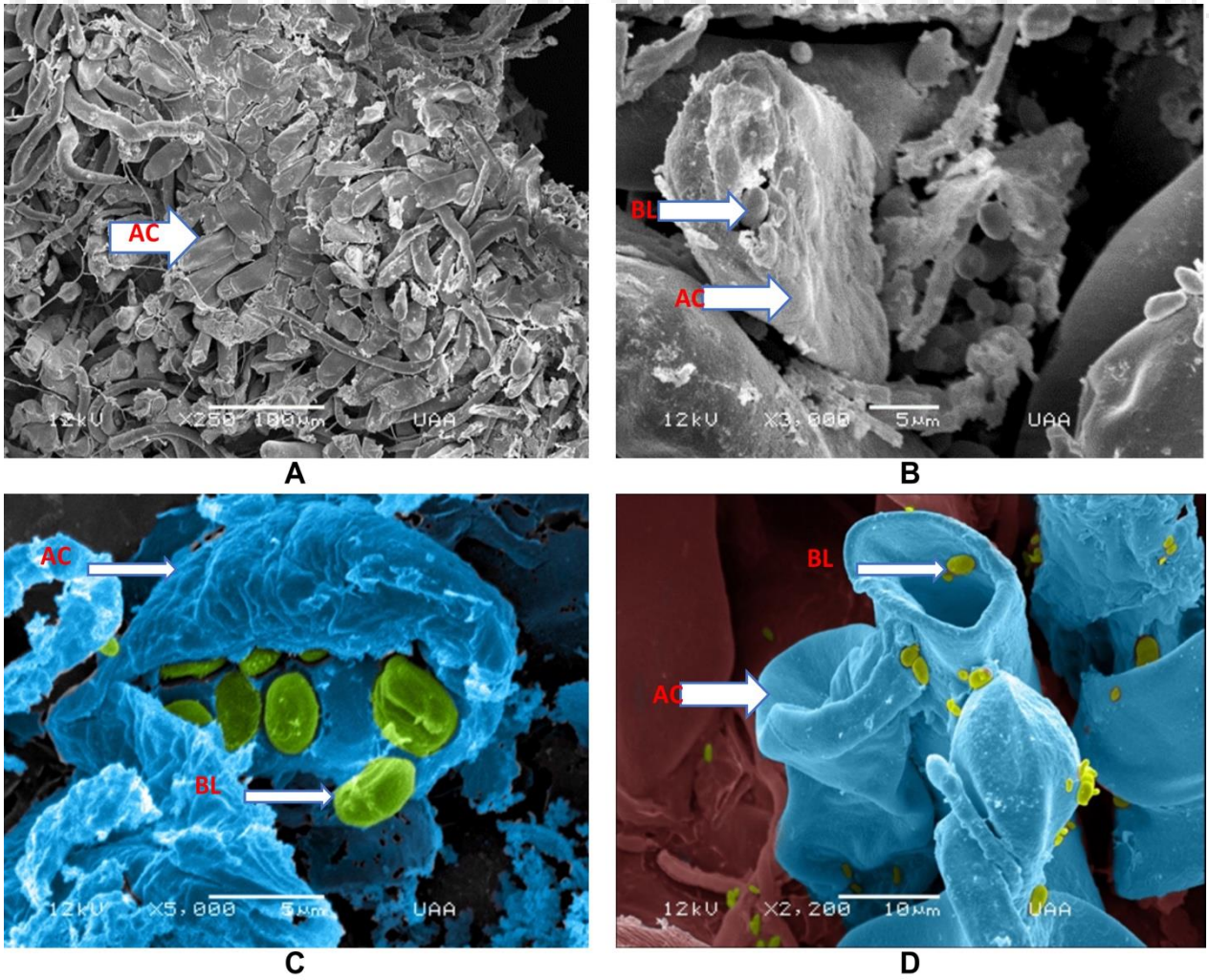

Figure 13: Asci developing on the lower epidermis of *Q. eduardii* (Trel.): A, B, C and D) Asci of *T. caerulescens* (Desm. & Mont.) on the abaxial surface of *Q. eduardii* (Trel.). (BL = Blastospores; AC = Ascus).

On penetrating the leaves of its host, the fungus begins to aggressively modify host cells, beginning with cells of the lower epidermis (Figure 2B, 3D), as fungus progresses through the leaf, most of the cells of the spongy mesophyll are destroyed (Figure 2B, 2C, 2D), cells of the palisade layer increased significantly in length and number and displayed a thinning of their cell walls, protoplast appeared less dense than in the control, the nuclei and other organelles, modified or destroyed. Cells of the upper epidermis also drastically increased in number and size. These changes provoked by *T. caerulescens* (Desm. & Mont.) in the leaves of its host are very similar to changes observed in peach leaves infected with *T. deformans*, like

T. deformans it causes hypertrophy and hyperplasia, which then result in deformation, premature death and premature abscission of leaf blades (Syrop, 1975)

There were no differences in the anatomical parts of the leaf blade near the blisters and other unmodified parts of the leaf. In both cases, in the cross section of the leaf blade, the typical leaf cell system was visible and unchanged.

2.4. CONCLUSIONS

This study provided evidence that *T. caerulescens* (Desm. & Mont.) causes hypertrophy and hyperplasia of cells of the upper epidermis, lower epidermis and of the cells of the palisade layer of *Q. eduardii* (Trel.) leaves. The morphometric and statistical analysis confirmed that the differences observed in health cells and infected cells were significant, and that these differences were directed related to the infection by *T. caerulescens* (Desm. & Mont.). The plant - pathogen interaction also resulted in the destruction of cells in spongy mesophyll layer. Eventually they also destroyed the nucleus and other organelles in the infected cells. This study, also, for the first time describes the atypical symptoms (lesions) also formed on leaves infected with the pathogen. These symptoms do not involve the formation of blisters on *Q. eduardii* (Trel.) leaves.

2.5. ACKNOWLEDGEMENTS

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CONCLUSIONES GENERALES

1. Las pruebas realizadas en estos estudios demuestran sin lugar a dudas que el patógeno responsable de las ampollas foliares de *Q. eduardii* en la Sierra Fría de Aguascalientes, México, es *T. caerulescens*.
2. Estos estudios proporcionaron evidencia adicional de que los síntomas de la enfermedad de la ampolla de la hoja causada por el patógeno *T. caerulescens* son el resultado de cambios a nivel celular. Estos cambios incluyen, pero no se limitan a hipertrofia e hiperplasia de células de la epidermis superior, epidermis inferior y de las células de la capa de empalizada de las hojas del huésped infectadas. La interacción planta - patógeno también resultó en la destrucción de células en la capa espesa de mesófila y la destrucción del núcleo y otros orgánulos de las células infectadas. Este estudio también describe por primera vez los síntomas atípicos que también se forman en las hojas infectadas con el patógeno. Estos síntomas no implican la formación de ampollas en *Q. eduardii.*

GENERAL CONCLUSIONS

1. The tests realized in these studies prove without a doubt that the pathogen responsible for the leaf blisters of *Q. eduardii* in Sierra Fria of Aguascalientes, Mexico, is *T. caerulescens*.
2. These studies provided further evidence that the disease symptoms of leaf blister caused by the pathogen *T. caerulescens* are as a result of changes at the cellular level. These changes include, but are not limited to hypertrophy and hyperplasia of cells of the upper epidermis, lower epidermis and of the cells of the palisade layer of the infected host leaves. The plant - pathogen interaction also resulted in the destruction of cells in spongy mesophyll layer and destruction of the nucleus and other organelles of the infected cells. This study, also, for the first time describes the atypical symptoms also formed on leaves infected with the pathogen. These symptoms do not involve the formation of blisters on *Q. eduardii* leaves.

RECOMENDACIONES

1. Se deben realizar estudios moleculares adicionales para determinar qué genes se modifican (activan o desactivan) dentro del patógeno y el huésped durante su interacción.
2. También sería interesante utilizar técnicas de análisis molecular para determinar qué factor o factores inician el cambio de la fase de levadura no patógena a la fase patógena del hongo.
3. Que continúen los estudios sobre las enfermedades de *Quercus* spp. en la Sierra Fría y que se le brinden más recursos financieros.
4. Los investigadores también deben ampliar su investigación para incluir otras especies importantes de árboles forestales, específicamente *Pinus*, ya que los pinos junto con los encinos constituyen las dos especies de árboles más importantes no solo en la Sierra Fría de Ags. pero en todos los bosques templados de México.

RECOMMENDATIONS

1. Further molecular studies should be carried out to determine what genes are modified (activated or deactivated) within the pathogen and host during their interaction.
2. It would also be interesting to use molecular analysis techniques to determine what factor or factors initiate the switch from the non-pathogenic yeast phase to the pathogenic phase of the fungus.
3. That the studies into the diseases of *Quercus* spp the Sierra Fria should continue and more financial resources she be made available.
4. The investigators should also expand their research to include other main forest tree species specifically *Pinus*, since pine together with oak species make up the two most important tree species not only in the Sierra Fria of Ags. but in all the temperate forests of Mexico.