
El Colegio de la Frontera Sur

# Interacciones interespecíficas en la estructuración de las comunidades de hongos sobre roca calcárea

Tesis
presentada como requisito parcial para optar al grado de
Doctor en Ciencias en Ecología y Desarrollo Sustentable
Con orientación en Conservación de la Biodiversidad

Por

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# El Colegio de la Frontera Sur

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Las personas abajo firmantes, miembros del jurado examinador de **Sergio Alberto Gómez Cornelio** hacemos constar que hemos revisado y aprobado la tesis titulada: **"Interacciones interespecificas en la estructuración de las comunidades de hongos sobre roca calcárea"** para obtener el grado de **Doctor en Ciencias en Ecología y Desarrollo Sustentable**

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## CONTENIDO

## Resumen

La estructura de las comunidades fúngicas está determinada por las condiciones ambientales y la bioreceptividad de la roca calcárea, pero se desconoce el papel de las interacciones en la composición de las especies. Por ello, en este trabajo se estudió la importancia de las interacciones entre especies de hongos, tomando como modelo de estudio la estructura de las comunidades fúngicas de biopelículas asociadas a roca calcárea, en paredes con 1 (joven), 5 (intermedia) y 10 (avanzada) años de exposición al ambiente en Campeche, México. El aislamiento se realizó mediante el lavado y la filtración de partículas y se identificaron 69 géneros y 202 especies de 844 aislados. En la biopelícula intermedia se encontró alta abundancia, pero baja riqueza y diversidad de especies, mientras que en la biopelícula avanzada, se registró alta riqueza y diversidad. Se observó la dominancia de algunas especies en cada biopelícula. Los resultados sugieren que la composición de la comunidad fúngica cambia en función del tiempo de exposición de la roca al ambiente. Posteriormente con 11 especies dominantes se analizaron las interacciones duales en dos medios de contenido nutricional contrastantes (un medio oligotrófico y uno copiotrófico) y sobre cupones de roca. Se observó que los tipos de interacciones ocurrieron en función de las especies y del medio de cultivo. En el medio oligotrófico se encontró mayor frecuencia de inhibición entre las especies, y el crecimiento del Hyphomycete 1, prevaleciente en la biopelícula avanzada que fue estimulado por otras especies en ambos medios. Por su parte, en los cupones de roca se observaron diferentes niveles de colonización, *Phoma eupyrena* y *Curvularia lunata* mostraron altos porcentajes de colonización y dominancia en la roca respecto a otras especies; mientras que los porcentajes de colonización de *Cladosporium cladosporioides* y *Paraconiothyrium* sp. fueron bajos. En los controles, *Pestalotiopsis maculans* y *Paraconiothyrium* sp. provocaron la producción de cristales de oxalato de calcio, pero esta producción fue variable al coexistir con otras especies. Se concluye que las interacciones entre especies de hongos aislados de biopelículas sobre roca calcárea a diferentes tiempos de exposición al ambiente, influyen en la composición de la comunidad fúngica, particularmente en la dominancia de ciertas

especies, y las consecuencias biogeoquímicas que potencialmente se derivan de la producción de ácidos orgánicos.

**Palabras claves:** antagonismo, colonización fúngica, biopelículas epilíticas, diversidad fúngica, biomineralización.

# CAPÍTULO 1. INTRODUCCIÓN

Este trabajo de investigación se estructura en cuatro capítulos. En el presente capítulo se presenta una introducción general del tema de investigación, así como algunos conceptos básicos y se define la premisa de investigación. El capítulo dos muestra la composición de las comunidades fúngicas inmersas en biopelículas desarrolladas sobre roca calcárea a diferentes tiempos de exposición al ambiente, además de discutir los cambios a nivel de especies en las diferentes biopelículas con especial atención en las especies dominantes.

En el capítulo tres se presentan las diferentes interacciones intra e interespecíficas entre hongos dominantes en las diferentes biopelículas, además de las posibles implicaciones de estas interacciones en la estructura de la comunidad fúngica. Se muestran ensayos de interacciones duales en dos medios de cultivo contrastantes y sobre cupones de roca calcárea que fueron analizados después de cuatro meses de inoculados los hongos. En el capítulo cuatro se abordan los principales resultados obtenidos en este estudio y las conclusiones generales sobre las interacciones entre especies fúngicas provenientes de sustratos líticos, así como las posibles implicaciones de las interacciones fúngicas en la roca calcárea.

En los sustratos líticos habitan microorganismos adaptados a este ambiente hostil. Estos microorganismos se encuentran inmersos en entidades microbianas denominadas biopelículas, que se caracterizan por presentar una matriz heterogénea de microorganismos fuertemente unidos por sustancias poliméricas extracelulares, producto de su metabolismo, las cuales son de gran importancia para las etapas de máximo estrés ambiental como el déficit hídrico y las altas radiaciones de luz ultravioleta (UV), ya que funcionan como protección para los microorganismos que ahí habitan (Ortega-Morales et al. 2000; Gorbushina 2007; Gadd 2007). Estas biopelículas están compuestas por bacterias (heterótrofas y quimiolitotróficas), hongos (filamentosos y levaduras), organismos fotótrofos (cianobacterias y algas microeucarióticas), líquenes y protozoarios (Warscheid y Braams 2000; Gorbushina 2007; Aptroot y Herk 2007).

La colonización microbiana en las rocas es un fenómeno complejo. Las especies que integran estas comunidades y su permanencia en ellas está determinada por las características intrínsecas del sustrato como la porosidad, rugosidad, pH de la superficie, retención del agua y composición mineralógica (Guillitte 1995; Kumar y Kumar 1999; Gadd 2007), así como por factores ambientales extremos como la temperatura, humedad, radiación solar, disponibilidad de oxígeno y nutrimentos (Sterflinger 2000; Burford et al. 2003; Burford et al. 2006; Miller et al. 2006; Gorbushina y Broughton 2009; Gómez-Cornelio et al. 2012), el tiempo de exposición en la interacción roca-medio ambiente (Gómez-Cornelio et al. 2012; Adamson et al. 2013) y el microhábitat donde se desarrollen en la roca (Gadd 2007). Además, la influencia humana juega un papel importante, ya que la presencia y composición de las comunidades microbianas pueden ser alteradas (Moroni y Pitzurra 2008).

El sustrato rocoso es generalmente heterogéneo y por ende contiene múltiples microhábitats que pueden ser epilíticos (sobre la superficie), hipolíticos (debajo de la roca), euendolíticos (los que penetran activamente dentro de la roca), y los endolíticos (habitan en la subsuperficie de la roca) que pueden ocurrir como casmoendolíticos (en grietas y fisuras observados desde la superficie) y criptoendolíticos (con crecimiento en cavidades no observadas desde la superficie) (Gadd 2007). Los microorganismos de estos hábitats juegan un papel importante en la transformación de la roca a suelos minerales y en el ciclaje de los elementos en ambientes naturales (Sterflinger 2000; Gadd 2007; Gorbushina 2007; Scheerer et al. 2009).

Entre los diferentes grupos de microorganismos que colonizan los sustratos rocosos, los fotótrofos son los más estudiados (Gaylarde y Gaylarde 2005; Ortega-Morales et al. 2005; Miller et al. 2008; Macedo et al. 2009). En las zonas tropicales, las biopelículas epilíticas están dominadas por poblaciones fototróficas (Gaylarde y Gaylarde 2005), que además de servir de protección contra la radiación solar, proporcionan sus desechos metabólicos para ser utilizados como fuente de carbono por las comunidades heterotróficas asociadas, como los hongos filamentosos, las levaduras y las bacterias

heterótrofas (Ortega-Calvo et al. 1995; Gadd 2004; Gadd 2007). Esto promueve la sucesión y conduce al establecimiento de plantas inferiores (Warscheid y Braams 2000).

Las comunidades fúngicas líticas es un grupo de organismos poco estudiados y explorados, a pesar de que crecen en un ambiente oligotrófico sobre residuos orgánicos e inorgánicos, como los desechos de microalgas y bacterias, células muertas, polvo y agua de lluvia (Sterflinger 2000; Gadd 2007), son capaces de proliferar bajo factores climáticos extremos como altas temperaturas, déficit hídrico y la exposición directa y continua a altas intensidades de radiación UV por lo que son llamados poiquilotróficos (Gadd 2004; Rindi 2007).

En la península de Yucatán, las rocas que soportan a estas comunidades son de origen calcáreo, y puede encontrarse una gran variedad de litotipos desde compactos hasta altamente porosos, y mecánicamente pueden ser desde débiles hasta muy fuertes. Todos estos litotipos son susceptibles al deterioro por diferentes tipos de mecanismos, como el deterioro físico por desprendimiento superficial de la roca por una combinación de factores climáticos y desarrollo filamentoso de organismos, deterioro químico por disolución cárstica, así como por la degradación microbiana (Scheerer et al. 2009; González-Gómez et al. 2015), principalmente por efecto del metabolismo de los hongos. Debido a que las hifas penetran dentro de la roca calcárea (Ortega-Morales et al. 2000; Ortega-Morales et al. 2016), su capacidad para producir y excretar ácidos orgánicos los posiciona como uno de los principales agentes biológicos meteorizantes de los sustratos rocosos (Burford et al. 2003; Gadd 2007). Dicha capacidad se revela incluso a niveles oligotróficos, que son típicos del ambiente lítico (Ortega-Morales et al. 2016).

Los estudios sobre roca calcárea en la península de Yucatán demuestran que las comunidades mixtas de fotótrofos (principalmente cianobacterias) y heterótrofos penetran activamente en los sustratos rocosos, provocando la redeposición de calcio sobre la superficie (Ortega-Morales et al. 1999; Videla et al. 2000; Herrera y Videla

2004; Ortega-Morales et al. 2005). Esto sugiere un efecto de movilización del material hacia el exterior, con el consecuente debilitamiento de la estructura interna del sustrato (Ortega-Morales et al. 2005). En el sitio arqueológico de Edzná, se ha comprobado tanto el deterioro no biológico como el biológico, este último asociado a la presencia del alga *Trentepohlia* sp., que es capaz de formar micropozos en la superficie de la piedra (Gaylarde et al. 2006; Ortega-Morales et al. 2013). Por otro lado, en cupones de roca previamente esterilizados, se determinó que las variaciones en la estructura y sucesión de las comunidades de hongos en un periodo de corto plazo, están influenciadas principalmente por el tiempo de exposición de las rocas, las características ambientales que prevalecen durante el tiempo de exposición y las características intrínsecas de la roca como su porosidad (Gómez-Cornelio et al. 2012).

Las interacciones microorganismo-roca son complejas, ya que además del biodeterioro, los microorganismos inmersos en las biopelículas pueden proteger los sustratos pétreos contra los factores ambientales adversos, funcionando como una biobarrera. Esto se manifiesta debido al grado de conservación que exhiben las superficies colonizadas por biopelículas, las cuales contrastan con el deterioro observado en superficies adyacentes desprovistas de éstas (Viles y Goudie 2004; Miller et al. 2012), lo que sugiere posibles funciones contrastantes de los microorganismos en las superficies líticas.

La colonización de microorganismos sobre la roca es el inicio del proceso conocido como sucesión primaria, que empieza cuando las células microbianas se depositan sobre la superficie. Como ocurre en otros sustratos, el proceso de sucesión primaria sobre la roca podría estar determinado principalmente por la disponibilidad de nutrimentos, por las características del sustrato (bioreceptividad) y las condiciones ambientales. A nivel general, la colonización de los sustratos rocosos se describe a través de grandes grupos de organismos, sin considerar la composición y frecuencia de las especies que integran a las comunidades microbianas (Viles 1995; Warscheid y Braams 2000). En la mayoría de los estudios o modelos de colonización y sucesión vegetal en los ecosistemas terrestres, el componente microbiano pasa generalmente

desapercibido, empezando a describir el proceso de colonización a partir de los líquenes y las briofitas como colonizadores primarios, sin embargo, existe evidencia actual que refuta esta visión, ya que se muestra que los colonizadores primarios son principalmente los microorganismos, ya sean heterótrofos por su alta velocidad de crecimiento o los fotótrofos de crecimiento lento (Chertov et al. 2004; Roeselers et al. 2007).

Se ha documentado que en el proceso de sucesión sobre las rocas, cuando los fotótrofos y quimiolitótrofos se han establecido en una comunidad microbiana e inician la formación de una biopelícula, la materia orgánica que resulta de sus actividades metabólicas y restos celulares, se utiliza como fuente de nutrimentos para continuar promoviendo el proceso de sucesión. De esta manera se consolidan las comunidades heterotróficas, que en conjunto pueden incrementar el deterioro físico y químico del material pétreo, con la acción sinérgica de los factores bióticos y abióticos (Gloer 1995; Ortega-Calvo et al. 1995; Tomaselli et al. 2000). El establecimiento de líquenes y briofitas sobre el sustrato rocoso, ocurre al final de la colonización de la roca, normalmente se establecen bajo condiciones de degradación extrema, cuando la actividad microbiana junto con otros fenómenos de deterioro dan origen al protosuelo (Gadd 2004; Gleeson et al. 2005). Este proceso tiene importantes implicaciones en el funcionamiento del ecosistema terrestre, ya que 60 % de la superficie continental está constituida de zonas áridas donde el componente microbiano está presente en etapas tempranas y en algunos casos, es la única manifestación biológica (Belnap et al. 2004).

En la sucesión fúngica, definida como la ocupación secuencial de un mismo sitio por micelios de diferentes especies o asociaciones de hongos (Frankland 1998), las interacciones entre especies integrantes de la comunidad, son el factor biótico que determina los cambios en la composición, estructura y complejidad de las comunidades fúngicas por mecanismos tales como la facilitación, tolerancia e inhibición (Boddy 2000; Fryar 2002; Falconer et al. 2008). La comunidad es la que controla la sucesión, aunque el patrón y la velocidad del cambio están determinados por los factores espaciales y temporales, el medio ambiente y los recursos nutrimentales, a menudo induciendo o

limitando el grado de desarrollo de los organismos (Odum y Warret 2006; Prosser et al. 2007).

Las interacciones fúngicas se han clasificado de dos formas. Por un lado, se encuentran las nutritivas, en las cuales un organismo obtiene sus nutrimentos directamente del otro, tales como la biotrofia, la necrotrofia y la saprotrofia. Por otro lado, las interacciones no nutritivas, corresponde a aquellas donde la fuente de interrelación biótica se asocia a otro tipo de mecanismo, como el antagonismo o la interacción competitiva. La interacción competitiva se lleva a cabo mediante dos mecanismos, la competencia por explotación y la competencia por interferencia. La primera ocurre cuando el sustrato está dominado por especies de rápida germinación y el crecimiento micelial compite por digerir las fuentes de nutrimentos disponibles en el sustrato, además de tolerar los factores ambientales. Mientras que en la competencia por interferencia, mediante antibiosis o por interferencia hifal, donde se obstaculiza directamente las actividades del otro organismo en un hábitat natural (Shearer 1995; Moore-Landecker 1996).

En la interferencia por antibiosis, un hongo produce metabolitos volátiles o difusibles, lo que origina un ambiente tóxico y restrictivo para otras especies (Wicklow 1992; Shearer 1995; Wheatley 2002; Hynes et al. 2007; Evans et al. 2008; Gaylarde et al. 2015; El-Ariebi et al. 2016). Mientras que en la interferencia hifal, se producen cambios citológicos por contacto y fusión entre hifas de especies diferentes, provocando la lisis de uno de los interactuantes y finalmente la muerte (Boddy 2000). Por lo tanto, los hongos con capacidad de expresar interacciones competitivas (antagonistas) tienen cierta ventaja en la sucesión y en la competencia por recursos o espacio (Moore-Landecker 1996; Carlsoon et al. 2014).

Por las características intrínsecas de las rocas, este tipo de interacción puede ocurrir frecuentemente, y ha sido la más estudiada sobre sustratos como la hojarasca (Shearer y Zare-Maivan 1988; Treton et al. 2004; Duarte et al. 2013), madera (Strongman et al. 1987; Shearer y Zare-Maivan 1988; Yuen et al. 1999; Wald et al. 2004; Carlsoon et al.

2014; Hiscox et al. 2015a; Hiscox et al. 2015b; Hiscox et al. 2016), y el suelo (Sthal y Christensen 1992), sin embargo sobre roca es nulo el conocimiento de las interacciones.

Otras interacciones, como la facilitación no son estudiadas comúnmente o se omiten en los trabajos sobre interacciones (Boege y Carmona 2012). Tal es el caso del comensalismo, donde una especie recibe beneficio en su aptitud biológica mientras que la otra no se beneficia ni se perjudica; o el mutualismo donde ambas especies se benefician y pueden mejorar su aptitud (Tuininga 2005). Cabe mencionar que todos los tipos de interacciones entre especies fúngicas pueden estar influenciadas por la temperatura, humedad, pH, disponibilidad de nutrimentos, entre otros, además del tiempo que transcurre para que ocurra la interacción (Frankland 1998).

En el proceso de estructuración de las comunidades fúngicas, las principales ventajas que pueden tener algunas especies son las implicadas en la colonización del sustrato, como la capacidad para germinar y dominar un espacio, la cual va a depender de la concentración de propágulos en el ambiente (Jones 1994). Sin embargo, en algunos sustratos como la hojarasca, la interacción fúngica puede no ocurrir, ya que los nutrimentos podrían agotarse antes de que las colonias se acerquen lo suficiente como para interactuar y las comunidades puedan estructurarse (Shearer 1995). En sustratos como madera y rocas, la descomposición o el biodeterioro son lentos, por lo que las interacciones entre especies pueden ocurrir y por ende influir en la estructura de las comunidades. Los hongos que invaden, colonizan y dominan un nuevo sustrato disponible son los colonizadores primarios, posteriormente colonizan los secundarios, los cuales arriban en forma de esporas y fragmentos de micelio dando inicio a una interacción con los primarios; si ningún colonizador primario es capaz de excluirlos, sus hifas empiezan a entremezclarse en el sustrato (Jones y Hyde 2002). Por ejemplo, en las rocas se ha documentado un incremento de $9 \times 10^2$ a $7.5 \times 10^5$ unidades formadoras de colonias en tan solo 11 semanas (Gorbushina y Krumbein 2000). Pero no todos los propágulos tienen la capacidad de desarrollarse, y son las interacciones entre especies junto con diversos factores bióticos y abióticos los que podrían

determinar qué especies pueden desarrollarse, integrarse y permanecer en la comunidad a través del tiempo.

El reemplazo en una comunidad fúngica ocurre parcial o totalmente y puede ser originado por el agotamiento de los nutrimentos o debido a cambios físicos y ambientales. Este reemplazo puede afectar solo a una población fúngica, o puede ser secuencial donde se reemplacen dos o más poblaciones (Moore-Landecker 1996; Falconer et al. 2008). La comunidad de hongos que reemplaza probablemente utiliza los recursos orgánicos que la comunidad reemplazada no puede asimilar, o bien pueden ser capaces de tolerar las condiciones extremas prevalecientes en el sustrato, como las que se presentan en la roca.

La predominancia y permanencia de los hongos en la comunidad fúngica sobre cualquier sustrato, incluidas las rocas, se debe a los mecanismos de penetración a través de sus hifas (Chertov et al. 2004), y a su tasa de crecimiento micelial. La tasa de crecimiento de cada especie podría influir en las interacciones, por una parte, existe evidencia de que en las etapas sucesionales medias y tardías, los hongos con tasas de crecimiento lento tienen una fuerte actividad antagonista hacia los competidores de crecimiento rápido como los colonizadores primarios (Wicklow 1992; Lockwood 1992). Mientras que, otros autores afirman que los hongos de crecimiento rápido pueden captar de forma eficiente los recursos, dominando, excluyendo o desplazando a las especies de crecimiento lento en la utilización de los nutrimentos (Yuen et al. 1999; Falconer et al. 2008). Por lo tanto, un estudio detallado de las tasas de crecimiento radial debe tomarse en cuenta para determinar el tipo y la respuesta de la interacción entre las especies de hongos.

La mayoría de las investigaciones sobre interacciones ecológicas entre especies de hongos están enfocadas a solo dos organismos, es decir a las interacciones duales (Strongman et al. 1987; Shearer y Zare-Maivan 1988; Sthal y Christensen 1992; Yuen et al. 1999; Treton et al. 2004; Wald et al. 2004). Gran parte de estos estudios se han realizado bajo condiciones de laboratorio (Heilmann-Clausen y Boddy 2005; Hynes et

al. 2007; Licyayo et al. 2007; Evans et al. 2008; Ferreira et al. 2010), donde es poco probable que los nutrimentos se agoten, debido al uso de medios de cultivo con fuente de carbono disponible y corta duración de los experimentos (Shearer 1995; Yuen et al. 1999). Aunque los resultados obtenidos en laboratorio distan de lo que ocurre en la naturaleza, son una buena aproximación al conocimiento de los tipos de interacción (Boege y Carmona 2012).

Los estudios sobre interacciones de hongos y otros microorganismos realizados en condiciones *in situ* son escasos (Fryar et al. 2001; Fryar et al. 2005) y de ellos, en pocos se relacionan las interacciones con la estructuración de la comunidades fúngicas (Yuen et al. 1999; Jones y Hyde 2002; Hiscox et al. 2015b). Estos estudios son objeto de constantes críticas, debido a las dificultades técnicas en la cuantificación e identificación de las especies inoculadas, así como de aquellas especies que arriban al sustrato en el ambiente natural donde son expuestos (Boddy 2000; Hiscox et al. 2015a). Además, las comunidades de hongos no se presentan en poblaciones definidas, por lo que se han encontrado dificultades metodológicas en la investigación de las interacciones, particularmente sobre los sustratos estudiados, así como problemas en la subsecuente aplicación de modelos e índices (Wardle et al. 1993). Un análisis de las interacciones entre especies de hongos cercano al ideal es la observación y seguimiento del crecimiento hifal en el espacio y tiempo sobre el sustrato en cuestión, para determinar los procesos sucesionales y la estructura de la comunidad. Las técnicas que se utilizan en este tipo de estudio son destructivas, pero un buen diseño experimental y replicas apropiadas podrían aportar patrones generales de la sucesión (Fryar 2002).

En las comunidades fúngicas, la sucesión va a generar cambios en la estructura de las comunidades, y conforme avanzan, la composición y la frecuencia de ocurrencia de las especies en la comunidad es variable (Hiscox et al. 2015a). Idealmente la coexistencia de estas especies en la estructura de la comunidad, implica que cada especie se encuentra bajo condiciones óptimas de supervivencia y reproducción. Sin embargo, la baja ocurrencia de una especie, podría indicar que se encuentra resistiendo a las

condiciones ambientales que se presentan en ese espacio y tiempo, en espera de condiciones idóneas (Duarte et al. 2013). Lo que explicaría las variaciones en el aislamiento y particularmente la dominancia de algunas especies, como se demuestra en un estudio sucesional a corto plazo sobre roca calcárea (Gómez-Cornelio et al. 2012).

Actualmente, los estudios sobre sucesión son un tema clásico en ecología de comunidades que ha presentado debates sustanciales, por lo que es difícil obtener una teoría general a partir de los patrones encontrados en ambientes particulares, ni que decir del ambiente microbiano. Tal es el caso de las comunidades vegetales, donde el número de especies incrementa durante las etapas iniciales e intermedias de la sucesión y conforme las etapas avanzan, el número de individuos por especie y el número de especies disminuyen o desaparecen debido a la exclusión competitiva (Connell y Slatyer 1977). Mientras que, en ambientes con condiciones de estrés, se ha sugerido que ocurre una autosucesión, debido a que las especies de las fases tardías colonizan progresivamente el espacio o sustrato, iniciando con una menor frecuencia e incrementando durante todo el proceso sucesional (Svodoba y Henry 1987), este evento podría ocurrir en la roca con microorganismos.

Se ha sugerido ampliar el conocimiento de las interacciones entre las especies fúngicas y evaluar la relevancia de las interacciones en la estructuración de las comunidades (Shearer 1995); para esto se deben realizar estudios en hábitats completamente diferentes a los que normalmente se han estudiado (hojarasca, madera y suelo), involucrar a más de dos especies de hongos en las interacciones y evaluar las diferentes formas de interacción entre ellas, no solo la antagonista. Aunque no se tiene un consenso en los patrones sucesionales de las comunidades fúngicas y debido a que los hongos son participantes activos en el grupo de colonizadores primarios de cualquier ecosistema, resulta de gran interés conocer la dinámica de la comunidad de hongos en diferentes sustratos, entre ellos la roca calcárea, para que hasta donde sea posible, a través de la experimentación y técnicas adecuadas, se intenten explicar los patrones sucesionales en estos ambientes.

A la fecha, no se tiene conocimiento de estudios sobre interacciones entre especies de hongos que provengan de sustratos líticos. En el único estudio realizado hasta hoy, se investigaron las interacciones del tipo mutualista en co-cultivos entre un hongo y un alga proveniente de roca, donde ambos organismos crecen entremezclados, y después de cierto periodo las hifas crecen sobre las algas (Gorbushina et al. 2005). Entre las posibles implicaciones que las interacciones entre especies de hongos puedan presentar sobre el sustrato rocoso, podrían optimizar el proceso de biomineralización de las rocas para la formación del suelo o en contraparte contrarrestar el impacto de estos en el patrimonio cultural edificado, tal y como ocurre en las interacciones entre especies de la madera, donde la producción de la enzima lacasa es mayor en las interacciones que la reportada para monocultivos (Ma y Ruan 2015). Cabe mencionar que el enfoque de preservación del patrimonio cultural es el más estudiado, con diversos organismos que ahí habitan, entre ellos los hongos en diferentes ambientes y tipos de rocas (Gorbushina et al. 2002; Scheerer et al. 2009; Sterflinger 2010; De la Rosa-García et al. 2011; Gómez-Ortiz et al. 2014; Ortega-Morales et al. 2016).

La importancia de los hongos en los procesos geológicos, principalmente en la pedogénesis, se debe a su capacidad de realizar transformaciones orgánicas e inorgánicas, biometeorizar las rocas y transformar minerales, gracias al polimorfismo y su capacidad para adaptarse a las condiciones ambientales extremas (Sterflinger 2000; Gadd 2007). No obstante, el conocimiento sobre la ecología de los hongos sobre la roca presenta grandes interrogantes, entre ellas el conocimiento de las interacciones entre especies y el papel que estas juegan en los diferentes procesos ecológicos, por lo que en este trabajo la pregunta planteada fue: ¿las interacciones entre las especies de hongos epilíticos dominantes son las que podrían influir en la estructura de la comunidad fúngica inmersa en biopelículas que se desarrollan sobre roca calcárea a diferentes tiempos de exposición? Para responder esta pregunta se plantearon los siguientes objetivos:

a) caracterizar la composición de la comunidad fúngica que se desarrolla en biopelículas sobre sustratos rocosos calcáreos expuestos a diferentes tiempos de colonización,

b) evaluar los tipos de interacciones intra e interespecíficas que presentan los hongos aislados de roca calcárea que son representativos por su abundancia,
c) determinar si estas interacciones podrían tener influencia en la estructura de la comunidad fúngica de las biopelículas en diferentes fases sucesionales.

La colonización microbiana de roca es un proceso biológico complejo, la mayor parte de las investigaciones apuntan a la importancia de las características del sustrato (composición química, porosidad y rugosidad), del clima (tropical *versus* templado) y el microclima, en particular el régimen lumínico, la disponibilidad de agua, la temperatura y la presencia de contaminantes y otros compuestos químicos atmosféricos. Pocos estudios se enfocan en el papel que juegan las interacciones bióticas entre los constituyentes microbianos en la dinámica de colonización de biopelículas epilíticas. La hipótesis de este trabajo de tesis se centró en que las especies dominantes presentarían interacciones antagónicas sobre aquellas especies con menor abundancia y por consiguiente podrían influir en la estructuración de la comunidad. En principio, las biopelículas recientes promoverían un bajo nivel de interacción biótica ya que se propiciaría la colonización de especies primarias resistentes a las condiciones adversas; en la medida en que estas biopelículas maduran y se incrementan las especies, las interacciones bióticas se intensificarían por el arribo de nuevas especies y con ello la partición del recurso que finalmente va a conducir al desplazamiento de especies.

## CAPÍTULO 2.

# Changes in fungal community composition of biofilms on limestone across a chronosequence in Campeche, México

Artículo publicado en la revista Acta Botánica Mexicana (Anexo general 1).

# Cambios en la composición de la comunidad fúngica de biopelículas sobre roca calcárea a través de una cronosecuencia en Campeche, México

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Running Title: Fungal communities composition in biofilms on limestone

ABSTRACT:

Background and Aims:

The colonization of lithic substrates by fungal communities is determined by the properties of the substrate (bioreceptivity) and climatic and microclimatic conditions. However, the effect of the exposure time of the limestone surface to the environment on fungal communities has not been extensively investigated. In this study, we analyze the composition and structure of fungal communities occurring in biofilms on limestone walls of modern edifications constructed at different times in a subtropical environment in Campeche, Mexico.

Methods:

A chronosequence of walls built one, five and 10 years ago was considered. On each wall, three surface areas of 3 × 3 cm of the corresponding biofilm were scraped for subsequent analysis. Fungi were isolated by washing and particle filtration technique and were then inoculated in two contrasting culture media (oligotrophic and copiotrophic). The fungi were identified according to macro and microscopic characteristics.

Key results:

We found 73 genera and 202 species from 844 isolates. Our results showed that fungal communities differed in each biofilm. In the middle-aged biofilm a high number of isolates was found, but both species richness and diversity were low. In contrast, in the old biofilm species richness and diversity were high; Hyphomycete 1, *Myrothecium roridum* and *Pestalotiopsis maculans* were abundant. The dominant species in the middle-aged biofilm were *Curvularia lunata*, *Curvularia pallescens*, *Fusarium oxysporum* and *Fusarium redolens*, and in the young biofilm were *Cladosporium cladosporioides*, *Curvularia clavata*, *Paraconiothyrium* sp. and *Phoma eupyrena*.

Conclusions:

Our results suggest that the composition of the fungal community in each biofilm varies according to time of exposure to the environment. Furthermore, the fungal community

was composed of a pool of uncommon species that might be autochthonous to limestone.

Key words: fungal diversity, succession, dominant species, trophic preference, fungal colonization.

RESUMEN:
Antecedentes y Objetivos:
La colonización de los sustratos líticos por comunidades fúngicas esta determinada por las propiedades del sustrato (bioreceptividad) y las condiciones climáticas y microclimáticas. Sin embargo, los efectos del tiempo de exposición de la superficie de la roca calcárea al ambiente sobre la composición de las comunidades fúngicas no se ha investigado. En este estudio, analizamos la composición y estructura de las comunidades fúngicas inmersas en biopelículas asociadas a roca calcárea, en paredes de edificaciones modernas construidas a diferentes tiempos en un ambiente subtropical en Campeche, México.

Métodos:
Se consideró una cronosecuencia de paredes construidas a uno, cinco y diez años. Sobre cada pared, tres superficies de 3 x 3 cm para cada biopelícula fue raspada. Los hongos se aislaron por la técnica de lavado y filtración de partículas, posteriormente se inocularon en dos medios de cultivo contrastantes (un medio oligotrófico y uno copiotrófico). Los hongos se identificaron de acuerdo a sus características macro y microscópicas.

Resultados clave:
Encontramos 73 géneros y 202 especies de 844 aislados. Los resultados mostraron que las comunidades fúngicas son diferentes en las tres biopelículas. En la biopelícula de desarrollo intermedio encontramos un alto número de aislados, pero tanto la riqueza como la diversidad fueron bajas. En contraste, en la biopelícula avanzada, los valores de riqueza de especies y diversidad fueron altos, y las especies abundantes fueron

Hyphomycete 1, *Myrothecium roridum* y *Pestalotiopsis maculans*. Las especies dominantes en la biopelícula intermedia fueron *Curvularia lunata*, *Curvularia pallescens*, *Fusarium oxysporum* y *Fusarium redolens*, y en la biopelícula joven fueron *Cladosporium cladosporioides*, *Curvularia clavata*, *Paraconiothyrium* sp. y *Phoma eupyrena*.

Conclusiones:
Nuestros resultados sugieren que la composición de la comunidad fúngica en cada biopelícula cambia de acuerdo al tiempo de exposición de la roca calcárea al ambiente. Además, como parte de la composición de la comunidad fúngica, encontramos un conjunto de especies poco comunes que podrían ser autóctonas en la roca calcárea.

Palabras clave: diversidad fúngica, sucesión, especies dominantes, preferencia trófica, colonización fúngica.

# INTRODUCTION

It is well known that rocks, either in natural geological settings or as part of monuments, are common habitats for a wide range of microorganisms (Scheerer et al., 2009; Miller et al., 2012). The colonization of lithic substrates by microbial communities is influenced by the properties of the substrate, such as porosity, surface roughness and mineralogical composition –bioreceptivity– (see review in Miller et al., 2012), in addition to climate and microclimatic conditions (Guillitte, 1995; Ortega-Morales et al., 1999; Gaylarde and Gaylarde, 2005; Barberousse et al., 2006). Furthermore, communities of microorganisms growing on lithic substrates, including fungi, may respond differentially to environmental conditions over time based on their ecophysiological requirements (Scheerer et al., 2009; Mihajlovski et al., 2014).

In the tropics and subtropics, rocks are capable of being colonized by microorganisms due to high levels of relative humidity and particular bioreceptivity of the limestone (Kumar and Kumar, 1999; Gómez-Cornelio et al., 2012). Gaylarde and Gaylarde (2005) found that the macro- and micro-environments of different geographical regions play an important role in the biomass and composition of the microorganism groups that compose biofilms. For example, microbial biomass in Latin America is dominated by cyanobacteria and fungi, while in Europe phototrophs, including algae and cyanobacteria, are the most common organisms. Furthermore, the development of biofilms on rocks represents an important stage in the primary succession of terrestrial ecosystems (Chertov et al., 2004; Gorbushina, 2007). In this process, fungi that form part of biofilms physically and chemically deteriorate rock, and thus actively participate in the formation of protosoil and minerals and also accelerate this process, enabling subsequent colonization of the substrate by mosses, lichens or plants (Gorbushina and Krumbein, 2000; Sterflinger, 2000; Gadd, 2007). Although molecular techniques are commonly used to study communities in the field of environmental microbiology, the traditional techniques of isolation and identification of fungi are of vital importance in order to phenotypically characterize fungi and to determine their role on epilithic substrates (Ruibal et al., 2005; Gleeson et al., 2010).

Fungal epilithic communities have been studied in a wide range of environments and for several lithotypes (Sterflinger and Krumbein, 1997; Sterflinger and Prillinger, 2001; Urzì et al., 2001; Gorbushina et al., 2002; Ruibal et al., 2005; Ruibal et al., 2009; Tang and Lian, 2012). However, most research has not considered the influence of time on the colonization patterns of fungal communities. One exception was the study of Lan et al. (2010), in which fungal communities of young and old biofilms on sandstone were found substantially different. Furthermore, the importance of filamentous fungi as rock colonizers and their ecological role in environments are not well-understood, especially in tropical and subtropical climates. More attention has been placed on the microcolonial fungi, meristematic fungi and yeasts of temperate climates (Sterflinger and Krumbein, 1997; Gorbushina et al., 2002; Chertov et al., 2004; Gorbushina et al., 2005; Ruibal et al., 2005; 2009; Sterflinger et al., 2012). Therefore, in order to expand our current understanding of the fungal community associated with limestone, we studied the culturable subset of fungi in biofilms exposed to similar environmental conditions and substratum properties. A chronosequence was considered by examining the biofilms of three walls constructed one, five and 10 years ago.

## MATERIALS AND METHODS

### *Study area and climate variables*

The coastal city of Campeche, Mexico has a subtropical climate and an altitudinal range of 3-10 m. The studied biofilms were relatively categorized as young, middle-aged and old, corresponding to walls that were constructed with limestone rock fragments 1, 5 and 10 years ago, respectively, according to historical documentation (Fig. 1). The colonization of buildings by fungi may initiate shortly after construction but the formation of biofilm usually takes several years (Barberousse et al., 2006; Gómez-Cornelio et al., 2012; Adamson et al., 2013). Hence, the monthly climate data were obtained from the local meteorological observatory in Campeche, in order to calculate the annual means of climate variables as well as the mean conditions corresponding to the number of years since walls were constructed and exposed to the environment. The considered climatic

variables were: minimum and maximum temperature, minimum, mean and maximum relative humidity and mean rainfall (Table 1).

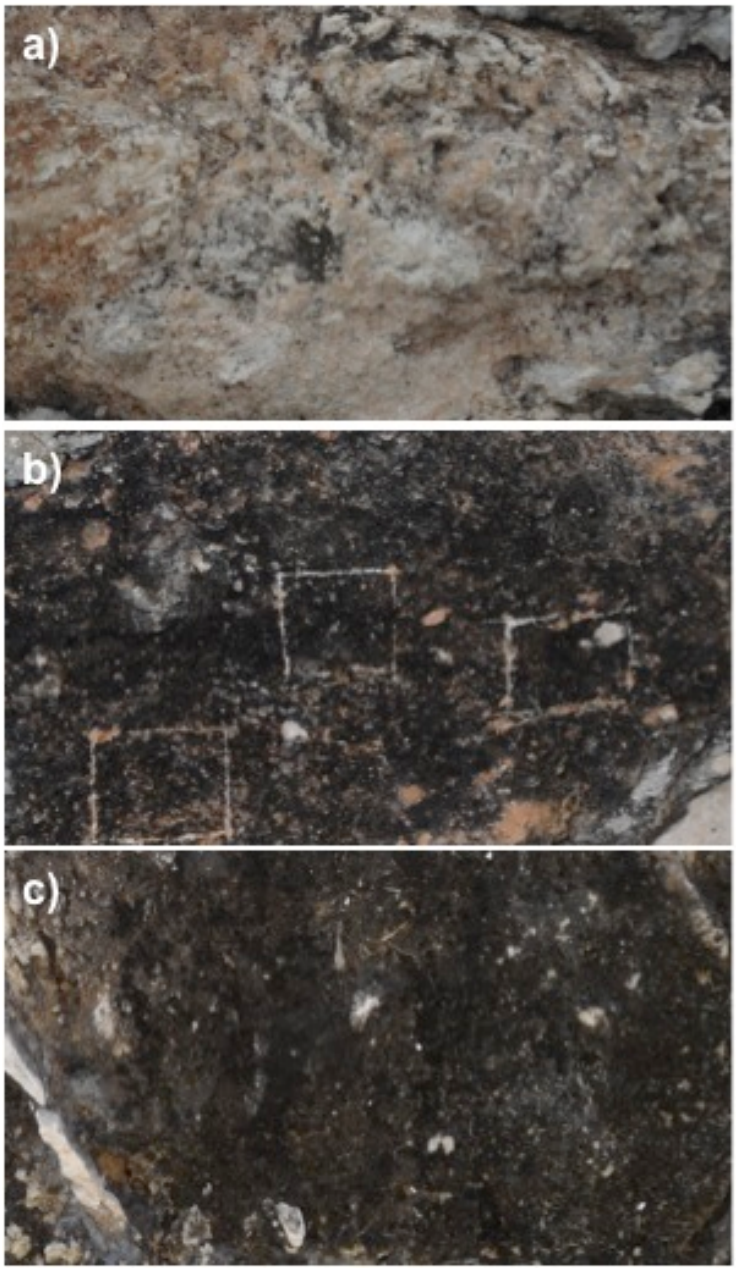


**Figure 1:** Biofilm samples from rock fragments of limestone buildings (time elapsed since construction): a) young biofilm (1 year), b) middle-aged biofilm (5 years) and c) old biofilm (10 years).

**Table 1:** Geographical location, color and climatic parameters of biofilms developed on the surface of sampled limestone walls. (Values are expressed as means ± 1SD).

| | | Young biofilm | Middle-aged biofilm | Old biofilm |
|---|---|---|---|---|
| Geographical location | | 19º49'30.7" N 90º32'51.2" W | 19º49'30.5" N 90º33'16.3" W | 19º49'29.4" N 90º33'14" W |
| Mean temperature (ºC) | Minimum | 16.5 ± 2.5 | 16.4 ± 4.7 | 16.7 ± 2.5 |
| | Maximum | 37.1 ± 4.3 | 37.6 ± 2.6 | 37.2 ± 4.5 |
| Mean relative humidity (%) | Minimum | 43.8 ± 10.7 | 39.9 ± 9.4 | 39.6 ± 9.4 |
| | Mean | 78.6 ± 5.6 | 75 ± 5.8 | 74.5 ± 6.6 |
| | Maximum | 97.9 ± 1.2 | 97.8 ± 1.1 | 97.4 ±1.4 |
| Mean rainfall (mm) | Rainy season | 224.9 ± 11.3 | 192.6 ± 54 | 194.8 ± 29 |
| | Dry season | 20.4 ± 19 | 19.8 ± 17 | 24.4 ± 20 |
| Degree of colonization (visual inspection) | | Not observed | Dark green | Black |

Limestone walls with comparable characteristics of exposure to the surrounding environment were chosen. All walls were composed of rock blocks and had similar substratum properties (or bioreceptivity), vertical surfaces and homogeneous coverage of biofilms. In addition, all walls were oriented towards the north where low solar irradiation and high relative humidity prevail in comparison to facades oriented towards other directions (Adamson et al., 2013; Ortega-Morales et al., 2013). Further criteria for selecting the walls included the absence of surrounding vegetation and low levels of human disturbance; sites with automobile traffic or post-construction remodeling works were avoided. Samples were taken from above the height of one meter in order to avoid confounding factors such as potential microbial colonization due to splashing water. Hence, time elapsed since construction of the walls and establishment of biofilms was the main influential variable considered in the analysis of fungal community structure.

*Biofilm sampling and fungal isolation*

In the dry season of January 2014, we sampled biofilms on the three selected limestone surfaces. Mean maximum and minimum temperatures in January were 34 °C and 11.4 °C, respectively. Mean rainfall was 23 mm, and the mean relative humidity was 83 %. On each wall, we scraped three surface areas of 3 × 3 cm to a maximum depth of 3 mm,

using a sterile scalpel. Scraping were performed by the same person to avoid bias. The biomasses of the biofilms scraped from the wall were placed in sterile Petri dishes and transferred to the laboratory for processing.

Fungi were isolated by washing and filtration of particles technique (Bills et al., 2004). One gram of each scraped biofilm was placed in a washing apparatus with micro-sieves with pores of 250, 125, 100 and 75 µm (MINI-SIEVE INSERT-ASTD from Bel-Art Products, Pequannock, New Jersey), and was consequently washed and filtered for 10 min using bi-distilled water. This technique reduces the isolation of propagules from spores, favoring only the isolation of fungi attached to rock particles (Bills et al., 2004; Arias-Mota and Heredia-Abarca, 2014) that may present active bioweathering or serve a protective function on the surface of the limestone.

Particles trapped on the 75 µm sieve were transferred to sterile filter paper and incubated for 24 h at 27 °C to remove excess water. In order to isolate the greatest number of species, we used two culture media: a copiotrophic medium composed of 2 % malt extract, 2 % agar and 0.2 % $CaCO_3$ (MEAC) and an oligotrophic medium of 0.2 % $CaCO_3$ and 2 % agar (CCOA). Both media were adjusted to pH 7.7 and supplemented with chloramphenicol (200 mg $L^{-1}$) to inhibit the growth of bacteria. Media were prepared with $CaCO_3$, since it is the main component of limestone (Burford et al., 2003). Under a stereomicroscope, 50 particles were transferred to 10 plates with MEAC (5 particles per plate); this procedure was repeated for the CCOA medium. Plates were incubated at 27 °C in darkness. After the fourth day, plates were inspected daily for a period of four weeks. All fungal colonies that emerged from the particles were purified in inclined tubes with MEAC.

*Morphological identification of fungi*

The fungal isolates were identified according to macroscopic characteristics, such as coloration, diameter, texture, pigmentation, margin appearance, zonality and production of exudates in the culture medium, in addition to the morphological characteristics of their reproductive and vegetative structures, including color, conidiogenesis, spore type

and size. Fungal isolates that sporulated were identified using the taxonomic keys of Booth (1971), Ellis (1971; 1976), Sutton (1980), Pitt (2000), Klich (2002), Boerema et al. (2004), Domsch et al. (2007) and Seifert et al. (2011). The identity of the species with more than eight isolates was confirmed by performing genomic DNA extraction and sequencing the ITS region (data not shown).

Fungal colonies that did not sporulate were inoculated into the following culture media: cornmeal agar, oatmeal agar, potato-carrot agar, Czapek dox agar, potato dextrose agar and V8 agar. Plates were then subjected to cyclical periods of light/darkness (12/12 h) to promote sporulation (Bills et al., 2004) and incubated at 27 °C. Every fourth day for up to six weeks, plates were checked for signs of reproductive structures. Isolates that not produced spores were separated into morphotaxa, according to their macroscopic and microscopic morphology in the different culture media. All fungal isolates were conserved in malt extract broth supplemented with glycerol (20 % [vol/vol]) at -80 °C; agar plugs with mycelium were conserved in sterile distilled water at room temperature.

*Data analysis*

The fungal communities of the sampled biofilms were analyzed according to species richness, defined as the number of different fungi species per biofilm, in addition to species abundance as the number of fungal isolates per identified species. The colonization frequency of the particles was determined as number of emerged fungal species (one or two per particle) from particles divided by the number of inoculated particles, multiplied by 100 in order to obtain the percentage of particles with adhered mycelium (Bills et al., 2004). In order to determine the substrates that have been reported for the fungi that were identified at the species level, we used the literature previously employed in the identification of fungi and performed a search in Summon system. Fungal diversity was calculated with the Simpson´s (D') and Shannon´s (H') diversity indices, in addition to the Shannon (J') evenness index, which were performed in the EstimateS 9.1 software (Colwell, 2013). In order to determine the similarity and composition of the fungal species found in the three biofilms, the Jaccard index was calculated, and a Venn diagram was created.

RESULTS

*Analysis of fungal composition and diversity*

In the mycological analysis 844 isolates were recovered, distributed in 73 genera and 202 species (Table 2). The identified species were grouped as follows: 149 Ascomycota, one Basidiomycota and 52 Mycelia sterilia. Hyphomicetous asexual species of Ascomycota (108 species) dominated, while Coelomycetous species represented 21% of the isolates (38 species). The genera with highest number of species (>4) or isolates (>17) in the fungal community were *Aspergillus* P. Micheli ex Haller, *Cladosporium* Link, *Curvularia* Boedijn, *Fusarium* Link, *Myrothecium* Tode, *Nodulisporium* Preuss, *Paraconiothyrium* Verkley *Pestalotiopsis* Steyaert *Phoma* Sacc. and *Trichoderma* Pers. (Table 2).

**Table 2:** Epilithic fungal community in terms of abundance of species isolated from biofilms on limestone at different stages of development and color of their reproductive structures. (M: melanized and H: hyaline).

| Epilithic fungi | Coloration | Young biofilm | Middle-aged biofilm | Old biofilm | Total |
|---|---|---|---|---|---|
| **Ascomycota** | | | | | |
| *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not. | M | | 2 | 1 | 3[a,b] |
| Xylariales sp. 1 | M | | | 1 | 1[b] |
| Xylariales sp. 2 | M | | | 1 | 1[a] |
| Xylariales sp. 3 | M | | | 1 | 1[b] |
| **Coelomycetous asexual species of Ascomycota** | | | | | |
| *Ascochyta carpathica* (Allesch.) Keissl. | M | | | 2 | 2[a] |
| *Clypeopycnis* sp. | M | 2 | 1 | 3 | 6[a,b] |
| *Coleophoma* sp. | M | 7 | | | 7[a,b] |
| *Colletotrichum crassipes* (Speg.) Arx | H | 1 | | | 1[a] |
| *Colletotrichum dematium* (Pers.) Grove | M | 1 | | | 1[a] |
| *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. | H | 1 | | | 1[b] |
| *Coniothyrium* multiporum (V.H. Pawar, P.N. Mathur & Thirum.) Verkley & Gruyter | M | | 1 | | 1[b] |

| | | | | | |
|---|---|---|---|---|---|
| *Cytospora polygoni-sieboldii* Henn. | M | | | 1 | 1[a] |
| *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. | M | | | 7 | 7[a] |
| *Microsphaeropsis arundinis* (S. Ahmad) B. Sutton | M | | 1 | 2 | 3[a,b] |
| *Microsphaeropsis* sp. 1 | M | | | 1 | 1[b] |
| *Microsphaeropsis* sp. 2 | M | | 2 | | 2[a,b] |
| *Neosetophoma samararum* (Desm.) Gruyter, Aveskamp & Verkley | M | | | 1 | 1[a] |
| *Paraconiothyrium* sp. | M | 19 | 3 | | 22[a,b] |
| *Paraphoma chrysanthemicola* (Hollós) Gruyter, Aveskamp & Verkley | M | 1 | | | 1[b] |
| *Paraphoma fimeti* (Brunaud) Gruyter, Aveskamp & Verkley | M | 2 | | 3 | 5[a,b] |
| *Pestalotiopsis maculans* (Corda) Nag Raj | M | 5 | 3 | 10 | 18[a,b] |
| *Peyronellaea aurea* (Gruyter, Noordel. & Boerema) Aveskamp, Gruyter & Verkley | M | 2 | | | 2[a,b] |
| *Peyronellaea gardeniae* (S. Chandra & Tandon) Aveskamp, Gruyter & Verkley | M | 1 | | | 1[b] |
| *Phlyctema lappae* (P. Karst.) Sacc. | M | 1 | | | 1[a] |
| *Phoma adianticola* (E. Young) Boerema | M | 4 | | | 4[a] |
| *Phoma crystallifera* Gruyter, Noordel. & Boerema | M | | | 1 | 1[b] |
| *Phoma eupyrena* Sacc. | M | 23 | 12 | 15 | 50[a,b] |
| *Phoma herbarum* Westend. | M | 4 | 1 | 2 | 7[a,b] |
| *Phoma heteroderae* Sen Y. Chen, D.W. Dicks. & Kimbr. | M | 2 | | | 2[a,b] |
| *Phoma leveillei* Boerema & G.J. Bollen | M | | | 1 | 1[b] |
| *Phoma multirostrata* (P.N. Mathur, S.K. Menon & Thirum.) Dorenb. & Boerema | M | | 1 | 1 | 2[b] |
| *Phoma paspali* P.R. Johnst. | M | 5 | | | 5[a,b] |
| *Phoma pratorum* P.R. Johnst. & Boerema | M | | 1 | | 1[a] |
| *Phoma proteae* Crous | M | | | 1 | 1[b] |
| *Phoma putamina* Speg. | M | 1 | | 2 | 3[a,b] |
| *Phoma* sp. 1 | M | 1 | | | 1[a] |
| *Phoma* sp. 2 | M | 2 | | | 2[a,b] |
| *Phoma tropica* R. Schneid. & Boerema | M | | 2 | | 2[a,b] |
| *Phomopsis putator* (Nitschke) Traverso | M | | | 1 | 1[a] |

| | | | | | |
|---|---|---|---|---|---|
| *Pleurophomopsis lignicola* Petr. | M | 1 | 1 | | 2[a] |
| *Pyrenochaetopsis pratorum* (Berk. & M.A. Curtis) M.B. Ellis | M | 1 | | | 1[b] |
| *Westerdykella minutispora* (P.N. Mathur) Gruyter, Aveskamp & Verkley | M | 4 | 1 | 1 | 6[a,b] |
| **Hyphomicetous asexual species of Ascomycota** | | | | | |
| *Acremoniella velutina* (Fuckel) Sacc. | M | 1 | | | 1[a] |
| *Acremonium brachypenium* W. Gams | H | | 2 | | 2[b] |
| *Acremonium fusidioides* (Nicot) W. Gams | H | | | 1 | 1[b] |
| *Acremonium rutilum* W. Gams | H | | | 2 | 2[b] |
| *Acremonium sordidulum* W. Gams & D. Hawksw. | H | | | 1 | 1[a] |
| *Agaricodochium* sp. | H | 1 | | | 1[a] |
| *Alternaria longipes* (Ellis & Everh.) E.W. Mason | M | | | 1 | 1[a] |
| *Alternaria tenuissima* (Kunze) Wiltshire | M | 1 | 1 | | 2[a,b] |
| *Arxiella terrestris* Papendorf | H | | | 1 | 1[b] |
| *Aspergillus aculeatus* Iizuka | M | | | 2 | 2[a] |
| *Aspergillus alliaceus* Thom & Church | H | | | 1 | 1[a] |
| *Aspergillus awamori* Nakaz. | M | | | 2 | 2[a] |
| *Aspergillus foetidus* Thom & Raper | M | | | 2 | 2[a] |
| *Aspergillus fumigatus* Fresen. | M | | | 2 | 2[a] |
| *Aspergillus japonicus* Saito | M | | | 1 | 1[a] |
| *Aspergillus niger* Tiegh. | M | | | 7 | 7[a] |
| *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud | M | | | 1 | 1[a] |
| *Badarisama* sp. | M | | 1 | | 1[a] |
| *Baudoinia* sp. | M | | | 1 | 1[b] |
| *Calcarisporium* sp. | M | 1 | | | 1[a] |
| *Capnobotryella antalyensis* Sert & Sterfl. | M | | | 1 | 1[a] |
| *Chaetasbolisia falcata* V.A.M. Mill. & Bonar | M | 1 | | | 1[b] |
| *Chromelosporium* sp. | H | | | 1 | 1[a] |
| *Cladosporium cladosporioides* (Fresen.) G.A. de Vries | M | 22 | 10 | 8 | 40[a,b] |
| *Cladosporium oxysporum* Berk. & M.A. Curtis | M | 10 | | 8 | 18[a,b] |
| *Cladosporium sphaerospermum* Penz. | M | 9 | | 2 | 11[a,b] |
| *Cladosporium tenuissimum* Cooke | M | 2 | | 2 | 4[a,b] |
| *Corynespora citricola* M.B. Ellis | M | 1 | | | 1[a] |
| *Corynesporella pinarensis* R.F. Castañeda | M | | | 1 | 1[b] |
| *Curvularia australiensis* (Tsuda & Ueyama) Manamgoda, L. Cai & K.D. | M | 1 | 1 | 5 | 7[a,b] |

| | | | | | |
|---|---|---|---|---|---|
| Hyde | | | | | |
| *Curvularia brachyspora* Boedijn | M | | 2 | | 2[a] |
| *Curvularia clavata* B.L. Jain | M | 18 | 3 | 1 | 22[a,b] |
| *Curvularia fallax* Boedijn | M | 1 | | | 1[b] |
| *Curvularia hawaiiensis* (Bugnic. ex M.B. Ellis) Manamgoda, L. Cai & K.D. Hyde | M | | 1 | | 1[b] |
| *Curvularia lunata* (Wakker) Boedijn | M | 10 | 105 | 26 | 141[a,b] |
| *Curvularia pallescens* Boedijn | M | 6 | 13 | 11 | 30[a,b] |
| *Curvularia* sp. | M | | | 2 | 2[a,b] |
| *Curvularia spicifera* (Bainier) Boedijn | M | 1 | | | 1[a] |
| *Curvularia verruculosa* Tandon & Bilgrami ex. M.B. Ellis | M | | 7 | 1 | 8[a,b] |
| *Echinocatena* sp. | M | | | 1 | 1[b] |
| *Exochalara longissima* (Grove) W. Gams & Hol.-Jech. | M | | | 2 | 2[a] |
| *Friedmanniomyces simplex* Selbmann, de Hoog, Mazzaglia, Friedmann & Onofri | M | 1 | 3 | 2 | 6[b] |
| *Fusarium camptoceras* Wollenw. & Reinking | H | 1 | | | 1[b] |
| *Fusarium equiseti* (Corda) Sacc. | H | 1 | | | 1[a] |
| *Fusarium flocciferum* Corda | H | 1 | 1 | | 2[a,b] |
| *Fusarium incarnatum* (Desm.) Sacc. | H | | | 1 | 1[b] |
| *Fusarium oxysporum* Schltdl. | H | 8 | 40 | 4 | 52[a,b] |
| *Fusarium redolens* Wollenw. | H | 14 | 41 | 9 | 64[a,b] |
| *Fusarium sacchari* (E.J. Butler & Hafiz Khan) W. Gams | H | 1 | | | 1[a] |
| *Fusarium solani* (Mart.) Sacc. | H | 2 | | 2 | 4[a,b] |
| *Fusarium subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas | H | 1 | | | 1[b] |
| *Fusarium tabacinum* (J.F.H. Beyma) W. Gams | H | | 1 | | 1[b] |
| *Fusarium ventricosum* Appel & Wollenw. | H | 1 | | | 1[a] |
| *Gabarnaudia* sp. | H | | | 1 | 1[b] |
| *Geotrichum candidum* Link | H | | | 2 | 2[a,b] |
| *Gilmaniella subornata* Morinaga, Minoura & Udagawa | M | | 1 | | 1[a] |
| *Graphium penicillioides* Corda | M | | | 2 | 2[a,b] |
| Hyphomycete 1 | M | | | 11 | 11[a,b] |
| *Microdochium dimerum* (Penz.) Arx | H | | 1 | 2 | 3[b] |
| *Microdochium nivale* (Fr.) Samuels & I.C. Hallett | H | 2 | | | 2[b] |
| *Monodictys fluctuata* (Tandon & Bilgrami) M.B. Ellis | M | 1 | | | 1[b] |

| | | | | | |
|---|---|---|---|---|---|
| *Monodictys paradoxa* (Corda) S. Hughes | M | 3 | | | 3[a,b] |
| *Myrothecium cinctum* (Corda) Sacc. | M | 3 | | | 3[a] |
| *Myrothecium roridum* Tode | M | | 12 | 16 | 28[a,b] |
| *Myrothecium* sp. 1 | M | 1 | 3 | | 4[a,b] |
| *Myrothecium* sp. 2 | M | | 1 | | 1[a] |
| *Myrothecium* sp. 3 | M | | 1 | | 1[a] |
| *Nalanthamala madreeya* Subram. | H | | 1 | | 1[b] |
| *Nigrospora oryzae* (Berk. & Broome) Petch | M | 3 | 1 | 1 | 5[a] |
| *Nodulisporium acervatum* (Massee) Deighton | M | | | 1 | 1[b] |
| *Nodulisporium ochraceum* Preuss | M | 1 | | | 1[a] |
| *Nodulisporium puniceum* (Cooke & Ellis) Deighton | M | 3 | | 2 | 5[a,b] |
| *Nodulisporium radians* (Berk.) Deighton | M | | | 1 | 1[b] |
| *Nodulisporium* sp. 1 | M | 1 | | | 1[b] |
| *Nodulisporium* sp. 2 | M | | | 1 | 1[a] |
| *Nodulisporium sylviforme* Deighton | M | 1 | | 4 | 5[a,b] |
| *Nodulisporium thelenum* (Sacc.) G. Sm. | M | 1 | | 1 | 2[a,b] |
| *Ochroconis tshawytschae* (Doty & D.W. Slater) Kiril. & Al-Achmed | M | | | 1 | 1[b] |
| *Penicillium citreonigrum* Dierckx | H | | | 1 | 1[a] |
| *Penicillium dierckxii* Biourge | H | | | 1 | 1[b] |
| *Penicillium islandicum* Sopp | H | | | 1 | 1[a] |
| *Penicillium oxalicum* Currie & Thom | H | | | 1 | 1[a] |
| *Periconia igniaria* E.W. Mason & M.B. Ellis | M | 1 | | | 1[a] |
| *Periconiella mucunae* M.B. Ellis | M | | | 1 | 1[a] |
| *Prathoda longissima* (Deighton & MacGarvie) E.G. Simmons | M | 1 | | | 1[b] |
| *Pseudohelicomyces albus* Garnica & E. Valenz. | H | | | 2 | 2[a,b] |
| *Pseudopithomyces chartarum* (Berk. & M.A. Curtis) J.F. Li, Ariyawansa & K.D. Hyde | M | 1 | | | 1[a] |
| *Pseudoramichloridium brasilianum* (Arzanlou & Crous) Cheew. & Crous | M | | 1 | | 1[a] |
| *Ramichloridium apiculatum* (J.H. Mill., Giddens & A.A. Foster) de Hoog | M | | 2 | | 2[b] |
| *Sarocladium kiliense* (Grütz) Summerb. | H | | 1 | | 1[a] |
| *Sarocladium strictum* (W. Gams) Summerb. | H | 1 | | | 1[b] |
| *Scolecobasidium constrictum* E.V. | M | 3 | 4 | 1 | 8[a,b] |

| | | | | | |
|---|---|---|---|---|---|
| Abbott | | | | | |
| *Sepedonium* sp. | H | | | 1 | 1[b] |
| *Stachybotrys microspora* (B.L. Mathur & Sankhla) S.C. Jong & E.E. Davis | M | | | 1 | 1[b] |
| *Stachybotrys nephrospora* Hansf. | M | | | 1 | 1[b] |
| *Stachybotrys renispora* P.C. Misra | M | | | 1 | 1[b] |
| *Tolypocladium* sp. | H | 1 | | | 1[b] |
| *Torula herbarum* (Pers.) Link | M | | | 1 | 1[a] |
| *Trichobotrys* sp. | M | | 1 | | 1[a] |
| *Trichocladium* sp. | M | | 1 | | 1[b] |
| *Trichoderma aggressivum* Samuels & W. Gams | H | 1 | | 1 | 2[a] |
| *Trichoderma harzianum* Rifai | H | | 2 | 1 | 3[a,b] |
| *Trichoderma longibrachiatum* Rifai | H | | | 2 | 2[a] |
| *Trichoderma ovalisporum* Samuels & Schroers | H | | | 1 | 1[a] |
| *Trichoderma strigosum* Bissett | H | | | 1 | 1[a] |
| *Veronaea musae* M.B. Ellis | M | | 1 | | 1[a] |
| *Verruconis verruculosa* (R.Y. Roy, R.S. Dwivedi & R.R. Mishra) Samerp. & de Hoog | M | | 1 | | 1[a] |
| **Basidiomycota** | | | | | |
| *Geotrichopsis* sp. | H | | | 1 | 1[a] |
| **Mycelia sterilia** | | | | | |
| Mycelia sterilia (Morphotaxon 01) | H | 1 | 1 | 1 | 3[b] |
| Mycelia sterilia (Morphotaxon 02) | M | 2 | | | 2[a,b] |
| Mycelia sterilia (Morphotaxon 03) | H | 1 | 1 | | 2[b] |
| Mycelia sterilia (Morphotaxon 04) | M | 1 | | 1 | 2[b] |
| Mycelia sterilia (Morphotaxon 05) | M | 1 | | 1 | 2[a,b] |
| Mycelia sterilia (Morphotaxon 06-10*) | M | 1 | | | 5[a] |
| Mycelia sterilia (Morphotaxon 11-15*) | M | 1 | | | 5[b] |
| Mycelia sterilia (Morphotaxon 16) | H | 1 | | | 1[b] |
| Mycelia sterilia (Morphotaxon 17-18*) | M | | 3 | | 6[a,b] |
| Mycelia sterilia (Morphotaxon 19-20*) | M | | 2 | | 4[a,b] |
| Mycelia sterilia (Morphotaxon 21) | M | | 2 | | 2[b] |
| Mycelia sterilia (Morphotaxon 22-23*) | M | | 1 | | 2[a] |
| Mycelia sterilia (Morphotaxon 24-25*) | H | | 1 | | 2[b] |
| Mycelia sterilia (Morphotaxon 26) | M | | 1 | | 1[b] |
| Mycelia sterilia (Morphotaxon 27) | H | | | 3 | 3[a,b] |
| Mycelia sterilia (Morphotaxon 28) | M | | | 2 | 2[b] |
| Mycelia sterilia (Morphotaxon 29-34*) | M | | | 1 | 6[a] |
| Mycelia sterilia (Morphotaxon 35-39*) | H | | | 1 | 5[a] |
| Mycelia sterilia (Morphotaxon 40-50*) | M | | | 1 | 11[b] |
| Mycelia sterilia (Morphotaxon 51-52*) | H | | | 1 | 2[b] |

[a]Copiotrophic medium (MEAC).

[b]Oligotrophic medium (CCOA).

*Mycelia sterilia with one or more isolated, which are added in the total.

We found the largest number of isolates (322) in the middle-aged biofilm, followed by the old and young biofilms with 268 and 254 isolates, respectively. However, species richness and diversity were higher in the old biofilm and lower in the middle-aged biofilm (Table 3). The Shannon evenness index of the old biofilm generated a value close to 1, and in the middle-aged biofilm, a value of 0.47 (Table 3). The Jaccard's similarity index showed a low degree of similarity among the young, middle-aged and old fungal communities inhabiting the biofilms. The resulting index values were similar for the comparisons of young and middle-aged (0.18), young and old (0.16) and middle-aged and old (0.15) biofilms.

**Table 3:** Abundance, richness, diversity and evenness of the fungal epilithic community colonizing biofilms on limestone at different stages of development.

| State of biofilms | Abundance (number of isolates) | Richness (number of species) | Simpson Diversity index (D') | Shannon Diversity index (H') | Shannon evenness index (J') | Particle colonization (%) |
|---|---|---|---|---|---|---|
| Young | 254 | 83 | 25.8 | 3.7 | 0.67 | 73 |
| Middle-aged | 322 | 59 | 6.8 | 2.7 | 0.47 | 86 |
| Old | 268 | 117 | 37.2 | 4.1 | 0.75 | 75 |

Overall, of the 202 species identified, 26 % were isolated from inoculated particles in both culture media (52 species), while 36 % (73 species) were found exclusively in the oligotrophic medium (CCOA) and 38 % in the copiotrophic medium (MEAC). Additionally, in the analysis of colonization frequency of particles from all three biofilms, we observed a high number of particles with adhered mycelia (Table 3). The middle-aged biofilm showed the highest percentage (80 %) of colonization; however, most mycelium that emerged from these particles belonged to the species *Curvularia lunata* (Wakker) Boedijn, *Fusarium oxysporum* Schltdl. and *Fusarium redolens* Wollenw. (Table 2). The young and old biofilms presented a minor colonization frequency of particles (Table 3).

In the old biofilm, a high number of isolated species (35 %) was specific to only one of the media, and a slightly lower proportion was present in both media (30 %). In young

and middle-aged biofilms, 41 % of species were indistinctly isolated from both media (Table 2). Isolates of *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. and *Nigrospora oryzae* (Berk. & Broome) Petch were obtained in the MEAC medium. A high percentage (>70 %) of species from the genera *Aspergillus*, *Penicillium* Link and *Trichoderma* Pers. were also isolated. Meanwhile, in the CCOA medium the lithic specie *Friedmanniomyces simplex* Selbmann, de Hoog, Mazzaglia, Friedmann & Onofri (6 isolates) was found as well as *Stachybotrys* Corda species and a large number of uncommon species (Table 2).

Of the 124 fungi identified at the species level, many were associated with numerous substrates, based on the literature review to determine with which substrates identified fungal species had been previously associated (Fig. 2). Thirty-one species were identified as cosmopolitan and belonged to the genera *Cladosporium*, *Curvularia*, *Fusarium* and *Penicillium*, including several common species, such as *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud and *Geotrichum candidum* Link (Domsch et al., 2007). Most of the identified species (81) have been reported in soil, mainly species of the genera *Aspergillus*, *Microdochium* Syd., *Monodictys* S. Hughes, *Myrothecium*, *Phoma*, *Scolecobasidium* E.V. Abbott and *Trichoderma*. Many species (78) were also associated with plants, including the genera *Alternaria*, *Colletrotrichum* Corda, *Microsphaeropsis*, *Monodictys*, *Myrothecium*, *Phoma* and *Sarocladium* W. Gams & D. Hawksw. Forty-four of the identified species have been found in litter, corresponding to the genera of *Myrothecium*, *Scolecobasidium* and *Stachybotrys*. Forty species (genus *Monodictys*) have been associated with air, and 39 species with wood (genera *Nodulisporium* and *Phlyctema* Desm). Finally, 40 and 31 species have been reported on rocks and in water, respectively.

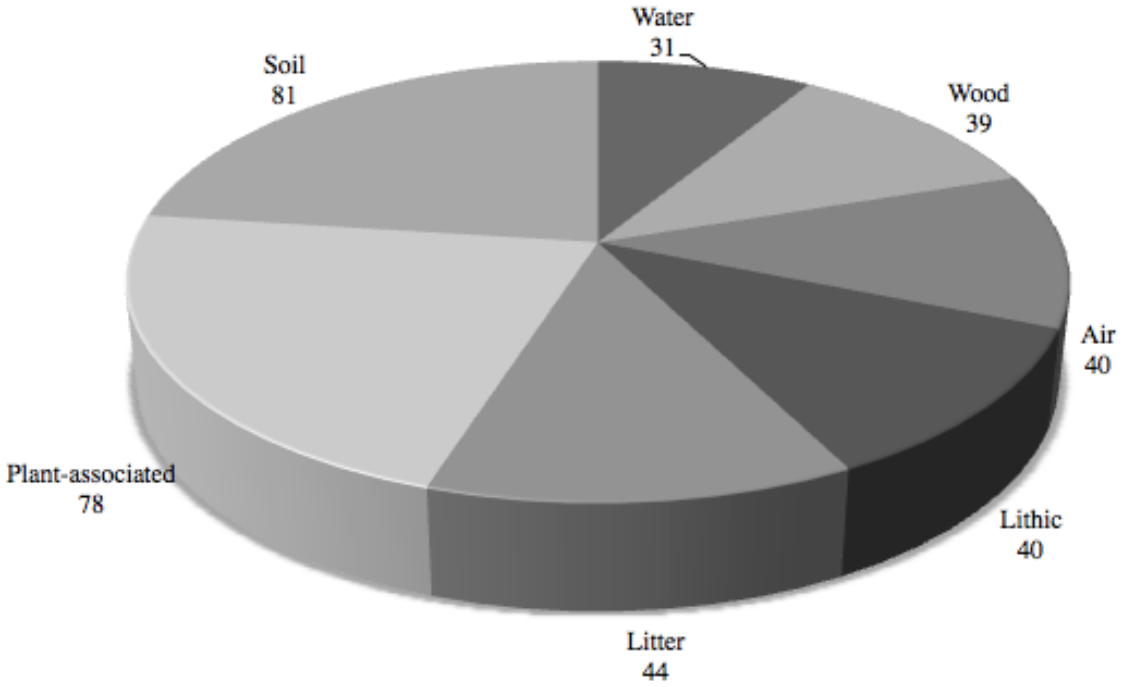


**Figure 2:** Frequency of substrate types reported for the fungal species isolated from biofilms according to the literature.

*Species dominance*

With respect to species abundance, 61 % of all fungal species were isolated only once, and 6 % were isolated more than 10 times. The remaining fungi (33 %) were isolated from 2 to 9 times. The encountered fungal community was mainly dominated by fungi that contain melanin at some or all of their reproductive stages (149 species). Only 53 species (26 %) were found with hyaline structures without pigments (Table 2). In all biofilms, we found that species composition has an approximate ratio of 4:1 of melanized fungi to hyaline species.

In regard to the fungal communities, most co-existing groups of species may be associated with a particular biofilm, characterized by time of exposure of the substrate (limestone) to the environment; this is shown in the Venn diagram (Fig. 3). In the young biofilm, we found 49 exclusive species, including several from the genera *Colletotrichum* and *Coleophoma* Höhn.; the species *Monodyctis paradoxa* (Corda) S. Hughes, *Myrothecium cinctum* (Corda) Sacc., *Phoma adianticola* (E. Young) Boerema and

*Phoma paspali* P.R. Johnst. were also prominent. In the middle-aged biofilm, only 30 exclusive species were found, although these were not frequent (> 2 isolates). The old biofilms had the highest number of exclusive species (82), among these species of the genera *Aspergillus*, *Lasiodiplodia* Ellis & Everh., *Penicillium* and *Stachybotrys*, in addition to Xylariales spp. and Hyphomycete 1.

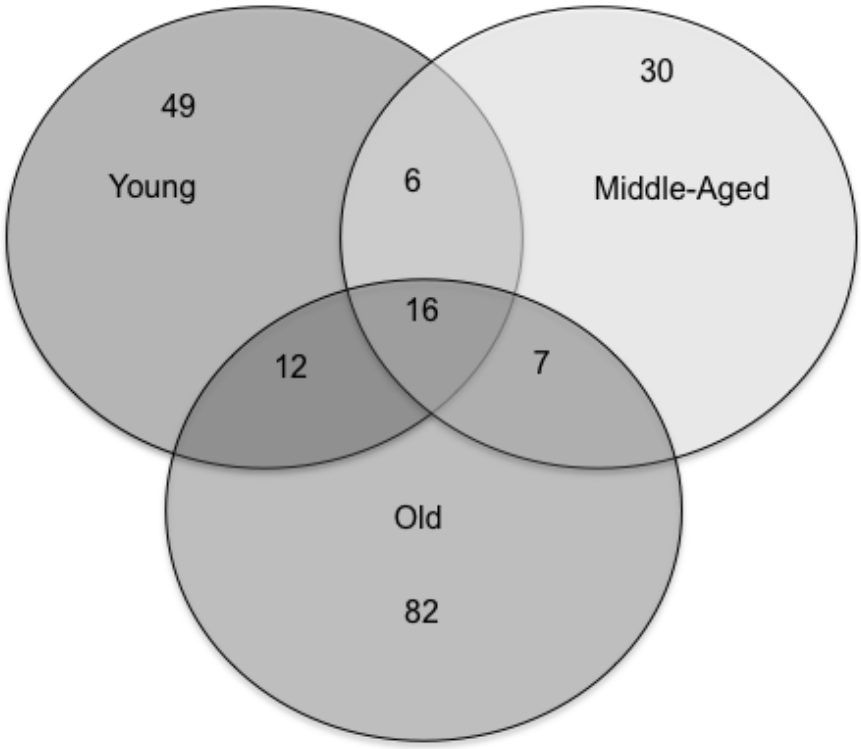


**Figure 3:** Venn diagram indicating number of common and exclusive fungal species to the studied biofilms.

The Venn diagram shows 16 species in the core group (three biofilms) of the fungal community (Fig. 3 and Table 2). Meanwhile, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Curvularia clavata* B.L. Jain, *Nigrospora oryzae*, *Phoma eupyrena* Sacc., *Phoma herbarum* Westend. and *Westerdykella minutispora* (P.N. Mathur) Gruyter, Aveskamp & Verkley were abundant in the young biofilm. In the middle-aged biofilm *Curvularia lunata*, *Curvularia pallescens* Boedijn, *Friedmanniomyces simplex*, *Fusarium oxysporum*, *Fusarium redolens* and *Scolecobasidium constrictum* E.V. Abbott, and in

the old biofilm *Clypeopycnis* sp., *Curvularia australiensis* (Tsuda & Ueyama) Manamgoda, L. Cai & K.D. Hyde and *Pestalotiopsis maculans* (Corda) Nag Raj were frequent (Fig. 4 and Table 2). Twenty-five species were present in at least two biofilms; the young and old biofilms had the highest number of shared species (12). *Paraconiothyrium* sp. was dominant and was isolated 19 times in the young biofilm, although its abundance diminished in the middle-aged biofilm. *Cladosporium oxysporum* Berk. & M.A. Curtis, *Cladosporium sphaerospermum* Penz. and *Nodulisporium puniceum* (Cooke & Ellis) Deighton were found at a higher frequency in the young biofilm in comparison to the old biofilm. *Curvularia verruculosa* Tandon & Bilgrami ex. M.B. Ellis was also more frequent in the middle-aged biofilm than the old biofilm. Meanwhile, the species *Nodulisporium sylviforme* Deighton and *Paraphoma fimeti* (Brunaud) Gruyter, Aveskamp & Verkley were dominant in the old biofilm than in the young biofilm. Finally, *Myrothecium roridum* Tode was more frequent in the old biofilm than in the middle-aged.

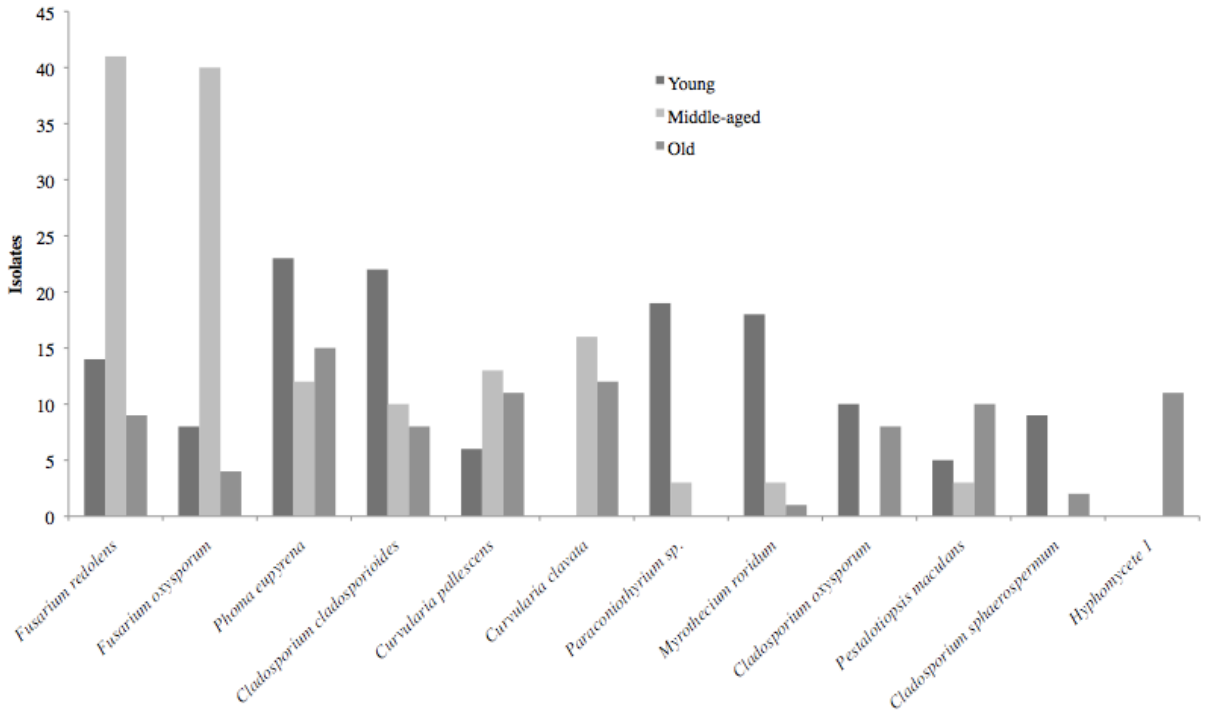


**Figure 4:** Fungal species with the highest number isolates of the three biofilms at different stages of development on limestone.

## DISCUSSION

The fungal communities of biofilms occurring on limestone walls, considering time elapsed since wall construction, presented different degrees of microbial colonization. In the 5- and 10-year-old samples, colonization was evident by the green and black biomass of the biofilms that were visibly observed (Fig. 1), which is consistent with the findings of Adamson et al. (2013). The phototrophs colonizing such substrates are mainly composed of filamentous cyanobacteria and cocoidal bacteria (Scheerer et al., 2009); these have been found on Mayan buildings in the Yucatan peninsula (Ortega-Morales et al., 1999; 2013). The metabolic products of these organisms provide nutritional support for the establishment of heterotrophic communities, including fungi, allowing for their colonization (De la Torre et al., 1991).

The fungal composition was dominated by species of the Ascomycota class, which are common in rock substrates; these Hyphomicetous asexual species are able to colonize rocks during the first year of exposure (Ruibal et al., 2008; Gleeson et al., 2010; Hallman et al., 2011; Gómez-Cornelio et al., 2012). In contrast, Coelomycetous species are known to colonize limestone in Mediterranean regions (Wollenzien et al., 1995). The identification of only one taxon belonging to Basidiomycota may indicate that this group of organisms is not common in biofilms developing on limestone, as confirmed by Tang and Lian (2012), who used culture-independent methods; however, species of this group may exist in sterile form. The genera *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Microsphaeropsis*, *Myrothecium*, *Nodulisporium, Paraconiothyrium* and *Phoma* are common in subtropical environments and in this study (Table 2). For other regions, some of the most common, dominant genera that have been reported on epilithic substrates include *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, *Phoma* and *Trichoderma* (Wollenzien et al., 1995; Kumar and Kumar, 1999; Gorbushina and Krumbein, 2000; Urzì et al., 2001; Gorbushina et al., 2002). All these genera were also identified in this study, but only *Cladosporium*, *Fusarium* and *Phoma* were common and dominant. These differences may be dictated by environmental factors and the bioreceptivity of rocks.

Some isolates showed no reproductive structures (8 %) and were identified as Mycelia sterilia (25 % of morphospecies). However, few isolates belonged to these morphospecies, indicating that they are rare in the community (Table 2). The finding of sterile mycelia commonly occurs in microbiological studies of other substrates (Arias-Mota and Heredia-Abarca, 2014; Rocha et al., 2014; Khirilla et al., 2015), including limestone substrates (Gómez-Cornelio et al., 2012). These fungi may be classified according to morphotype based on their morphological characteristics (Paulus et al., 2003). The production of less complex structures and poorly elaborated reproduction systems may represent an adaptation strategy in order to conserve energy on certain substrates (Ruibal et al., 2005) due to lack of nutrients or water.

In this study, greater species richness was found in the old biofilm. However, in another study on sandstone, the highest number of species was reported for fresh biofilms in comparison to older biofilms (Lan et al., 2010). Meanwhile, in this study the Shannon evenness index showed that the species identified from isolates were almost equitable in the old biofilm. The lowest evenness was obtained in the middle-aged biofilm, probably due to the presence of several dominant species, such as *Curvularia lunata* and *Fusarium redolens* (Table 3). We found higher diversity values in comparison to other studies on fungal communities colonizing distinct substrates of plant litter and endophytes (Collado et al., 2007; Reverchon et al., 2010). This diversity may be due to the establishment and accumulation of propagules on bare rock at a constant and rapid speed. For example, an increase of $9 \times 10^2$ to $7.5 \times 10^5$ colony-forming units was documented in only 11 weeks (Gorbushina and Krumbein, 2000). Under ideal environmental conditions, a high number of fungi could colonize and grow on limestone.

These results, in addition to those of a previous study that investigated the fungal communities on bare limestone (Gómez-Cornelio et al., 2012), suggest that the species richness of limestone in subtropical environments is high in comparison to other rock surfaces, that have been studied in Europe, for example, and in particular in the Mediterranean (De la Torre et al., 1991; Sterflinger and Prillinger, 2001; Gorbushina et

al., 2002; Ruibal et al., 2005; Hallman et al., 2011). Although the intrinsic characteristics of rock, such as its mineral composition, porosity and roughness, have been reported to influence the colonization of microbial communities (Guillitte, 1995; Burford et al., 2003; Lan et al., 2010), the study of Tomaselli et al. (2000), did not find a relationship among existing organisms and the petrographic characteristics of rock. In this study, the high values of species richness and diversity may be attributed to favorable environmental factors in the subtropics (Table 1) and time elapsed since initial colonization (Gaylarde and Gaylarde, 2005; Mihajlovski et al., 2014).

Furthermore, the Jaccard's similarity index showed low values of similarity among fungal communities corresponding to different ages. This is notable in considering that the middle-aged and old biofilms were located less than 1 km from each other. These biofilms also had a similar chromatic aspect; therefore, one might expect a high degree of similarity. Meanwhile, the young biofilm was located at an approximate distance of 10.5 kilometers from the other biofilms and had a visible although incipient colonization of microorganisms. Microclimatic differences present at each site may contribute towards the formation of a unique and particular mycobiota (Mihajlovski et al., 2014). Our results differed from those reported for sandstone, in which no differences in eukaryotic composition were found between fresh and young biofilms (Lan et al., 2010). However, to the contrary, on a serpentine substrate fungal communities were found to have low similarity (Daghino et al., 2012). These findings highlight that the community composition and diversity of fungi are not always determined by substrate bioreceptivity.

Based on the fungi that emerged from the analyzed particles that were scraped from biofilms on limestone, we were able to evaluate the fungal network and find a high proportion of active mycelium. The use of an oligotrophic culture medium (CCOA) allowed for the characterization of the cultivable fungi, which represented a complete, diverse and functional community (Ruibal et al., 2005). Although many of these species require water to grow, such as species of the *Stachybotrys* genus (Jain et al., 2009), the products of the extracellular matrix and the retention of water in rock pores could allow the growth water-demanding species. A large variety of specialized fungi were observed

in this study; these species could represent an autochthonous community specific to limestone, a substrate with limited nutrients. However, due to the intrinsic environmental conditions of the tropics, nutrients may be transported by air and deposited on the rock as dust (Kumar and Kumar, 1999; Gorbushina and Krumbein, 2000; Ruibal et al., 2009), thereby providing conditions for the colonization of specialized fungal species in the production of exopolymers allowing fungi to adhere to the surfaces; some fungi are also capable of biomineralizing limestone. Furthermore, many fungi are able to successfully establish on limestone since they precipitate calcium; these represent the main sink of toxic forms of calcium in the soil or other environments (Sterflinger, 2000). Hence, fungi are essential members of the microbial communities that develop in the biofilms of limestone.

The composition of fungal communities may also be influenced by surrounding substrates (Urzì et al., 2001; Hallman et al., 2011). As previously mentioned, we identified the substrates previously reported in the literature for the 124 fungi that were identified in our study at the species level. A high proportion of fungi may have originated from soil or plants, and to a lesser extent, from decomposing litter and/or wood. Also, these fungal species could come from other rocks or from spores suspended in air or water (Fig. 2). However, the establishment and development of fungi over a period of time may be determined by the interactions of species with their environmental conditions, such as relative humidity and temperature (Gorbushina and Krumbein, 2000; Gorbushina et al., 2005), in addition to the bioreceptivity of the substrate. The variety of fungi reported for other surrounding substrates confirms that a large quantity of propagules may potentially reach and colonize rock surfaces. Therefore, according to our findings, limestone surfaces could act as a reservoir of fungal species and function as a fungal source via the dispersion of species under ideal conditions.

Most species isolated from biofilms occurred only once or twice, and few species showed dominance. The isolation technique used in this study may promote the growth of rare and uncommon species; these results do concur with those found by other

authors that used both taxonomic and molecular identification techniques (Collado et al., 2007; Ruibal et al., 2008; Gómez-Cornelio et al., 2012). Additionally, the abundance of dominant species of the three analyzed biofilms was variable and may be determined by time of exposure, similar to the variations observed in Lan et al. (2010) in the microorganism community of old and fresh biofilms.

The fungal community contained melanin at some or all of their reproductive stages (74 % of the species); this concurs with the reports of other authors, in which the dominant fungi isolated from monuments also contained pigmentation (Sterflinger and Krumbein, 1997; Gorbushina et al., 2002; Lan et al., 2010). Pigmentation in fungi may have different functions or result from the environmental conditions experienced on the rock surface (Scheerer et al., 2009; Hallman et al., 2011). Therefore, melanized communities of fungi have been shown to occur at a high frequency on rock surfaces, although composition may differ from one community to another, as mentioned in the results.

Moreover, fungal propagules are capable of quickly colonizing rock surfaces. Gorbushina and Krumbein (2000) have suggested that fluctuations in environmental conditions, such as those that lead to deficits in nutrients and water on the rock surfaces, promote changes in the diversity of the fungal community. In our results the environmental conditions and the characteristics of the limestone substrate of the biofilms were similar; thus one would expect to find a relationship among the studied fungal communities. However, it may be necessary to study the roles of dominant species during succession of fungal communities. For example, it has been shown that the hyphae of common fungal species biomineralize the surface of the limestone (Burford et al., 2006) and thus lead to subsequent changes. In this study the composition of the fungal community is likely determined by time of exposure to the environment and species interactions, which may then facilitate or inhibit colonization by other species, leading to changes in the composition of the fungal chronosequence associated with limestone (Fryar, 2002). During this process, the functional properties of fungi on the limestone may also be potentially affected.

## CONCLUSIONS

In this study, the fungal communities immersed in biofilms were different at each stage of development, defined by the time of exposure of the limestone substrate to the environment. Although the mineral composition of rock substrates has been found to have a certain degree of influence on the structure of fungal communities, in this study limestone samples with similar characteristics of bioreceptivity, such as rock color, roughness and porosity, were selected. Environmental conditions were also similar across sites in the city of Campeche, Mexico. Therefore, in addition to time of exposure, the differentiation in the community structure and diversity of fungi in this study may be determined by the interactions among the species of each biofilm; this should be confirmed by subsequent studies. A particular species composition was isolated in each biofilm corresponding to a different developmental stage, although a common pool of hyaline and melanized fungi appear to colonize rock with great success and may have specific functions on the rock substrate. In future studies, biotic factors, including interactions among bacterial, fungal and algal species, should be studied in order to determine their influence on the structure of the fungal community.

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## CAPÍTULO 3.

## Influence of interactions among dominant fungi on the structure of the fungal community in limestone

Artículo sometido a Fungal Ecology (Anexo general 2).

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RESEARCH HIGHLIGHTS

- Fungal dominance on limestone is determined by the competitive capacity of the species.
- Dominant fungi determine the colonization and establishment of other species on the limestone.
- Dominant fungi colonize limestone coupons with no addition of external nutrients.
- The interactions between species affect calcium oxalate production on limestone.

ABSTRACT

The assembly of fungal communities on stone materials is determined by multiple factors. However, little is known about the role that fungal interactions play in the colonization and establishment of species. We analyzed both the intra- and interspecific interactions among 11 fungal species dominant on limestone in two contrasting culture media and assessed the effects of the interactions on limestone coupons. The results varied across species and between culture media. In the oligotrophic medium, we found greater frequency of intra- and interspecific inhibitorios effects. On the surface in limestone coupons, fungi exhibited different colonization percentages. *Cladosporium cladosporioides* had a lower colonization alone than when coexisting with other species, whereas *Phoma eupyrena* exhibited the highest colonization percentage and dominance of all species. XRD and SEM analyses revealed that *Pestalotiopsis maculans* and *Paraconiothyrium* sp. produced calcium oxalate crystals during growth alone and in interactions on coupons. Our results demonstrate the role that interactions between dominant fungi on limestone walls play in the composition of fungal community growing on limestone biofilms.

Keywords: epilithic biofilm, community interactions, antagonism, fungal colonization, limestone, calcium oxalate

## INTRODUCTION

Studies on fungal communities that colonize lithic substrates have revealed a diverse assemblage of species (Gorbushina et al., 2002; Burford et al., 2003; Ruibal et al., 2008; Gleeson et al., 2010; Gómez-Cornelio et al., 2012; Ortega-Morales et al., 2016). This diversity is the result of different biotic and abiotic factors, including duration of exposure of the substrate, climatic and microclimatic variables, availability of nutrients, substrate characteristics, and migration of fungal propagules (Guillitte, 1995; Gorbushina and Krumbein, 2000; Sterflinger, 2000; Miller et al., 2006; Gadd, 2007; Gómez-Cornelio et al., 2012; Ortega-Morales et al., 2016). Interactions among species are also believed to play an important role in epilithic diversity (Gorbushina et al., 2005; Gómez-Cornelio et al., 2012).

Most studies on fungal interactions have been conducted with species isolated from wood, soil and aquatic environments, such as leaf litter and submerged wood (Stahl and Christensen, 1992; Yuen et al., 1999; Boddy, 2000; Fryar et al., 2001; Heilmann-Clausen and Boddy, 2005; Tiunov and Scheu, 2005; Duarte et al., 2013; Hiscox et al., 2015; Hiscox et al., 2016). The broad range along competitive and facilitative interactions have the potential to influence community composition and organization at a given time and space (Jones and Hyde, 2002; Fryar et al., 2005; Tiunov and Scheu, 2005; Hiscox et al., 2016).

Responses to such interactions are dependent on nutrient and specific environmental conditions (Stahl and Christensen, 1992; Yuen et al., 1999; Ferreira et al., 2010; Schöneberg et al., 2015). It is therefore desirable to use culture media that simulates both the components of the natural substrate and the environmental conditions under which these interactions developed, in addition to studying the interactions on the natural substrates from which the fungi were isolated. Stone surfaces are usually colonized by hundreds of fungi (Ruibal et al., 2005; 2008; Li et al., 2009; Gómez-Cornelio et al., 2012; Ortega-Morales et al., 2016), but only one out of four species dominates the community, thus accounting for more than 62% of the total number of

isolated species both by taxonomic and molecular methods (De la Torre et al., 1991; Ruibal et al., 2008). In the environments, the coexistence between fungal species implies they have reached a balance in which co-existing species survive and reproduce; therefore, the low occurrence of a given species could be attributed to colonization under non-ideal conditions (HilleRisLambers et al., 2012).

Interspecific interactions on wood allow the optimal degradation of the substrate (Boddy, 2000), and on stone, interactions in the community likely contribute to biomineralization leading to soil formation (Gadd, 2007). To the best of our knowledge, interactions among fungal species on lithic substrates have not been studied. We reasoned that, as is the case with other substrates, the interactions among species that inhabit a given time and space are important in structuring communities, in particular those species that dominate limestone biofilms. Based on studies on wood and stone, we hypothesized that dominant/abundant fungi from lithic biofilms behave antagonistically towards less abundant species. Thus, the objective of this study was to describe and assess the interactions that occur between dominant fungal species isolated from limestone biofilms, both on culture media and on limestone coupons.

## MATERIALS AND METHODS

### *Selection and identification of fungal species*

We selected 11 dominant species, distinguishable by their morphological characteristics (e.g., growth rate, colony morphology, pigmentation, etc.), from a community of 202 fungal species isolated from representative biofilm samples developed on limestone walls at different times points [after 1 (young biofilm), 5 (middle-aged biofilm), and 10 (old biofilm) years (Cámara et al., 2011)] in a subtropical climate in Campeche, Mexico (Table 1).

**Table 1:** Fungal isolates used for interaction studies in culture medium and in rock coupons. Testing criteria included representability in stone environments and dominance in biofilms at different times of colonization. Y: young biofilm, M: Middle-aged biofilm, O: Old biofilm, NR: Not reported.

| Species | GenBank accession | Number of isolates recovered by biofilm | | | Lithotypes reported |
|---|---|---|---|---|---|
| | | Y | M | O | |
| *Cladosporium cladosporioides* | KX610321 | 22 | 10 | 8 | Marble (2, 3, 4), Sandstone (1, 4, 7), Granite (4), Limestone (3, 8), Andesite (4, 5); Mortars (6) |
| *Curvularia clavata* | KX610320 | 18 | 3 | 1 | Limestone (8) |
| *Curvularia lunata* | KX610322 | 10 | 105 | 26 | Marble (4), Sandstone (4), Andesite (4); Limestone (8) |
| *Fusarium oxysporum* | KX610330 | 8 | 40 | 4 | Sandstone (7); Limestone (8) |
| *Fusarium redolens* | KX610323 | 14 | 41 | 9 | Limestone (8) |
| Hyphomycete 1 | KX610326 | - | - | 11 | NR |
| *Myrothecium roridum* | KX610325 | | 12 | 16 | NR |
| *Paraconiothyrium* sp. | KX610324 | 19 | 3 | - | NR |
| *Pestalotiopsis maculans* | KX610327 | 5 | 3 | 10 | Mortars (6), Limestone (9) |
| *Phoma eupyrena* | KX610328 | 23 | 12 | 15 | Sandstone (1), Marble (2) |
| *Scolecobasidium constrictum* | KX610329 | 3 | 4 | 1 | Limestone (8) |

[1]De la Torre et al., 1991; [2]Gorbushina and Krumbein, 2000; [3]Gorbushina et al., 2002; [4]Burford et al., 2003; [5]Miller et al., 2008; [6]Giannantonio et al., 2009; [7]Jain et al., 2009; [8]Gómez-Cornelio et al., 2012; [9]Ortega-Morales et al., 2016

The fungi were identified by their micro- and macroscopic characteristics (Nag-Raj, 1993; Domsch et al., 2007; Ariyawansa et al., 2014) and by sequencing of their internal transcribed spacer (ITS) regions. We performed DNA extraction following the protocol described by Nicholson et al. (2001). The ITS region was amplified by polymerase chain reaction (PCR) in a 20-µl reaction volume containing 10X PCR buffer without $MgCl_2$, 10 mM dNTPs, 25 mM $MgCl_2$, 1 *U* of Taq DNA polymerase, 2% DMSO, 2 ng/µl of DNA as template, and 5 µM of primers ITS-F KYO2 and ITS-R 4GC (Toju et al., 2012). The amplification was performed in a BIO-RAD T100 (Hercules, CA, USA) thermocycler (3 min at 94ºC, 36 cycles [45 s at 94ºC, 45 s at 53ºC, 1 min at 72ºC], and 7 min at 72ºC). Subsequently, we purified PCR products with the GeneAll Expin Gel Gp kit (GeneAll Biotechnology, Seoul, South Korea).

We cloned the purified PCR products following the CloneJET™ PCR Cloning Kit protocol (Fermentas, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Sanger sequencing was conducted at LANGEBIO (CINVESTAV, Mexico). The data derived

from the automatic sequencing analysis were analyzed with Sequence Scanner v1.0 (Applied Biosystems, Foster City, CA, USA). The sequences were deposited in GenBank under Accession Numbers **KX610320-KX610330** (Table 1).

*Culture media and assessment of radial growth rate*

Malt extract agar is the most commonly used medium for the isolation of fungi from lithic substrates (Gorbushina et al., 2002; Gleeson et al., 2010). However, the rocky substrate generally has oligotrophic conditions (Sterflinger, 2000; Ruibal et al., 2005; Gadd, 2007). Therefore, assessments of the growth rate and fungal interactions were carried out in two different media, a copiotrophic medium, MEAC (1 g of malt extract, 0.2 g of $CaCO_3$, and 20 g of agar per liter), and an oligotrophic medium, CACO (0.2 g of $CaCO_3$ and 20 g of agar per liter), as limestone is mainly composed of calcium carbonate ($CaCO_3$). The pH of the media was adjusted to 7.7, similar to pH in limestone.

We assessed the growth rate of the 11 species in quintuplicate by inoculating a 5-mm disk with each species and placing it in the center of a 90 mm Petri dish in both culture media. Fungal development and macroscopic morphology were inspected daily. Colony growth measurement were stopped either when the edges of the Petri dish were reached by the mycelium or, in the case of slow-growing fungi, 17 days after inoculation. The fungal growth rate was classified as slow (0-1 mm $d^{-1}$), moderate (1-3 mm $d^{-1}$), or fast (3-6 mm $d^{-1}$) (Asthana and Shearer, 1990). The radial growth rate determination and fungal interactions assays were incubated at 28ºC and 81% relative humidity, conditions that reflect the annual means for the last 10 years in the region from which the fungi were isolated (data provided by the Comisión Nacional del Agua in January 2014).

*Paired interactions on culture media*

Fungi were first grown in MEAC. We performed dual interactions (inter- and intraspecific) between the 11 species by testing all possible combinations and using both culture media (CACO and MEAC) to observe the variations in their growth. Disc of 5-mm diameter of an actively growing colony of each fungi were inoculated for each paired combination of species, these discs were placed 35 mm apart on the same 90-

mm Petri dish. To avoid spore dispersal over culture media by fungi with abundant sporulation, we placed the discs in a 20% glycerol solution for 5 minutes. Each assay was performed in quintuplicate. As a control, we inoculated each single species in both culture media with the corresponding replica. Moderate- and slow-growing fungi were inoculated one and two weeks in advance to fast-growing fungi, respectively, and fungi with similar growth rates were inoculated simultaneously (Yuen et al., 1999).

We analyzed the results of the assays after 21 days or when one of the species reached the edge of the Petri dish. For all interactions, we measured the rate of radial growth of the species on each medium by drawing a line from the center of the inoculation discs towards the challenge strains. We then compared the growth of each strain with the growth of its respective control. In addition, we prepared slides of the area of interaction between fungal species. We assessed the antagonism index (AI), defined as the ability of a species to compete with and dominate over a competitor, by assigning a category to each reaction evidenced by the interaction and then adding the values of each category listed in Table S1:

$AI = A(n \times 1) + B_1(n \times 1) + B_2(n \times 1) + C(n \times 2) + D(n \times 3) + E_1(n \times 4) + E_2(n \times 4)$; where n= number of times that a fungus corresponds to a given category.

The overall ability of a species to inhibit the radial growth of its competitors was determined by summing the percent inhibition of all the species with which it was paired, and with itself with respect to the control (Yuen et al., 1999), as shown below:

% Inhibition= (growth of the opposing species in the control dish (I1) – growth of the species in the presence of the challenge species (I2)) x 100/growth in the control dish (I1).

The overall ability of each species to resist inhibition was determined by summing the percent inhibition of growth of a particular species by each species with which it was paired, where lower values indicate greater resistance to the presence of other species (Asthana and Shearer, 1990). The percent resistance was determined as follows:

% Resistance= (growth of the fungal species in the control dish (R1) – growth of the same species when interacting with the challenge species (R2)) x 100/growth in the control dish (R1).

*Dual interactions on limestone coupons*

The assay was performed with seven of the 11 species used in the previous section to differentiate the morphological growth on limestone coupons. The species with the largest number of isolates from the genera *Curvularia* and *Fusarium* were selected. Inoculation was performed with spores. Thus, Hyphomycete 1 and *Scolecobasidium constrictum* were excluded due to their weak sporulation. The limestone used as a substrate was a hard rock of low porosity (~5%) with red shades due to goethite traces found in soil 0.3 to 2.5 meters deep. The limestone was characterized by a high occurrence of pellets and the presence of foraminifera, undetermined bivalves, and echinoderm remains embedded in a sparitic matrix (Gónzalez-Gómez et al., 2015). We cut 2 x 1 x 0.5 cm coupons of this lithotype and sterilized them three times at 121ºC for 60 min.

We then prepared a spore suspension in a saline solution (0.85%) using fungi previously inoculated in MEAC. To minimize the bias caused by density-dependent effects, the concentration was adjusted to $1.2 \times 10^5$ conidia $mL^{-1}$ per species before inoculation of control coupons. In the case of interactions between two species, a $6 \times 10^4$ conidia $mL^{-1}$ suspension was prepared for each fungus, such that, the final inoculum concentration per coupon was of $1.2 \times 10^5$ conidia $mL^{-1}$. Fungi were inoculated on coupon surfaces in all possible combinations, and each combination was tested in quadruplicate. We also prepared a fungi-free control sample. After inoculation, coupons were placed on supports and slides in 10 x 10 cm square Petri dishes (Fig. 1A), which were incubated for 4 months and inspected every 15 days.

After 4 months, we photographed the coupons, examined surface colonization, and analyzed fungal morphology using a stereomicroscope (Carl Zeiss, Jena, Germany) (Fig. 1B). We applied a mycelium re-isolation technique by transfer to Petri dishes with

culture medium (Holmer and Stenlid, 1997; Hiscox et al., 2015). We then sectioned the surface of each coupon into 50 sections of approximately 20-mm per section, covering the entire surface (Figs. 1C and 1D). We picked the surface of each coupon with a sterile dissecting needle and transferred each section to Petri dishes containing CACO medium. Dishes were incubated at 28ºC and were inspected daily for 30 days. We identified the colonies that emerged from inoculation points by comparing them to pure cultures of known identity (Carlsson et al., 2014). The percent colonization and occupied territory for each species was calculated for the control and interactions.

*SEM and XRD analysis*

We used x-ray diffraction (XRD) to observe the production of biominerals such as calcium oxalate crystals produced by the fungal species interacting on the surface of limestone coupons, and scanning electron microscope (SEM) to observe fungal adhesion, colonization, and distribution on stone coupons. XRD was performed with a Bruker Advance D8 diffractometer (Karlsrushe, German) at room temperature with Cu-Kalpha radiation, at θ–θ configuration, and a graphite secondary beam monochromator. X-ray diffraction patterns were recorded between 10 – 50° (2θ), with a 2θ step of 0.02 for 1.0 s per point.

Morphological characterization of the coupon surfaces was performed after 4 month of incubation with single and mixed species using an environmental SEM (Philips XL30, Eindhoven, The Netherlands). In addition, we deposited fragments of coupons on standard carbon tape for observation, and the images were taken under low-vacuum conditions (secondary electrons). We determined the structure of the crystals using field emission scanning electron microscopy (FESEM, Model JSM-7600F, Jeol Ltd., Tokyo, Japan).

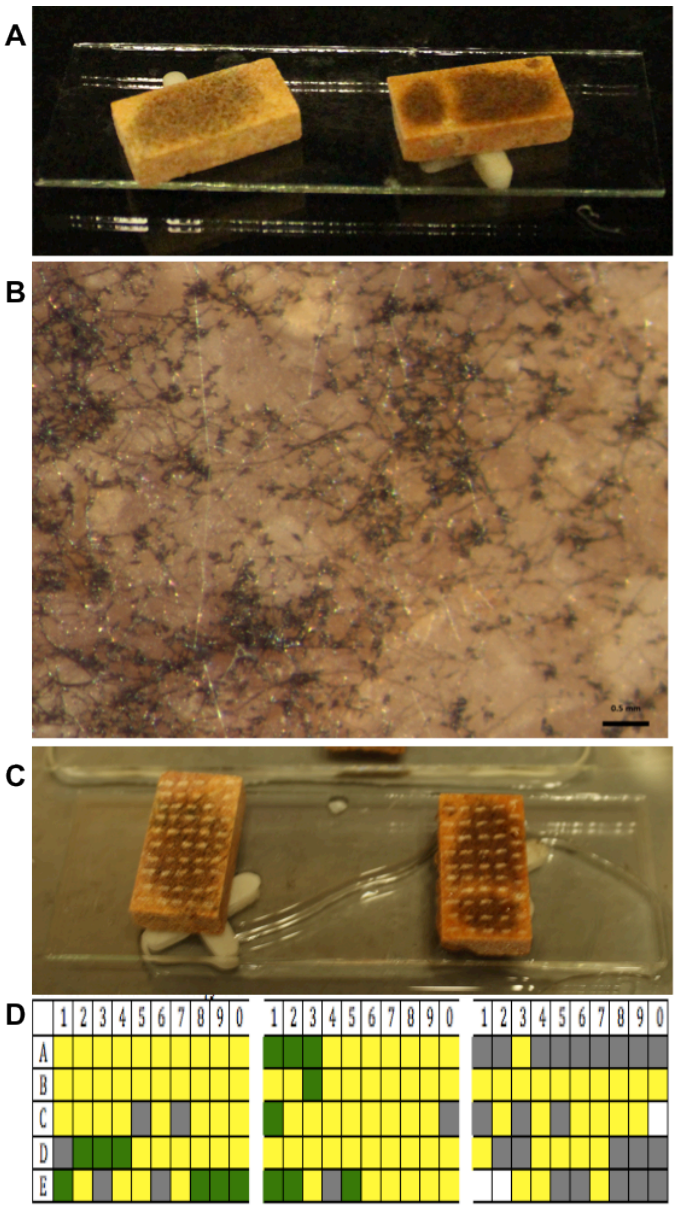


**Figure 1:** Interaction between *Curvularia lunata – Phoma eupyrena*. A) coupons 4 months after inoculation, B) microphotography of coupon, C) surface after sampling, D) area colonized by *C. lunata* (green), *P. eupyrena* (grey), both species (yellow) and not growth (white) in limestone coupons for triplicates, recovered in medium CACO.

*Statistical analysis*

The quantitative analyses of the responses of the interactions in each culture medium, between culture media and on limestone coupons, were subjected to analysis of variance (ANOVA) with a classification criterion followed by a Tukey's multiple

comparisons test to determine significant differences between treatment means (Schöneberg et al., 2015). We verified the homogeneity of variances using Levene's test and used the Shapiro-Wilk test to assess normality. Statistical analyses were performed with MiniTab 16.

## RESULTS

### *Dual interactions in Petri dishes*

In interaction assays, the growth of all fungi tested was influenced by the presence of their neighbor in the cultures. All species exhibited a full spectrum of interaction types, depending both on the species with which they interacted (Fig. 2 and Table S2) and on the culture medium (Table 2 and Table S3). Regarding intraspecific interactions in CACO medium, 91% of fungi displayed competitive interactions of categories C and D (Table 2 and S2), where the radial growth of both inocula was stopped just before coming into contact, or when there was mutual inhibition at a distance. An exception to this occurred with Hyphomycete 1, which involved hyphal intermingling. In fact, mutual intermingling was observed for four species in MEAC medium, whereas the other seven species displayed competitive interactions (Table S2, Fig. 2M and 2N).

A total of 220 interspecific interactions were recorded (110 for each culture medium), in which we observed different reactions (Table S1). The most frequent interaction observed was of two species growing radially until almost coming into contact with each other, and growth then stopped, both in MEAC (41%) and CACO (34%) media (Fig. 2L, 2N, 2R, 2T and Table S2). In 9% of the interactions in CACO, we observed hyphal intermingling (category A), especially for *Cladosporium cladosporioides* with its neighbors (Fig. 2A, Table 2) and 4% in MEAC (*Paraconiothyrium* sp. with *Pestalotiopsis maculans* and *Phoma eupyrena*). In 21% of the interactions in MEAC, one species overgrew the other ($B_1$ and $E_1$), and 32% of the species grew up to and around the challenge species ($B_2$ and $E_2$). Mutual inhibition at a distance was observed in 34% of the cases when few nutrients were available in the culture medium (CACO) (Fig. 2M, 2O and 2Q). However, in MEAC, this was only observed in 2.5% of cases (Table 2).

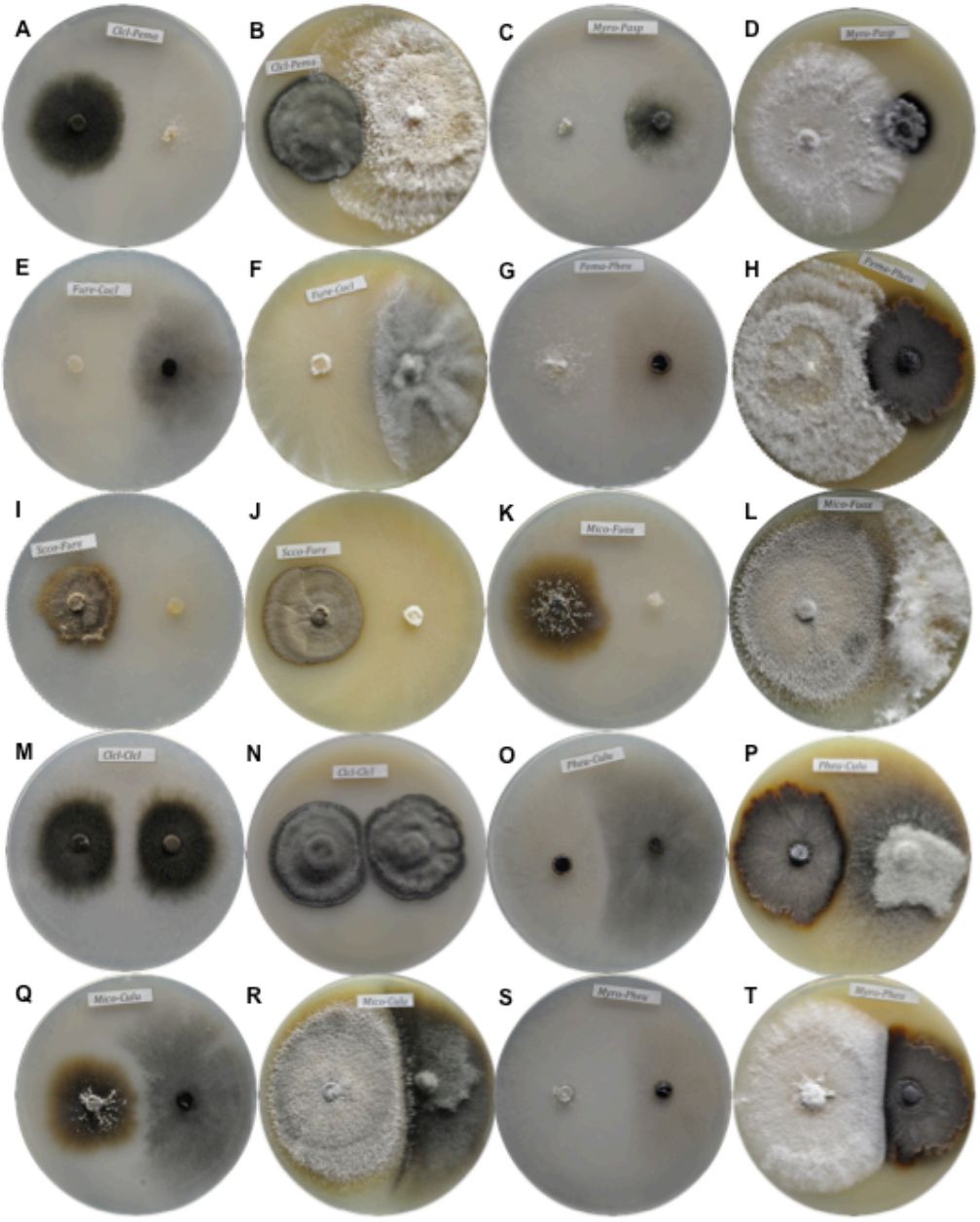


**Figure 2:** Interaction between paired fungi isolates of biofilms in limestone. Columns 1 and 3: CACO medium, columns 2 and 4: MEAC medium. A and B) *C*. *cladosporioides* – *P*. *maculans*, C and D) *M*. *roridum* – Hyphomycete 1, E and F) *F*. *redolens* – *C*. *clavata*, G and H) *P*. *maculans* – *P*. *eupyrena*, I and J) *S*. *constrictum* – *F*. *redolens*, K and L) *Paraconiotrhyrium* sp. – *F*. *oxysporum*, M and N) *C*. *cladosporioides* – *C*. *cladosporioides*, O and P) *P*. *eupyrena* – *C*. *lunata*, Q and R) *Paraconiothyrium* sp. – *C*. *lunata*, S and T) *M*. *roridum* – *P*. *eupyrena*.

In general, these reactions, which resulted from interactions, were reflected in the antagonism index (Table 2), where a low antagonism index was recorded for the slow-growing species *C*. *cladosporioides*, Hyphomycete 1 and *S*. *constrictum* in both culture media. *Fusarium redolens* was the only species that had a high antagonism index, hence presenting dominant reactions mostly of categories $E_1$ and $E_2$ in cultures (Table 3 and S1). For the remaining species, the antagonism index varied between culture media, such as for *P*. *maculans*, *P*. *eupyrena* and *M*. *roridum* (Table 2).

**Table 2:** Outcome in the interaction, index of antagonism, sum of percent of the inhibition and resistance ability for each fungal species paired with the other ten species and itself on copiotrophic medium (MEAC) and ologrotrophic medium (CACO). Categories in Table S1 of supplementry material.

| Species | Medium | Categories* | | | | | | | Index of antagonism | Inhibition (Σ % inhibition) | Resistance (Σ % inhibition) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | $B_1$ | $B_2$ | C | D | $E_1$ | $E_2$ | | | |
| *Cladosporium cladosporioides* | MEAC | 1 | 4 | 3 | 3 | - | - | - | 19 | 177.9 | 105.7 |
| | CACO | 4 | - | 1 | 2 | 4 | - | - | 45 | 179.5 | 188.6 |
| *Curvularia clavata* | MEAC | 1 | 1 | - | 5 | - | - | 4 | 85 | 370.7 | 603.8 |
| | CACO | - | - | 2 | 2 | 7 | - | - | 73 | 339.9 | 636.8 |
| *Curvularia lunata* | MEAC | - | - | - | 8 | 1 | - | 2 | 73 | 354.9 | 620.0 |
| | CACO | - | - | - | 3 | 8 | - | - | 84 | 358.3 | 592.6 |
| *Fusarium oxysporum* | MEAC | 1 | - | 1 | 7 | - | 2 | - | 61 | 322.3 | 429.6 |
| | CACO | 1 | - | - | 6 | 1 | - | 3 | 81 | 417.2 | 447.8 |
| *Fusarium redolens* | MEAC | - | - | - | 2 | - | 4 | 5 | 152 | 480.9 | 333.4 |
| | CACO | - | - | - | - | 4 | 1 | 6 | 148 | 422.6 | 507.4 |
| Hyphomycete 1 | MEAC | 1 | 2 | 4 | 4 | - | - | - | 22 | 151.0 | 72.4 |
| | CACO | 2 | 1 | 4 | 2 | 2 | - | - | 31 | 221.0 | 2.9 |
| *Myrothecium roridum* | MEAC | - | - | 1 | 5 | - | 4 | 1 | 101 | 542.1 | 247.3 |
| | CACO | 2 | - | 1 | 5 | - | 2 | 1 | 69 | 485.2 | 221.9 |
| *Paraconiothyrium* sp. | MEAC | 1 | - | - | 7 | - | - | 3 | 76 | 557.6 | 378.7 |
| | CACO | - | - | 2 | 4 | 5 | - | - | 63 | 363.1 | 186.7 |
| *Pestalotiopsis maculans* | MEAC | 2 | 2 | - | 4 | - | 2 | 1 | 66 | 347.6 | 435.8 |
| | CACO | 1 | 2 | - | 7 | - | - | 1 | 46 | 341.9 | 463.1 |
| *Phoma eupyrena* | MEAC | 1 | 1 | 3 | 3 | 2 | - | 1 | 50 | 206.3 | 362.2 |
| | CACO | - | - | - | 5 | 5 | - | 1 | 81 | 297.4 | 421.0 |
| *Scolecobasidium constrictum* | MEAC | - | 1 | 6 | 3 | 1 | - | - | 28 | 234.9 | 161.3 |
| | CACO | 1 | - | 2 | 4 | 4 | - | - | 54 | 260.8 | 27.6 |

* The values for each category indicate the sum of fungal species with which they interacted and presented that category.

With respect to inhibition ability, *M*. *roridum* and *F*. *redolens* inhibited the growth of other species in both culture media relative to controls (Table 2). Although *Paraconiothyrium* sp. exhibited the highest percent inhibition in MEAC, the inhibition in CACO was

moderate. *Fusarium oxysporum* showed a high percent inhibition in CACO but intermediate inhibition in MEAC. In both culture media, the species *Curvularia lunata*, *C*. *clavata*, and *P*. *maculans* present moderate inhibition, while *C*. *cladosporioides*, Hyphomycete 1, *P*. *eupyrena*, and *S*. *constrictum* exhibited the lowest ability to inhibit the growth of other species (Table 2).

*Cladosporium cladosporioides*, Hyphomycete 1, and *S*. *constrictum* displayed a high ability to resist mycelial growth inhibition in both culture media, whereas *M*. *roridum* displayed intermediate resistance. *Paraconiothyrium* sp. displayed high resistance when there were few nutrients (CACO) than when it interacted in the copiotrophic medium (MEAC). *F*. *redolens* displayed high resistance in MEAC (333.4 %) and low resistance when it interacted in CACO (507.4 %). *C*. *lunata*, *C*. *clavata*, and *P*. *maculans* displayed the lowest percent resistance, whereas *F*. *oxysporum* and *P*. *eupyrena* displayed intermediate resistance in both culture media (Table 2).

In the oligotrophic medium, the growth of Hyphomycete 1 increased significantly ($P<0.05$) in the presence of *C*. *cladosporioides* and *S*. *constrictum* compared to when it was grown alone (Table S3). In most interactions, a decrease in radial growth was observed in the presence of neighbors, with significant differences relative to the control. The species that did not exhibit significant differences in colony growth in the presence of at least five challenge species were *C*. *cladosporioides* and Hyphomycete 1 in MEAC; and *S*. *constrictum*, Hyphomycete 1, and *Paraconiothyrium* sp. in CACO. Furthermore, *M*. *roridum* and *Paraconiothyrium* sp. caused a decrease in the radial growth rate of all challenge species and of each other ($P<0.05$) in both culture media (Table S3).

*Fungal interactions on limestone coupons*

The colonization surface was heterogeneous, both for coupons inoculated with one species (control) and for those inoculated with two species (Tables 3 and 4). When species were grown alone, colonization was high (greater than 70%), with the exception of *C*. *cladosporioides* (8.7% colonization). Significant differences were observed

between *C*. *lunata*, *P*. *maculans*, and *P*. *eupyrena*, with respect to *F*. *redolens* and *C*. *cladosporioides* (Table 3).

**Table 3:** Percentage of surface colonized for fungi on coupons limestone (control) after 4 months of exposure.

| Species | Colonization % |
|---|---|
| *Cladosporium cladosporioides* | 8.7 ± 2.3[c] |
| *Curvularia lunata* | 99.3 ± 1.2[a] |
| *Fusarium redolens* | 74.7 ± 15[b] |
| *Myrotecium roridum* | 88.0 ± 7.2[ab] |
| *Paraconiothyrium* sp. | 87.3 ± 1.2[ab] |
| *Pestalotiopsis maculans* | 94.0 ± 6.0[a] |
| *Phoma eupyrena* | 98.0 ± 2.0[a] |

Means (± SE, n = 3) followed by the same letter do not differ significantly in the colonization fungal according to Tukey post hoc test at P≤ 0.05.

In dual interactions, *C*. *cladosporioides* did not dominate any species (Table 4); however, it displayed a high coexistence percentage with *C*. *lunata* and with *M*. *roridum* at the same point on the coupon. When combined with *F*. *redolens*, *P*. *maculans*, or *P*. *eupyrena* the percentage colonization of *C*. *cladosporioides* was very low or non-existent (P<0.05). In turn, *C*. *lunata* dominated the stone surface when it interacted with *F*. *redolens*, *Paraconiothyrium* sp., or *P*. *maculans* (P<0.05). However, *C*. *lunata* was able to coexist with *M*. *roridum*, *P*. *eupyrena*, and *F*. *redolens* at the same point on the stone surface (recovery rate higher than 54%) (Fig. 1). Furthermore, *F*. *redolens* displayed greater dominance of surface colonization than *M*. *roridum* or *P*. *maculans*. The opposite was observed for *P*. *eupyrena*, which dominated *F*. *redolens* in colonization (Table 4).

**Table 4:** Percentage of area colonized on limestone coupons product of the interaction between fungi inoculated after 4 months of exposure.

| Specie A | Specie B | Specie A | Specie B | Both species | Area without colonization |
|---|---|---|---|---|---|
| *C. cladosporioides* | *C. lunata* | – | 47.3 ± 28.0$^{a}$ | 52.0 ± 29.1$^{a}$ | 0.7 ± 1.2$^{a}$ |
| *C. cladosporioides* | *F. redolens* | 30.0 ± 7.2$^{b}$ | 52.0 ± 7.2$^{a}$ | 6.0 ± 4.0$^{c}$ | 12.0 ± 2.0$^{c}$ |
| *C. cladosporioides* | *M. roridum* | 10.7 ± 18.5$^{ab}$ | 34.0 ± 28.2$^{ab}$ | 54.0 ± 19.7$^{a}$ | 1.3 ± 2.3$^{b}$ |
| *C. cladosporioides* | *Paraconiothyrium* sp. | 2.0 ± 2.0$^{b}$ | 2.7 ± 2.3$^{b}$ | – | 95.3 ± 1.2$^{a}$ |
| *C. cladosporioides* | *P. maculans* | 0.7 ± 1.2$^{c}$ | 72.0 ± 8.7$^{a}$ | – | 27.3 ± 9.5$^{b}$ |
| *C. cladosporioides* | *P. eupyrena* | – | 98.0$^{a}$ | 1.3 ± 1.2$^{b}$ | 0.7 ± 1.2$^{b}$ |
| *C. lunata* | *F. redolens* | 90.7 ± 4.2$^{a}$ | 1.3 ± 1.2$^{b}$ | 0.7 ± 1.2$^{b}$ | 7.3 ± 5.0$^{b}$ |
| *C. lunata* | *M. roridum* | 21.3 ± 4.2$^{b}$ | 2.0 ± 3.5$^{c}$ | 75.3 ± 4.2$^{a}$ | 1.3 ± 1.2$^{c}$ |
| *C. lunata* | *Paraconiothyrium* sp. | 99.3 ± 1.2$^{a}$ | – | – | 0.7 ± 1.2$^{b}$ |
| *C. lunata* | *P. maculans* | 92.0 ± 6.0$^{a}$ | – | 3.3 ± 2.3$^{b}$ | 4.7 ± 6.4$^{b}$ |
| *C. lunata* | *P. eupyrena* | 10.0 ± 8.7$^{b}$ | 19.3 ± 23.4$^{b}$ | 68.7 ± 18.1$^{a}$ | 2.0 ± 3.5$^{b}$ |
| *F. redolens* | *M. roridum* | 48.7 ± 11.4$^{a}$ | 28.7 ± 7.0$^{b}$ | 2.7 ± 2.3$^{c}$ | 20.0 ± 4.0$^{bc}$ |
| *F. redolens* | *Paraconiothyrium* sp. | 2.7 ± 1.2$^{b}$ | 2.0 ± 2.0$^{b}$ | – | 95.3 ± 2.3$^{a}$ |
| *F. redolens* | *P. maculans* | 89.3 ± 3.1$^{a}$ | – | 1.3 ± 1.2$^{c}$ | 9.3 ± 3.1$^{b}$ |
| *F. redolens* | *P. eupyrena* | – | 88.7 ± 8.1$^{a}$ | 0.7 ± 1.2$^{b}$ | 10.7 ± 9.0$^{b}$ |
| *M. roridum* | *Paraconiothyrium* sp. | 95.3 ± 4.6$^{a}$ | – | 0.7 ± 1.2$^{b}$ | 4.0 ± 5.3$^{b}$ |
| *M. roridum* | *P. maculans* | 44.0 ± 30.2$^{a}$ | 19.3 ± 33.5$^{a}$ | 28.0 ± 17.1$^{a}$ | 8.7 ± 8.3$^{a}$ |
| *M. roridum* | *P. eupyrena* | 39.3 ± 3.1$^{a}$ | 39.3 ± 5.0$^{a}$ | 12.7 ± 3.1$^{b}$ | 8.7 ± 5.0$^{b}$ |
| *Paraconiothyrium* sp. | *P. maculans* | 6.0 ± 5.3$^{bc}$ | 82.7 ± 4.2$^{a}$ | 0.7 ± 1.2$^{c}$ | 10.7 ± 3.1$^{b}$ |
| *Paraconiothyrium* sp. | *P. eupyrena* | – | 88.7 ± 12.9$^{a}$ | 4.7 ± 5.0$^{b}$ | 6.7 ± 8.1$^{b}$ |
| *P. maculans* | *P. eupyrena* | – | 98.7 ± 1.2$^{a}$ | 0.7 ± 1.2$^{b}$ | 0.7 ± 1.2$^{b}$ |

Means (± SE, n = 3) followed by the same letter do not differ significantly for each interaction according to Tukey post hoc test at P≤ 0.05. –: without colonization

When interacting with its neighbor, *M. roridum* dominated *Paraconiothyrium* sp., whereas colonization dominance was not clear when it was inoculated next to *P. maculans*. In the interaction between *M. roridum* and *P. eupyrena*, we observed competition for space during colonization, though to a lesser proportion, coexistence at a same point was also observed (Table 4). *P. maculans* dominated the stone surface when interacting with *Paraconiothyrium* sp. and *C. cladosporioides*, whereas the remaining species, including *P. eupyrena*, dominated *P. maculans*. Lastly, in the interactions between *Paraconiothyrium* sp. with *C. cladosporioides*, *F. redolens*, or *P. maculans*, surface colonization was less than 6%.

As expected, XRD analysis revealed a high proportion of calcite on all coupon surfaces. The presence of calcium oxalates was observed in *Paraconiothyrium* sp., with a greater proportion of whewellite than weddellite, however, in *P*. *maculans*, show a greater proportion of weddellite. In the interaction of *P*. *maculans* with *M*. *roridum* and *C*. *cladosporioides*, whewellite production increased compared with coupons inoculated with *P*. *maculans* alone, and weddellite was not detected. However, weddellite production increased in the interaction of *P*. *maculans* with *Paraconiothyrium* sp., whereas it decreased in the interaction with *P*. *eupyrena* (Table 5).

Through SEM analysis, we observed the preference of *C*. *cladosporioides* for colonizing the edge of limestone coupons (Fig. 3A), whereas *C*. *lunata* was a successful colonizer with conidia and hyphae production on the stone surface (Fig. 3B), and pycnidia formation was observed in the case of *P*. *eupyrena* (Fig. 3C). We observed that in most interactions, adhesion to the substrate was generated by the production of extracellular polymeric material (Fig. 3C and D, arrows). We also observed the presence of different types of crystals produced by fungi, listed in Table 5 (Fig. 3E-I). In addition, we observed calcium oxalate crystals in the interaction between *C*. *lunata* and *P*. *maculans*, which were not detected using the XRD technique.

**Table 5:** XRD analysis of coupons inoculated with fungal species individual and paired with calcium oxalate production.

| Fungal interaction | Calcita | Whewellita | Weddellita |
|---|---|---|---|
| *Paraconiothyrium* sp. | 73.0% | 25.2% | 1.8% |
| *Pestalotiopsis maculans* | 97.5% | 0.8% | 1.7% |
| *C. cladosporioides – P. maculans* | 97.9% | 2.1% | - |
| *M. roridum – P. maculans* | 97.2% | 2.8% | - |
| *Paraconiothyrium* sp.– P. *maculans* | 97.8% | - | 2.2% |
| *P. maculans – P. eupyrena* | 98.6% | - | 1.4% |

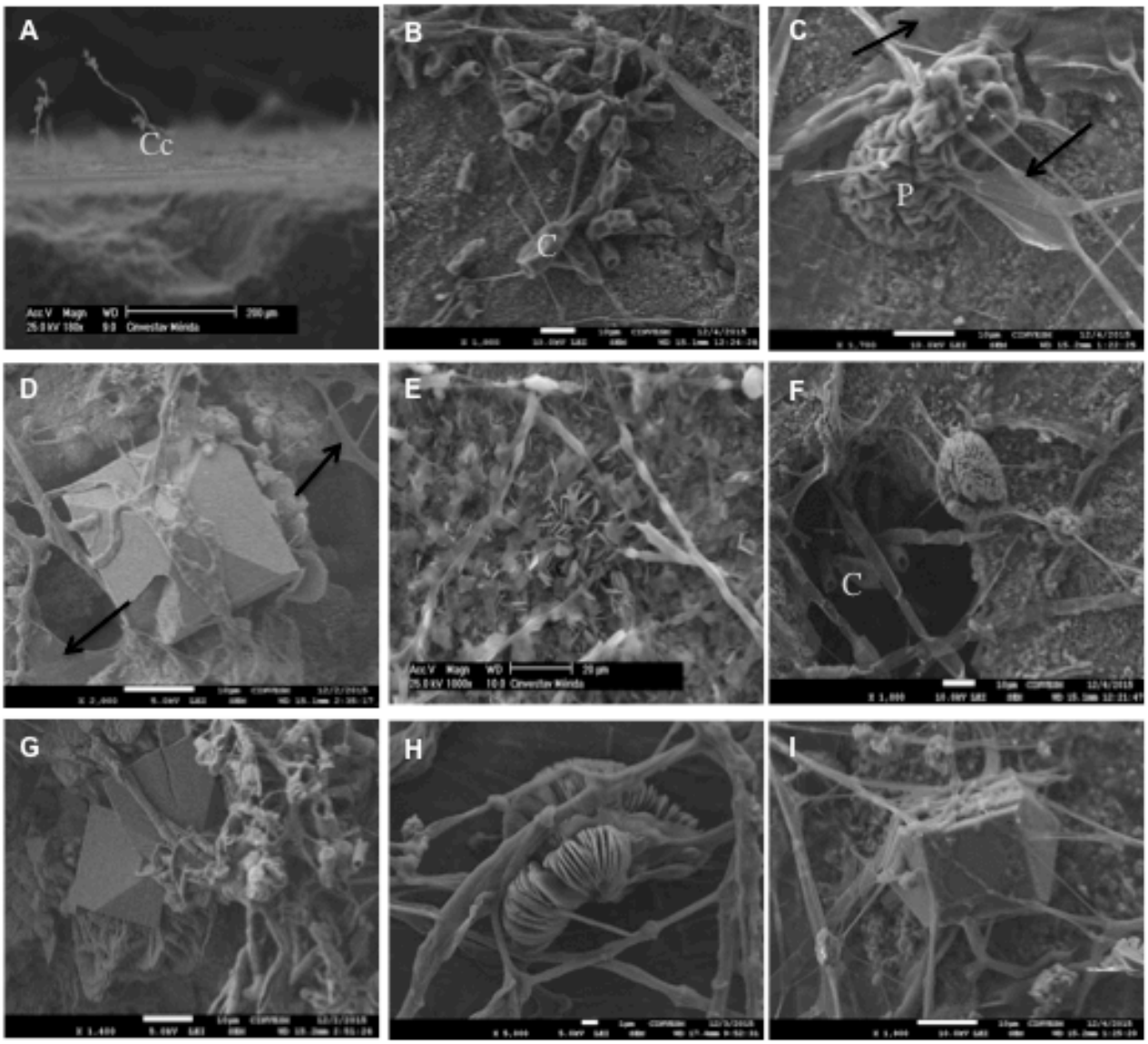


**Figure 3:** SEM images of surface of limestone coupons colonized by fungi paired after 4 months and calcium oxalate crystals produced by some species. A) *C*. *cladosporioides* – *Paraconiothyrium* sp., B) *C*. *lunata*, C) *Paraconiothyrium* sp. – *P*. *eupyrena*, D) *C*. *lunata* – *P*. *maculans*, E) *Paraconiothyrium* sp., F) *M*. *roridum* – *P*. *maculans*, G) *C*. *cladosporioides* – *P*. *maculans*, H) *P*. *maculans,* and I) *P*. *maculans* – *P*. *eupyrena*. Black arrows show the polymeric fungal production. Cc: Conidiophore; C: Conidia; P: Pycnidia.

## DISCUSSION

Fungi inhabiting lithic substrates can survive under high levels of osmotic, nutritional and thermal stress and can tolerate these conditions to colonize and establish themselves on stone surfaces (Gorbushina and Krumbein, 2000; Sterflinger, 2000; Gadd, 2007). Phototrophs and lichens are generally considered pioneers in the microbial communities that colonize bare rocks (Warscheid and Braams, 2000, Miller et al., 2008). Previous studies in a subtropical environment have shown that, in small limestone spaces, a high richness of fungal species coexists (Gómez-Cornelio et al., 2012; Gómez-Cornelio et al., unpublished data), including the 11 species selected for this study, which dominate the fungal community. These species have been reported on stony substrates (Table 1), with the exception of Hyphomycete 1, *M. roridum*, and *Paraconiothyrium* sp. The combination of morphologic and molecular identification techniques helped to identify nine fungi at the species level, and one at the genus level. Hyphomycete 1 could only be identified at the class level, as it displayed asexual reproductive structures in the taxonomy, in the molecular sequencing it was similar 94% to uncultured fungus.

Although the composition of a lithic fungal community is determined by biotic and abiotic factors (Guillitte, 1995; Miller et al., 2006; Gadd, 2007), our findings show that the interactions on limestone also influence the composition and structure of the community. Indeed, antagonistic responses were frequently observed between the species, especially from those with greater abundance as we hypothesized (Table 1), similar to what occurs in other substrates such as soil, wood and leaf litter (Boddy, 2000; Heilmann-Clausen and Boddy, 2005; Koide et al., 2005; Tiunov and Scheu, 2005). Interactions involving fungal species on stone have not been documented, with the exception of the mutualistic interaction between a fungus and an alga (Gorbushina et al., 2005).

Our study shows that epilithic fungi interact with their neighbors differently (Fig. 2) depending on the culture medium and the identity of the neighboring species, which is reflected in the antagonism index, percent inhibition, percent resistance and mycelium

growth towards the challenge species (Table 2 and S3). Therefore, the use of two nutritionally dissimilar culture media helped to uncover the responses of the interacting species (Stahl and Christensen, 1992; Treton et al., 2004). All the intraspecific interactions recorded in the oligotrophic medium and 73% of the interactions observed in the copiotrophic medium exhibited combative defense responses (categories C and D), suggesting the expression of metabolites that result in inhibition (Gaylarde et al., 2015).

In interspecific interactions, we frequently observed the inhibition of one or both fungal species (Table 2), which could indicate somatic incompatibility between the species (Bärlocher, 1991), as occurs with aquatic and soil fungi (Shearer and Zare-Maivan, 1988; Stahl and Christensen, 1992; Treton et al., 2004). In addition, the hyphal intermingling observed in the interactions of a low proportion of species (Table 2 and S2) could be the result of cooperative or neutral interactions (Duarte et al., 2013). While only the growth of Hyphomycete 1 was significantly stimulated during interactions (Table S3), this response appears to be unusual in other species grown on other substrates (Stahl and Christensen, 1992).

The success of *M*. *roridum*, *F*. *redolens*, and *Paraconiothyrium* sp. in inhibiting the radial growth of their neighbors as well as the moderate inhibition of *P*. *maculans*, *C*. *lunata*, and *C*. *clavata* (Table 2) could be explained by their rapid growth rate and high frequency (dominance in different biofilms). These fast-growing species can compete for nutrients and space, thus excluding slower-growing species (Bärlocher, 1991; Ferreira et al., 2010; Schöneberg et al., 2015).

The results of interactions in agar differ from what is observed in nature as well as on the substrate (Griffith and Boddy, 1991; Holmer and Stenlid, 1997; Fryar et al., 2005; Gaylarde et al., 2015; Schöneberg et al., 2015). Whereas most fungal species used here inhibited each other's growth on agar, fungi growing on limestone coupons develop different colonization and dominance strategies, as 90% of the interactions on limestone coupons colonized more than 70% of the surface (Table 4). This finding suggests that resource partitioning in the habitat and the abiotic preference of a species most likely

define its fundamental niche, whereas interactions allow for competition with other species to obtain their realized niche (Koide et al., 2005).

Taking into account that the coupons inoculated with fungi were not exposed to natural conditions where fungi might obtain nutrients from other sources such as dust and particulate environmental contaminants, rainwater and phototrophic biomass (Warscheid and Braams, 2000), we can infer that these fungal species lived at the expense of their metabolic waste, via cellular death or release of exopolymeric substances that form aggregates on the stone surface (Fig. 3 C and D) (Gadd, 2007). The production of exopolymeric substances reinforces the hypothesis that fungi gain a competitive advantage over those that do not produce such substances, as these substances allow for adherence to stones for colonization and establishment of biofilms (Harding et al., 2009; Fomina et al., 2010; Gadd et al., 2014).

In general, limestone coupons inoculated as controls displayed a high surface colonization (Table 2), with the exception of *C*. *cladosporioides*, which colonized less than 10% of the surface. This is due to the preference of *C*. *cladosporioides* to inhabit the edge of the limestone coupon (Fig. 3A) and to its need for association with other species to obtain nutrients from their metabolic waste, as observed when interacting with *C*. *lunata*, *F*. *redolens*, or *M*. *roridum* (Table 4). This was further corroborated by the interaction observed in the oligotrophic medium, where *C*. *cladosporioides* hyphae frequently mutual intermingling with other species. This interaction occurs because *C*. *cladosporioides* requires external nutrients for successful colonization, as was shown on concrete coupons by Giannantonio et al. (2009). Although *C*. *cladosporioides* causes inhibition at a distance when interacting with some species (Table 2), it has been demonstrated that such interaction is neither effective nor competitive (Schöneberg et al., 2015). However, on culture media, *C*. *cladosporioides* does produce inhibiting compounds (Wang et al., 2013).

The dominance of *P*. *eupyrena*, *C*. *lunata*, and *F*. *redolens*, in that order of importance, over other species sharing space in the coupons can be explained by their high

competitive ability to predominate on the stone, which results from high antagonism indices in the oligotrophic medium (CACO) and a greater frequency of isolation in the biofilms (Table 1 and 2), which it supports our hypothesis that the species with greater abundance/dominance in biofilms on limestone antagonize or inhibit the less abundant species. Additionally, these species may have morphologically adaptive mechanisms for surviving and competing on the surface, such as the melanized structures of *P*. *eupyrena* and *C*. *lunata*, or the hyaline structures of *F*. *redolens*, which preferentially colonizes small cracks and pores in the stone. The differential colonization reported here is consistent with a previous report (Gómez-Cornelio et al., 2012).

The selective dominance of *M*. *roridum* and *P*. *maculans* with two of the six species (Table 4) may have been due to their strategy to ensure permanence and dispersion over the coupon surface by means of sporodochia or acervuli production, respectively. This is not in line with a study by Giannantonio et al. (2009), who did not observe acervuli production in spite of having added nutrients to concrete coupons. Therefore, we believe that the rock and its oligotrophic condition could be a fundamental niche for *P*. *maculans*.

Studies on fungal succession and interactions between species on various substrates show that persistent species or late-stage species are strong competitors, while early-stage fungi are weak competitors (Griffith and Boddy, 1991; Holmer and Stenlid, 1997; Yuen et al., 1999), suggesting that competition determines the relative dominance of a species in the community (Fryar et al., 2001; Treton et al., 2004; Wald et al., 2004; Chagnon et al., 2016). In contrast to previous findings, our results suggest that dominant species in young and middle-aged biofilms on limestone are strong competitors. In advanced-stage biofilms in hostile environments such as limestone, competitive exclusion can occur via the addition of nutrients from autotrophic or heterotrophic organisms, resulting in an increase in the richness of species is observed (Gómez-Cornelio et al., 2012).

*Paraconiothyrium* sp. and *P. maculans* produced monohydrated (whewellite) and dihydrated (weddellite) calcium oxalate (Fig. 3F, 3H and Table 5). It is worth noting that the former was highly abundant in young biofilm, whereas the latter was highly abundant in old biofilm (Table 1), suggesting functional redundancy during succession on the natural substrate. For these fungi, weak competition was observed when interacting with other species on limestone coupons. However, the activity of *P. maculans* as a calcium oxalate producer in co-culture exceeded the percentage found in monoculture, though it produced only one of the two forms of calcium oxalate, suggesting a synergic and specialized interaction between fungi after four months of association (Table 5), favoring the functionality of some species on the substrates (Chagnon et al., 2016).

In the interactions for which calcium oxalate production was observed (Table 5), the interaction between *P. maculans* (no or low colonization) and *P. eupyrena* (dominant species in the coupon) stands out because at the beginning of the interaction, *P. maculans* was successful in colonizing the substrate and producing weddellite on the surface but was finally replaced by *P. eupyrena*. Moreover, *Paraconiothyrium* sp. inhibited its neighbors in both culture media, but when it grew on limestone coupons with other species, no colonization dominance was observed (<6%). This finding could indicate that the stone is a transitory substrate (fundamental niche), and soil is its realized niche (Ariyawansa et al., 2014).

The production of calcium oxalates by some fungi indicates that they are one of the main biodeteriorators of rocky substrates (Gorbushina and Krumbein, 2000; Gorbushina et al., 2005; Gadd et al., 2014). However, further studies are needed to understand what other mechanisms may also influence community composition and the interactions between community members. In addition, it is known that in multi-species communities, frequency and coexistence could be the result of interactions (Stohr and Dighton, 2004). Hence, performing tests with multiple species will help elucidate the different mechanisms that participate in interactions and how interactions can regulate the roles that fungi play on the stone, as well as to help understand the inhibition mechanisms in a community complex than a two-species community.

We conclude that the interactions between dominant fungi on limestone influence the colonization and overall composition of the fungal community in biofilms with different environmental exposure times, as dominant fungi displayed competitive interactions mainly on culture media and showed dominance in surface colonization of limestone coupons. Moreover, the species that produce calcium oxalates may be capable of modulating or regulating the biomineralization depending on the species with which they interact.

To our knowledge, this it is the first study providing evidence for the interactions between fungal species isolated from lithic substrates and their effect on colonization using both culture media and stone coupons under laboratory conditions. These findings could have possible implications in understanding how an ecosystem functions in terms of biodegradation and pedogenesis, as we demonstrated that fungi colonize limestone without the presence of autotroph pioneer organisms or nutrient addition. Furthermore, the interactions between species could alter the succession patterns, thus facilitating or inhibiting the growth of species that deteriorate the stone for their permanence on the substrate.

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SUPPLEMENTARY MATERIAL

**Table S1:** Types of reactions and values of the paired interactions between dominant fungal species in biofilms on limestone (Modified of Yuen et al., 1999).

| Categories | Reaction | Value |
|---|---|---|
| A | Mutual intermingling of the two species | 0 |
| $B_1$ | Response species overgrows challenge species, growth of challenge species is reduced | 1 |
| $B_2$ | Response species grows up to, on and around challenge species | 1 |
| C | Colonies of both species grow to nearly contact and then growth ceases | 2 |
| D | Mutual inhibition at a distance between both species | 3 |
| $E_1$ | Challenge species overgrows response species, growth of response species reduced | 4 |
| $E_2$ | Challenge species grows up to, on and around response species | 4 |

**Table S2:** Types of responses between paired fungal species on MEAC (M) and CACO (C). Top row= Response species. First column= Challenge species. Categories in Table S1.

| | Clcl | | Cucl | | Culu | | Fuox | | Fure | | Hyph | | Myro | | Para | | Pema | | Pheu | | Scco | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | C | M | C | M | C | M | C | M | C | M | C | M | C | M | C | M | C | M | C | M | C |
| Clcl | C | D | $B_2$ | D | $B_2$ | D | $B_1$ | A | $B_1$ | $B_2$ | C | A | $B_1$ | A | $B_2$ | D | A | A | $B_2$ | C | C | C |
| Cucl | $E_2$ | D | A | D | C | D | C | C | $B_1$ | $B_2$ | $E_2$ | D | C | $B_2$ | C | D | C | C | $E_2$ | D | $E_2$ | D |
| Culu | $E_2$ | D | C | D | C | D | C | D | C | C | C | D | C | C | C | D | C | C | D | D | $E_2$ | D |
| Fuox | $E_1$ | A | C | C | C | D | A | C | $B_2$ | C | C | $E_2$ | C | C | C | $E_2$ | C | C | C | C | $E_1$ | $E_2$ |
| Fure | $E_1$ | $E_2$ | $E_1$ | $E_2$ | $E_1$ | C | $E_2$ | C | C | C | $E_2$ | $E_2$ | $E_2$ | $E_2$ | C | $E_2$ | $E_1$ | $E_1$ | $E_2$ | C | $E_2$ | $E_2$ |
| Hyph | C | A | $B_2$ | D | $B_2$ | D | C | $B_2$ | $B_2$ | $B_2$ | A | A | $B_1$ | $B_1$ | $B_2$ | C | $B_1$ | $B_2$ | C | $B_2$ | C | C |
| Myro | $E_1$ | A | C | $E_2$ | $E_1$ | C | C | C | $B_2$ | $B_2$ | $E_1$ | $E_1$ | C | C | C | C | $E_1$ | $E_1$ | C | C | $E_2$ | A |
| Para | $E_2$ | D | C | D | C | D | C | $B_2$ | C | $B_2$ | $E_2$ | C | C | C | C | D | C | C | A | D | $E_2$ | C |
| Pema | A | A | C | C | C | C | C | C | $B_1$ | $B_1$ | $E_1$ | $E_2$ | $B_1$ | $B_1$ | C | C | A | C | $E_1$ | C | $E_2$ | C |
| Pheu | $E_2$ | C | $B_2$ | D | $B_2$ | D | C | C | $B_2$ | C | C | $E_2$ | C | C | A | D | $B_1$ | C | D | D | D | D |
| Scco | C | C | $B_2$ | D | $B_2$ | D | $B_1$ | $B_2$ | $B_2$ | $B_2$ | C | C | $B_2$ | A | $B_2$ | C | $B_2$ | C | D | D | C | D |

Clcl: *C. cladosporioides,* Cucl: *C. clavata*, Culu: *C. lunata,* Fuox: *F. oxysporum*, Fure: *F. redolens*, Hyph: Hyphomycete 1, Myro: *M. roridum*, Para: *Paraconiothyrium* sp., Pema: *P. maculans*, Pheu: *P. eupyrena*, Scco: *S. constrictum*.

**Table S3:** Effect of hyphal growth (cm) of each fungus without interacting (control) and interacting with their fungal pairs. M= MEAC medium; C= CACO medium.

| | Control | Clcl | Cucl | Culu | Fuox | Fure | Hyph | Myro | Para | Pema | Pheu | Scco |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clcl M | 17.4 ± 0.9$^{ab}$ | 13.6 ± 0.9$^{ef}$ | 16.4 ± 0.5$^{abcd}$ | 16.8 ± 0.8$^{abcd}$ | 15.8 ± 0.4$^{bcde}$ | 17.6 ± 2.2$^{ab}$ | 15.6 ± 0.5$^{bcde}$ | 13.6 ± 1.1$^{ef}$ | 14.8 ± 0.4$^{cde}$ | 16.6 ± 0.5$^{abcd}$ | 16.6 ± 0.5$^{abcd}$ | 15.6 ± 0.5$^{bcde}$ |
| Clcl C | 18.4 ± 0.9$^{a}$ | 12.3 ± 0.7$^{f}$ | 14.6 ± 1.8$^{de}$ | 17.4 ± 0.5$^{ab}$ | 17.0$^{abc}$ | 14.8 ± 1.3$^{cde}$ | 14.8 ± 0.4$^{cde}$ | 13.6 ± 1.1$^{ef}$ | 15.6 ± 0.9$^{bcde}$ | 16.8 ± 0.8$^{abcd}$ | 17.2 ± 0.8$^{ab}$ | 13.6 ± 0.5$^{ef}$ |
| Cucl M | 53.2 ± 1.3$^{a}$ | 36.8 ± 1.3$^{bc}$ | 17.8 ± 1.5$^{de}$ | 17.2 ± 0.4$^{de}$ | 17.8 ± 1.5$^{de}$ | 15.0 ± 1.9$^{de}$ | 39.2 ± 6.1$^{b}$ | 18.2 ± 1.1$^{d}$ | 12.6 ± 1.8$^{e}$ | 19.8 ± 2.7$^{d}$ | 36.4 ± 3.9$^{bc}$ | 33.2 ± 1.5$^{c}$ |
| Cucl C | 38.6 ± 2.6$^{b}$ | 17.4 ± 0.9$^{de}$ | 16.8 ± 1.3$^{de}$ | 15.4 ± 2.5$^{de}$ | 15.8 ± 1.5$^{de}$ | 16.0 ± 2.2$^{de}$ | 20.2 ± 2.6$^{d}$ | 12.6 ± 2.1$^{e}$ | 15.2 ± 2.3$^{de}$ | 17.4 ± 0.5$^{de}$ | 16.6 ± 0.9$^{de}$ | 15.4 ± 1.9$^{de}$ |
| Culu M | 53.0 ± 3.9$^{a}$ | 26.6 ± 1.1$^{ef}$ | 16.8 ± 0.8$^{hijklm}$ | 18.8 ± 0.8$^{hijkl}$ | 26.0 ± 0.7$^{efg}$ | 13.4 ± 1.1$^{lm}$ | 30.2 ± 5.7$^{de}$ | 14.8 ± 2.4$^{klm}$ | 13.6 ± 3.8$^{lm}$ | 22.6 ± 3.6$^{fgh}$ | 38.4 ± 3.6$^{bc}$ | 33.2 ± 0.8$^{cd}$ |
| Culu C | 43.2 ± 1.3$^{b}$ | 25.6 ± 1.1$^{efg}$ | 22.0 ± 2.0$^{fghi}$ | 17.2 ± 0.8$^{hijklm}$ | 15.6 ± 1.5$^{klm}$ | 16.0 ± 0.7$^{ijklm}$ | 22.6 ± 4$^{fgh}$ | 11.4 ± 2.6$^{m}$ | 21.2 ± 2.7$^{fghij}$ | 20.2 ± 2.3$^{ghijk}$ | 26.0 ± 4.3$^{efg}$ | 22.0 ± 1.2$^{fghi}$ |
| Fuox M | 27.0 ± 2.9$^{de}$ | 21.6 ± 0.9$^{efgh}$ | 17.0 ± 0.7$^{ghij}$ | 14.0 ± 1.0$^{hij}$ | 17.6 ± 2.3$^{fghij}$ | 11.0 ± 2.2$^{j}$ | 22.8 ± 2.2$^{defg}$ | 13.2 ± 1.9$^{ij}$ | 12.2 ± 3.3$^{j}$ | 16.6 ± 2.4$^{ghij}$ | 18.4 ± 1.7$^{fghij}$ | 16.6 ± 1.5$^{ghij}$ |
| Fuox C | 41.0 ± 8.3$^{a}$ | 37.4 ± 2.6$^{ab}$ | 16.4 ± 0.5$^{ghij}$ | 21.0 ± 2.3$^{efgh}$ | 15.6 ± 0.5$^{ghij}$ | 18.2 ± 1.1$^{fghij}$ | 36.8 ± 5.5$^{abc}$ | 17.4 ± 2.8$^{fghij}$ | 25.0 ± 6.3$^{def}$ | 20.4± 4.8$^{efghi}$ | 29.8 ± 4.0$^{bcd}$ | 29.4 ± 1.9$^{cd}$ |
| Fure M | 59.8 ± 1.1$^{ab}$ | 60.6 ± 0.9$^{a}$ | 36.0 ± 1.4$^{e}$ | 34.0 ± 3.8$^{ef}$ | 46.0 ± 2.7$^{d}$ | 16.0$^{h}$ | 53.6 ± 1.3$^{c}$ | 34.2 ± 4.3$^{ef}$ | 26.4 ± 2.5$^{g}$ | 41.6 ± 3.2$^{d}$ | 55.0$^{bc}$ | 55.0$^{bc}$ |
| Fure C | 51.4 ± 2.4$^{c}$ | 43.0 ± 1.2$^{d}$ | 29.6 ± 0.9$^{fg}$ | 18.0 ± 0.7$^{h}$ | 16.4 ± 1.3$^{h}$ | 17.2 ± 1.1$^{h}$ | 34.0 ± 2.9$^{ef}$ | 20.2 ± 1.5$^{h}$ | 33.2 ± 3.9$^{ef}$ | 32.2 ± 0.8$^{ef}$ | 30.8± 1.8$^{fg}$ | 30.0 ± 1.9$^{fg}$ |
| Hyph M | 15.2 ± 0.8$^{bcdef}$ | 15.0 ± 0.7$^{bcdef}$ | 15.2 ± 0.8$^{bcdef}$ | 16.2 ± 0.8$^{abcde}$ | 15.0 ± 1.9$^{bcdef}$ | 12.6 ± 0.5$^{efg}$ | 17.0 ± 1$^{abc}$ | 8.6 ± 1.9$^{h}$ | 12.0 ± 0.7$^{fgh}$ | 13.0 ± 1.6$^{defg}$ | 15.0 ± 1.6$^{bcdef}$ | 16.6 ± 2.8$^{abcd}$ |
| Hyph C | 13.8 ± 0.8$^{cdefg}$ | 19.0 ± 3.1$^{a}$ | 12.2 ± 0.4$^{fgh}$ | 13.0 ± 1.2$^{defg}$ | 10.2 ± 1.3$^{gh}$ | 12.2 ± 1.1$^{fgh}$ | 15.6 ± 2.5$^{abcdef}$ | 16.6 ± 1.5$^{abcd}$ | 12.0 ± 1.9$^{fgh}$ | 10.4 ± 0.9$^{gh}$ | 12.6 ± 1.5$^{efg}$ | 17.6 ± 1.5$^{ab}$ |
| Myro M | 33.0 ± 1.9$^{a}$ | 28.0 ± 1.9$^{bcd}$ | 27.2 ± 1.6$^{cde}$ | 26.6 ± 3.5$^{cdef}$ | 23.4 ± 0.5$^{efghi}$ | 25.0$^{cdefg}$ | 32.4 ± 1.8$^{ab}$ | 18.2 ± 1.3$^{j}$ | 19.2 ± 1.3$^{ij}$ | 29.2 ± 2.6$^{abc}$ | 25.4 ± 0.9$^{cdefg}$ | 26.8 ± 4.9$^{cde}$ |
| Myro C | 29.2 ± 0.8$^{abc}$ | 29.4 ± 0.5$^{abc}$ | 24.2 ± 0.8$^{defgh}$ | 23.4 ± 1.1$^{efghi}$ | 22.2 ± 1.3$^{fghij}$ | 21.0 ± 2.1$^{ghij}$ | 23.4 ± 1.5$^{efghi}$ | 18 ± 1.6$^{j}$ | 20.2 ± 1.1$^{hij}$ | 25.0 ± 1.6$^{cdefg}$ | 23.2 ± 1.8$^{efghi}$ | 26.4 ± 1.1$^{cdef}$ |
| Para M | 41.4 ± 1.5$^{a}$ | 35.0 ± 0.7$^{b}$ | 27.8 ± 1.3$^{d}$ | 29.0 ± 1.0$^{cd}$ | 31.6 ± 2.9$^{bc}$ | 25.6 ± 0.9$^{de}$ | 23.6 ± 1.3$^{ef}$ | 15.6 ± 1.1$^{ik}$ | 17.8 ± 1.1$^{ghij}$ | 26.8 ± 0.8$^{de}$ | 33.2 ± 1.3$^{b}$ | 32.6± 1.8$^{b}$ |
| Para C | 21.0 ± 1.6$^{fg}$ | 15.8 ± 1.3$^{ijk}$ | 18.8 ± 1.8$^{ghij}$ | 18.8 ± 1.6$^{ghij}$ | 19.2 ± 2$^{ghi}$ | 16.8 ± 0.8$^{hij}$ | 20.0 ± 1.9$^{gh}$ | 13.0 ± 0.7$^{k}$ | 17.0 ± 1.6$^{hij}$ | 17.4 ± 2.3$^{hij}$ | 16.8 ± 0.4$^{hij}$ | 18.2 ± 1.5$^{ghij}$ |
| Pema M | 37.4 ± 1.5$^{a}$ | 32.4 ± 1.9$^{bc}$ | 22.2 ± 3.3$^{ef}$ | 26.0 ± 5.3$^{de}$ | 18.8 ± 2.4$^{fgh}$ | 14.6 ± 0.9$^{hi}$ | 34.4 ± 1.5$^{ab}$ | 10.8 ± 1.5$^{ij}$ | 8.2 ± 1.1$^{j}$ | 17.6 ± 0.5$^{gh}$ | 31.6 ± 1.5$^{bc}$ | 31.8 ± 1.9$^{bc}$ |
| Pema C | 29.8 ± 0.4$^{cd}$ | 17.6 ± 1.5$^{gh}$ | 18.4 ± 1.1$^{fgh}$ | 16.2 ± 0.8$^{h}$ | 16.0 ± 1.4$^{h}$ | 15.4 ± 0.5$^{h}$ | 21.8 ± 0.4$^{efg}$ | 10.4 ± 0.9$^{ij}$ | 17.8 ± 2.6$^{fgh}$ | 16.4 ± 0.5$^{h}$ | 18.2 ± 1.9$^{fgh}$ | 21.6 ± 0.9$^{efg}$ |
| Pheu M | 23.8 ± 1.3$^{ab}$ | 24.4 ± 1.7$^{ab}$ | 14.4 ± 2.3$^{efgh}$ | 14.4 ± 1.1$^{efgh}$ | 18.2 ± 1.8$^{de}$ | 12.6 ± 0.9$^{hi}$ | 19.8 ± 0.4$^{cd}$ | 15.0 ± 3.2$^{efgh}$ | 10.2 ± 0.8$^{ij}$ | 18.2 ± 1.6$^{de}$ | 15.4 ± 0.5$^{efgh}$ | 13.0 ± 1.0$^{ghi}$ |
| Pheu C | 27.6 ± 0.9$^{a}$ | 22.4 ± 2.5$^{bc}$ | 14.4 ± 0.5$^{defg}$ | 15.2 ± 1.1$^{efgh}$ | 14.8 ± 0.4$^{efgh}$ | 14.0 ± 0.7$^{fghi}$ | 23.4 ± 2.9$^{bc}$ | 13.2 ± 1.9$^{ghi}$ | 15.6 ± 1.1$^{efgh}$ | 17.2 ± 1.6$^{def}$ | 16.8 ± 0.8$^{defg}$ | 18.2 ± 2.3$^{de}$ |
| Scco M | 18.6 ± 0.5$^{ab}$ | 16.0 ± 1.2$^{bcdefg}$ | 15.0 ± 1.0$^{cdefg}$ | 16.4 ± 1.1$^{bcde}$ | 16.4 ± 1.1$^{bcde}$ | 15.4 ± 0.5$^{cdefg}$ | 19.2 ± 0.8$^{a}$ | 13.4 ± 1.1$^{gh}$ | 13.8 ± 0.8$^{efgh}$ | 15.4 ± 0.5$^{cdefg}$ | 17.0 ± 0.7$^{abc}$ | 16.6 ± 0.9$^{abcd}$ |
| Scco C | 15.2 ± 0.8$^{cdefg}$ | 16.6 ± 0.5$^{abcd}$ | 16.2 ± 2.5$^{bcdef}$ | 16.4 ± 2.4$^{bcde}$ | 14.4 ± 1.1$^{cdefg}$ | 15.0 ± 1.4$^{cdefg}$ | 14.6 ± 1.8$^{cdefg}$ | 11.6 ± 0.9$^{h}$ | 13.6 ± 0.9$^{fgh}$ | 15.2 ± 0.4$^{cdefg}$ | 15.4 ± 1.1$^{cdefg}$ | 14.0 ± 0.7$^{defgh}$ |

Means (± SE, n = 5) followed by the same letter do not differ significantly for each interaction between the two media for each fungus according to Tukey post hoc test at P≤ 0.05. Clcl: *C. cladosporioides*, Cucl: *C. clavata*, Culu: *C. lunata*, Fuox: *F. oxysporum*, Fure: *F. redolens*, Hyph: Hyphomycete 1, Myro: *M. roridum*, Para: *Paraconiothyrium* sp., Pema: *P. maculans*, Pheu: *P. eupyrena*, Scco: *S. constrictum*.

# CAPÍTULO 4. DISCUSIÓN GENERAL Y CONCLUSIONES

Esta investigación se planteó con el objetivo de responder si las interacciones entre especies de hongos dominantes aislados de biopelículas desarrolladas sobre paredes de roca calcárea con diferentes tiempos de construcción (biopelícula joven-1 año; biopelícula intermedia-5 años; biopelícula avanzada-10 años), son capaces de influir en la estructura y la composición de la especies fúngicas en dicho sustrato. Para ello, primero se caracterizó la composición de la comunidad fúngica, donde se concluye que su estructura esta integrada por un conjunto de especies diferentes en cada biopelícula, la cual ocurre principalmente en función del tiempo transcurrido desde la edificación de las paredes con roca calcárea y su interacción con el ambiente.

De manera general, se obtuvieron 844 aislados agrupados en 202 especies y 69 géneros (Cuadro 2; Capítulo 2); el 74% de las especies presentaron estructuras melanizadas debido a las condiciones ambientales como la temperatura y radiación UV. Proporciones similares de especies con estructuras melanizadas fueron encontradas en cada biopelícula, lo que concuerda con estudios realizados para comunidades fúngicas de otros ambientes y otros litotipos de roca (Sterflinger y Krumbein 1997; Gorbushina et al. 2002; Ruibal et al. 2005; Ruibal et al. 2008; Lan et al. 2010; Gómez-Cornelio et al. 2012).

De las tres biopelículas, en la intermedia se presentó la mayor abundancia, pero los valores más bajos de riqueza, diversidad y equidad. Estos valores contrastantes son atribuibles a las especies *Curvularia lunata*, *Fusarium redolens* y *Fusarium oxysporum* que juntas representan el 58% del total de aislados. En la biopelícula joven se registraron valores medios en la abundancia, riqueza, diversidad y equidad, a pesar del desarrollo incipiente observado sobre la roca. Finalmente, en la biopelícula avanzada aunque la abundancia fue baja; en la riqueza, diversidad y equidad los valores fueron altos, consolidándola como la biopelícula con la estructura fúngica más equitativa y diversa (Cuadro 3; Capitulo 2).

Una alta proporción de las partículas inoculadas en los medios de cultivo presentaron micelio adherido, lo que indica que la biopelícula sobre la roca esta colonizada por la red micelial que ahí se desarrolla. Aunque las hifas no son visibles en las biopelículas, por la técnica de aislamiento empleada, es muy probable que estos hongos se encuentren funcionalmente activos sobre la roca y no sean derivados de esporas depositadas por el aire. Por otro lado, el uso de dos medios de cultivo permitió conocer una comunidad fúngica cultivable completa y diversa, ya que en el medio oligotrófico se aislaron exclusivamente 73 especies, como las del genero *Stachybotrys* y la especie *Friedmanniomyces simplex*. Mientras que en el medio copiotrófico 77 especies fueron exclusivas. Además, el $CaCO_3$ que es el componente principal de la roca calcárea, se adicionó a los medios de cultivo, permitiendo aislar hongos autóctonos de la roca.

Los ambientes y sustratos circundantes a la roca pueden influir en la composición de la comunidad fúngica. El 40% de los hongos identificados a nivel de especie en la comunidad estudiada, son hongos reportados en otros estudios para el suelo y asociados a plantas. Mientras que, para la hojarasca, la roca, los hongos del aire, la madera y el agua se han reportado entre 44 y 31 especies para las tres biopelículas. Aunque estos valores se relacionan con el número de estudios vinculados con estos ambientes (Domsch et al. 2007; Seifert et al. 2011). Con lo anterior se puede concluir que sobre la roca calcárea pueden arribar una gran variedad de propágulos y servir como reservorios de especies, las cuales en condiciones ideales pueden dispersarse, para colonizar y establecerse sobre sus hábitats frecuentes.

Un alto porcentaje de especies aisladas (79%) en las tres biopelículas fueron encontradas de una a dos veces en todo el estudio, lo que es consistente con lo reportado en otros estudios (Ruibal et al. 2005; Ruibal et al. 2008; Gómez-Cornelio et al. 2012), en donde muchas especies epilíticas son raras o poco comunes en las comunidades fúngicas líticas y pocas especies son dominantes. Entre los géneros frecuentes y comunes, destacaron *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Myrothecium*, *Nodulisporium, Paraconiothyrium* y *Phoma* (Cuadro 2; Capítulo 2). Las especies dominantes con más de siete aislados y que se encontraron exclusivamente

en la biopelícula joven fueron *Coleophoma* sp., mientras que en la biopelícula avanzada dominaron *Aspergillus niger*, *Lasiodiplodia theobromae* e Hyphomycete 1. Este último no se logró identificar por taxonomía morfológica, ni por biología molecular mediante la secuenciación del ITS, por lo que podría tratarse de una especie nueva.

De los hongos presentes en dos biopelículas, *Paraconiothyrium* sp. se encontró dominando en la biopelícula joven, disminuyendo su presencia en la intermedia y desapareciendo en la avanzada. *Myrothecium roridum* se encontró en la biopelícula intermedia y dominó en la avanzada; caso contrario ocurre con *Curvularia verruculosa*, que dominó en la biopelícula intermedia y casi desaparece en la avanzada. Los hongos *Cladosporium oxysporum* y *Cladosporium sphaerospermum* dominaron en la biopelícula joven, desaparecieron en la intermedia y reaparecieron con menor frecuencia en la avanzada.

Todas las especies encontradas en las tres biopelículas fueron dominantes en una de ellas y variables en cuanto a frecuencia para las otras dos. Tal es el caso de *Phoma eupyrena*, *Cladosporium cladosporioides*, *Curvularia clavata* y *Phoma herbarum* que dominaron en la biopelícula joven. En la biopelícula intermedia dominaron *C*. *lunata*, *F*. *redolens*, *F*. *oxysporum*, *Curvularia pallescens* y *Scolecobasidium constrictum*. Finalmente en la biopelícula avanzada *Pestalotiopsis maculans* y *Curvularia australiensis* fueron dominantes. Esta variación en la ocurrencia de las especies en las biopelículas con diferentes tiempo de exposición, permite determinar que la sucesión (tiempo) juega un papel importante en la estructura y composición de las comunidades fúngicas inmersas en la biopelículas, como se determina en sustratos como la madera (Hiscox et al. 2015a; Hiscox et al. 2016).

La dominancia y exclusividad de algunas especies se confirmó en el diagrama de Venn (Fig. 3; Capitulo 2), donde se observó que solo 16 especies se comparten entre las biopelículas estudiadas, en las biopelículas joven e intermedia se comparten 12 especies, mientras que en las otras combinaciones solo 6 y 7 especies (joven-intermedia e intermedia-avanzada, respectivamente). A pesar de que las paredes

estudiadas fueron edificadas con roca calcárea, y se encuentran a poca distancia entre ellas y por ende bajo influencia similar de las condiciones ambientales y características bioreceptivas (Cuadro 1; Capítulo 2), la alta exclusividad de especies encontradas en cada una de las biopelículas, permite concluir que el tiempo de exposición de las rocas al ambiente, las condiciones microclimáticas en cada roca, los sustratos circundantes a las rocas y las interacciones entre las especies que habitan en cada biopelícula, serían los factores determinantes en la estructura de la comunidad fúngica en cada biopelícula.

Para determinar si los hongos aislados de biopelículas sobre roca calcárea influyen en la colonización y desarrollo de otras especies, se realizaron interacciones intra e interespecíficas entre 11 especies dominantes, en dos medios de cultivo contrastantes y sobre cupones de roca calcárea (Cuadro 1; Capitulo 3). La respuesta observada en cada interacción fue determinada por la identidad de la especie con la que interactuó y por la composición de cada medio de cultivo (Fig. 2, cuadro 2, S2 y S3; capítulo 3). Con mayor frecuencia se observó el tipo de interacción competitivo (Categorías C y D) en ambos medios de cultivo. Mientras que el sobrecrecimiento de una especie sobre otra o el crecimiento hasta y alrededor de la especie (Categorías B y E) ocurrió con mayor frecuencia en el medio extracto de malta agar adicionado con carbonato de calcio. Las interacciones compatibles entre especies (entrecruzamiento de hifas; categoría A) que dieran indicios de facilitación en la interacción ocurrieron con menor frecuencia en ambos medios de cultivo.

Diferentes parámetros fueron evaluados para demostrar la capacidad antagónica e inhibitoria de los hongos dominantes en los medios de cultivo. Las especies de crecimiento rápido como *F*. *redolens*, *P*. *maculans*, *P*. *eupyrena* y *M*. *roridum* presentaron altos valores en el índice de antagonismo y en la capacidad inhibitoria, contrastando con las especies de crecimiento lento como *C*. *cladosporioides*, Hyphomycete 1 y *S*. *constrictum* (Cuadro 2; Capítulo 3). Sin embargo, las especies de crecimiento lento presentan un alto porcentaje de resistencia a la inhibición de las especies de crecimiento rápido, reflejado en el porcentaje de crecimiento radial (Cuadro

2; Capítulo 3). El incremento o estimulación de la tasa de crecimiento por la presencia de sus pares fue observado solo para el Hyphomycete 1, mientras que *M*. *roridum* y *Paraconiothyrium* sp. inhiben significativamente las tasas de crecimiento radial de las especies con las que interactuaron (Cuadro S3; Capítulo 3). Con lo anterior podemos concluir que en las interacciones, la tasa de crecimiento de cada especie podría influir en la dominancia u ocurrencia sobre la roca, haciéndolos aptos para antagonizar a las especies con menor tasa de crecimiento.

Por otro lado, en las interacciones duales sobre cupones de roca calcárea se seleccionaron siete especies fúngicas. En los cupones controles donde se inocularon solas, se encontró una colonización de la superficie superior al 70% para todas las especies (Cuadro 3; Capítulo 3), a excepción de *C*. *cladosporioides*, que solo coloniza la superficie cuando se encuentra asociada a algunas especies o en los bordes de los cupones (Fig. 3; Capítulo 3). En la Figura 1, se presentan los resultados de las interacciones duales de las especies probadas, donde *P*. *eupyrena* es la especie que demuestra mayor dominancia sobre sus pares (domina a cuatro especies), crece en asociación con *C*. *lunata* y tiene competencia por espacio sobre la superficie de los cupones con *M*. *roridum*. Mientras que *C*. *lunata* domina a tres especies y coloniza en asociación con las hifas de otras tres especies en el mismo espacio, caso similar ocurre en las interacciones de *F*. *redolens* que domina la superficie al interactuar con tres especies, coloniza en asociación con dos especies, pero con *Paraconiothyrium* sp. no hay crecimiento de ninguna de las dos especies (Cuadro 4; Capítulo 3).

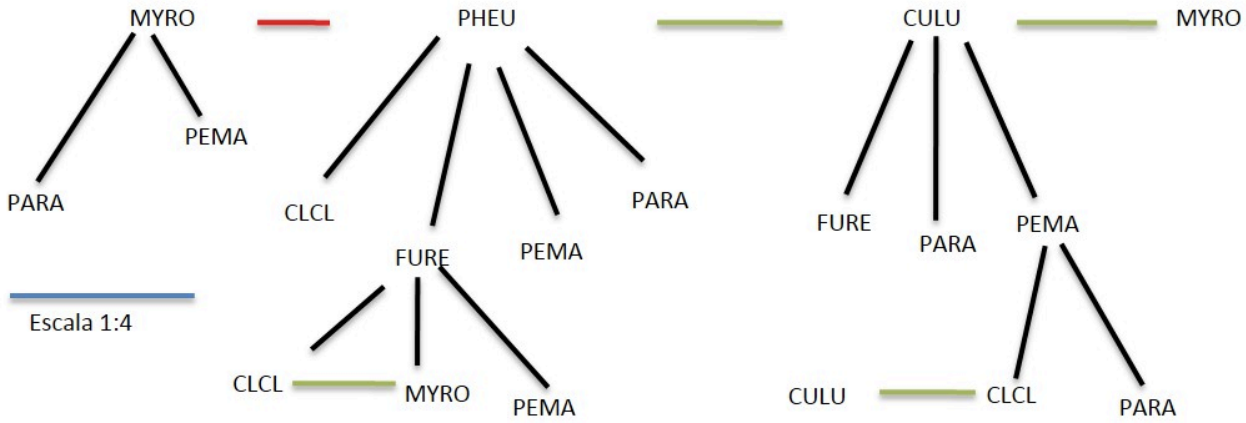


**Figure 1:** Interacciones entre especies de hongos inoculados sobre cupones de roca calcárea. Línea negra= dominancia. Línea verde= coexistencia. Línea roja= competencia. Longitud de línea azul= 100% de colonización de uno o ambos hongos sobre la superficie del cupón. Todas las líneas representan los promedios de colonización sobre los cupones (n=3). Donde CLCL: *C*. *cladosporioides,* CULU: *C*. *lunata,* FURE: *F*. *redolens*, MYRO: *M*. *roridum*, PARA: *Paraconiothyrium* sp., PEMA: *P*. *maculans*, PHEU: *P*. *eupyrena*.

*Myrothecium roridum* domina a dos especies y coloniza el mismo espacio de la superficie de roca con otras dos especies. El hongo *P*. *maculans* domina a *Paraconiothyrium* sp. y a *C*. *cladosporioides*. Las especies menos dominantes y competitivas sobre la roca son *C*. *cladosporioides* y *Paraconiothyrium* sp. La primera es dominada por tres especies y se asocia para colonizar el mismo espacio de la superficie con *M*. *roridum* y *C*. *lunata*, además en la interacción con *Paraconiothyrum* sp. no se observó colonización por parte de las dos especies. Mientras que *Paraconiothyrium* sp. es dominada por cuatro especies y no coloniza la superficie cuando interactúa con las otras dos especies.

En las observaciones mediante microscopía electrónica de barrido, se encontró la esporulación abundante de *C*. *lunata* y la formación de picnidios de *P*. *eupyrena* (Fig. 3; Capitulo 3). Además se registró la producción de material polimérico extracelular en la mayoría de las interacciones, lo cual estaría contribuyendo con la formación y mantenimiento de las biopelículas. Mediante difracción de rayos X sobre la superficie de los cupones de roca, se observó la presencia de oxalatos de calcio mono y dihidratado solo para las especies *P*. *maculans* y *Paraconiothyrium* sp. cuando fueron inoculadas

solas, lo que sugiere biomineralización (Burford et al. 2006; Scheerer et al. 2009; De la Rosa-García et al. 2011; Ortega-Morales et al. 2016) (Cuadro 5; Capítulo 3). Sin embargo, en los cupones con las interacciones donde se encontraban estas dos especie, soló se registro la presencia de uno de los dos tipos de cristales, pero nunca los dos. Por ejemplo para *P*. *maculans* se observó la producción de oxalato de calcio monohidratado cuando interactúo con *C*. *cladosporioides* y *M*. *roridum*; y dihidratado al interactuar con *Paraconiothyrium* sp. y *P*. *eupyrena*, por lo que se puede concluir que dependiendo la identidad de la especie con la que se encuentren interactuando, será el tipo de oxalato de calcio que encontraremos, sugiriendo una especialización producto de la interacción.

Por lo anterior, se concluye que las interacciones entre hongos dominantes sobre roca calcárea tienen influencia en la colonización y composición de la comunidad fúngica en biopelículas con diferentes tiempos de exposición al ambiente, ya que presentaron interacciones competitivas principalmente en los medios de cultivo (inhibición a distancia o inhibición del crecimiento), además demostraron su dominancia sobre otras especies menos abundantes. Lo anterior explica las variaciones en las abundancias encontradas en las biopelículas con diferentes tiempos de exposición, siendo las interacciones las que contribuyen con las variaciones en la estructura de la comunidad. Adicionalmente, las especies que producen oxalatos de calcio podrían ser capaces de modular o regular la biomineralización dependiendo de la especie con la que interactúan, siendo las especies dominantes posibles protectores de la superficie de la roca inhibiendo la colonización de las especies biodeteriorantes sobre el sustrato pétreo.

Este es el primer estudio que aporta evidencia de las interacciones entre las especies de hongos aislados de sustratos líticos y sus efectos en la colonización, utilizando tanto medios de cultivo, como cupones de roca bajo condiciones de laboratorio. Aquí se demuestra que los hongos colonizan la roca calcárea sin la presencia de organismos autótrofos pioneros y sin la adición de nutrientes, además de que las interacciones

entre especies alterarían los patrones de sucesión, facilitando o inhibiendo el crecimiento de especies que deterioran la roca para su permanencia sobre el sustrato.

Lo anterior podría tener posibles implicaciones desde dos puntos de vista contrastantes: el primero es utilizar las especies que al interactuar incrementan el potencial de biodegradación de la roca, como un modelo para la formación del suelo, particularmente en la biorremediación de áreas donde se realiza la extracción de roca calcárea para la construcción, además de que en la península de Yucatán existen zonas que tienen un suelo incipiente, que podrían contribuir en acelerar la formación del mismo. Por otro lado, la península de Yucatán es una zona con un patrimonio cultural rico y diverso donde el principal material de construcción es la roca calcárea, por lo que especies fúngicas que inhiban el crecimiento de las especies que biomineralizan la roca calcárea, podrían emplearse como modelo para la protección de dicho patrimonio.

Aunque la producción de oxalatos de calcio por parte de algunos hongos los coloca como uno de los principales agentes biodeteriorantes de la roca calcárea, es preciso realizar estudios posteriores que permitan comprender y complementar qué otros mecanismos podrían estar influyendo en la composición de la comunidad y las interacciones entre sus miembros sobre los sustratos líticos. Estos resultados y conclusiones se obtuvieron a partir de interacciones simples (duales), sin embargo en la naturaleza se sabe que las comunidades están integradas por múltiples especies de hongos y otros grupos de organismos, por lo que es necesario realizar estudios posteriores de interacciones entre múltiples especies de hongos y entre grupo de organismos que permitan comprender los principales factores que influyen en la formación, desarrollo y función de las biopelículas. Esta investigación sienta las bases para realizar estudios posteriores con organismos que crecen en la roca calcárea, que es un sustrato poco estudiado, además de comprender cómo, las especies de organismos que la colonizan y se establecen en ellas pueden coexistir y desempeñar sus funciones sobre la roca.

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# ANEXOS GENERALES

1. Carta aceptación de artículo en la revista Acta Botánica Mexicana (Capítulo 2).

2 de septiembre de 2016

M. en C. Sergio Alberto Gómez Cornelio
Estudiante de Doctorado
El Colegio de la Frontera Sur
24500 Campeche, Campeche
MÉXICO

Estimado M. en C. Sergio Alberto Gómez Cornelio:

Tengo el gusto de informarle que el trabajo ""Changes in fungal community composition of biofilms on limestone across a chronosequence in Campeche, México"", del cual es autor junto con Otto Ortega-Morales, Alejandro Morón-Ríos, Manuela Reyes-Estebanez y Susana De la Rosa-García, ha sido aceptado para su publicación.

El artículo aparecerá publicado en el número 117 a salir en el mes de octubre de 2016. En breve le enviaré las pruebas de página para su revisión y aprobación.

Saludos cordiales,

Patricia Mayoral Loera

Biól. Patricia Mayoral Loera
en nombre del Comité Editorial

*Acta Botanica Mexicana*

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2. Carta de artículo sometido en la revista Fungal Ecology (Capítulo 3)


**Sergio Alberto Gómez Cornelio <sgomez@ecosur.edu.mx>**

## Submission Confirmation

1 mensaje

**Fungal Ecology** <ees.funeco.0.3b8694.e86b573a@eesmail.elsevier.com> 24 de agosto de 2016, 9:41
Para: sgomez@ecosur.edu.mx, gocor25@yahoo.com.mx

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