UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA

CENTRO MULTIDISCIPLINARIO DE ESTUDIOS EN BIOTECNOLOGÍA

PROGRAMA INSTITUCIONAL DE DOCTORADO EN CIENCIAS BIOLÓGICAS
OPCIÓN: BIOTECNOLOGÍA MOLECULAR AGROPECUARIA

TESIS

ANÁLISIS DE LA PRODUCCIÓN DE METABOLITOS EXTRACELULARES CON ACTIVIDAD ANTIMICROBIANA EN *Irpex lacteus* y *Ganoderma* sp.

PRESENTA

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El presente trabajo se realizó en el Laboratorio de Conservación y Biotecnología de Microorganismos del Centro Multidisciplinario de Estudios en Biotecnología de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad Michoacana de San Nicolás de Hidalgo, bajo la dirección del D. en C. Gerardo Vázquez Marrufo. Se agradece al CONACyT el apoyo brindado mediante la beca número 477511 para la realización de estudios de posgrado. Un agradecimiento especial a los miembros del comité tutoral de esta tesis: Dra. Alejandra Ochoa Zarzosa, Dra. Ma. Soledad Vázquez Garcidueñas, Dr. Julio Cesar Villagómez Castro y Dr. Rafael Salgado Garciglia.

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RESUMEN

El grupo fúngico Basidiomycota incluye aproximadamente a 30.000 especies descritas, las cuales cumplen una gran variedad de roles ecológicos como mutualistas de otros grupos biológicos, como saprófitos que descomponen materia orgánica principalmente en ecosistemas terrestres, y como patógenos de plantas y animales. Los basidiomicetos producen una gran cantidad de metabolitos secundarios con actividad antimicrobiana y citotóxica, entre otras actividades biológicas. Así, los basidiomicetos son considerados como una fuente importante de compuestos para el combate de cepas resistentes a los antifúngicos y antibacterianos de uso convencional. Por lo anterior, el objetivo de este trabajo fue analizar la actividad antimicrobiana del micelio y los concentrados extracelulares basales de las cepas CMU-0113 de *Ganoderma* sp. y CMU-8413 de *Irpex lacteus*. Se evaluó la actividad antifúngica de los concentrados extracelulares de las cepas de estudio, así como el metaboloma extracelular en cultivos axénicos. Los terpenos destacaron entre los metabolitos detectados en los metabolomas obtenidos. Debido a esto, y a que un porcentaje importante de dichos metabolitos presentan actividad antimicrobiana se identificaron y caracterizaron bioinformáticamente los genes y enzimas asociadas a la producción de terpenos de *Ganoderma* spp., género del cual se han secuenciado los genomas de cuatro especies. El conocimiento de la biosíntesis de terpenos de *Ganoderma* spp. puede contribuir a optimizar su producción comercial, con impacto en el control de plagas agrícolas y el combate en patógenos de animales y humanos.

Palabras clave: Basidiomicetes, metabolitos secundarios, *Irpex lacteus*, *Ganoderma* sp., micelio.

ABSTRACT

The Basidiomycota is a fungal taxon that includes approximately 30,000 described species, which fulfill a wide variety of ecological roles as mutualists of other biological groups, as saprophytes that decompose organic matter mainly in terrestrial ecosystems, and as pathogens of plants and animals. Basidiomycetes produce a large number of secondary metabolites with antimicrobial and cytotoxic activity, among other biological activities. Thus, basidiomycetes are considered to be an important source of compounds for combating strains resistant to antifungal and antibacterial agents of conventional use. Therefore, the objective of this work was to analyze the antimicrobial activity of the mycelium and the basal extracellular concentrates of the CMU-0113 strains of *Ganoderma* sp. and CMU-8413 from *Irpex lacteus*. The antifungal activity of the extracellular concentrates of the study strains was evaluated, as well as the extracellular metabolome in axenic cultures. Terpenes stood out among the metabolites detected in the obtained metabolomes. Due to this, and because a significant percentage of these metabolites have antimicrobial activity, the genes and enzymes associated with the production of terpenes from *Ganoderma* spp., a genus for which the genomes of four species have been sequenced, were identified and bioinformatically characterized. Knowledge of the biosynthesis of terpenes from *Ganoderma* spp. can contribute to optimizing its commercial production, with an impact on the control of agricultural pests and the fight against animal and human pathogens.

Keywords: Basidiomycetes, secondary metabolites, *Irpex lacteus*, *Ganoderma* sp., mycelium.

I. INTRODUCCIÓN

I.1 Los pesticidas, herramientas agrícolas y agentes de toxicidad ambiental y humana

La producción agrícola ha sido una actividad relevante a lo largo de la historia de la humanidad (Bellwood, 2012; Fróna et al., 2019). Una de las razones por las que se ha podido incrementar la producción agrícola desde la segunda mitad del siglo pasado ha sido el empleo de pesticidas (Sharma et al., 2020). La OMS define a los pesticidas como como "compuestos químicos que se usan para matar plagas, incluidos insectos, roedores, hongos y plantas no deseadas (malezas) (FAO, 2021). Por su parte, la Agencia de Protección Ambiental de los Estados Unidos de América (EPA, por sus siglas en inglés), define a los pesticidas como cualquier sustancia o mezcla de sustancias destinadas a prevenir, destruir, repeler o mitigar a cualquier plaga, que puede ser empleado como regulador de plantas, defoliante o desecante, o bien como estabilizador de nitrógeno (EPA, 2021). Los pesticidas pueden dividirse de acuerdo con su grupo químico, su origen o el tipo de organismo sobre el que actúan. Así, los pesticidas pueden ser orgánicos, inorgánicos sintéticos y naturales; o bien, pueden adquirir nombres más específicos de acuerdo con la especie o grupo biológico sobre el que actúan, como herbicidas contra malezas, insecticidas contra insectos, fungicidas contra hongos, entre otros (Sharma et al., 2020).

Prácticamente de manera inmediata a su aplicación en la agricultura intensiva se documentaron los efectos adversos de los pesticidas en los ecosistemas y la salud humana (Sharma et al., 2020). Actualmente se encuentran bien documentada la relación de los agroquímicos de uso convencional a nivel global con diversas enfermedades en seres humanos, como cáncer, leucemia, diabetes, asma y enfermedades neurodegenerativas (Bourguet y Guillemaud, 2016; Kim et al., 2017). También se ha documentado la acumulación de pesticidas en alimentos y sus efectos nocivos en la diversidad biológica (Gomes et al., 2020; Sánchez-Bayo et al., 2016; Calatayud-Vernich et al., 2018; Uhl y Brühl, 2019). A pesar de estos efectos adversos en la salud humana y la biodiversidad, el uso de pesticidas se ha considerado necesario, ya que se ha documentado que pérdidas globales de aproximadamente entre el 23 y el 38% en cultivos de relevancia mundial como el maíz, el trigo y el algodón se debe a distintos tipos de plagas y enfermedades por diferentes especies de fitopatógenos (Popp et al., 2013). Sin

embargo, también se ha incrementado la resistencia a los plaguicidas más ampliamente empleados a nivel global (Bourguet y Guillemaud, 2016).

Se han descrito cuatro mecanismos principales de resistencia adquirida a los fungicidas de uso agrícola (Lucas et al., 2015), que incluyen la alteración de la proteína blanco por mutaciones en los genes codificantes respectivos, la disminución de la concentración del fungicida en el interior celular mediante su exportación por "bombas de eflujo", la sobre-expresión de la proteína blanco mediante el incremento de la transcripción del gen codificante respectivo, y la degradación del fungicida mediante acción enzimática (Figura 1).

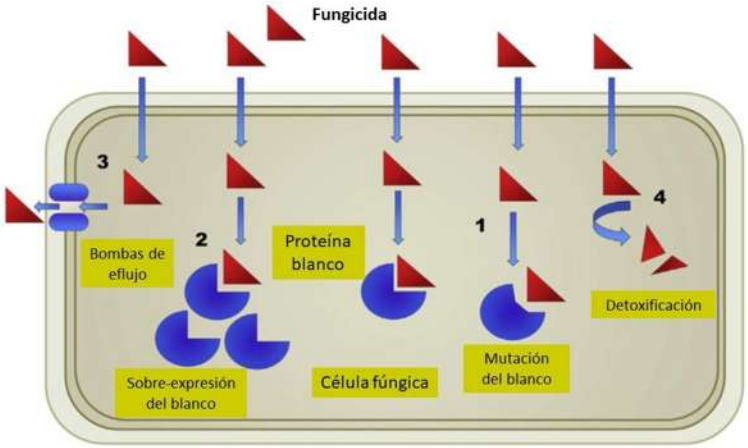

Figura 1. Mecanismos de Resistencia a fungicidas agrícolas. 1. Alteración del blanco, que previene la unión del fungicida; 2. Sobre-expresión de la proteína blanco, lo que incrementa la concentración del fungicida para ser eficaz; 3. Bombas de eflujo que exportan el fungicida del interior celular; 4. Degradación del fungicida mediante enzimas metabólicas (tomado y traducido de Lucas et al., 2015).

La alteración del blanco se ha reportado como mecanismo de resistencia a varios fungicidas de uso agrícola como metil-benzimidazoles e inhibidores de succinato deshidrogenasa. La acción de transportadores de membrana ABC y otras bombas de eflujo es un mecanismo ampliamente descrito en hongos patógenos de humano y hongos fitopatógenos. Aunque se ha reportado la sobre-expresión del blanco y la degradación del fungicida, estos dos mecanismos no parecen estar ampliamente distribuidos en hongos fitopatógenos (Lucas et al., 2015). El caso de este último mecanismo es sorpresivo ya que se conoce que la monooxigenesa P450 fúngica y diversas enzimas con capaces de degradar una gran cantidad de compuestos xenobióticos.

Entre los casos relevantes de resistencia a fungicidas de fitopatógenos de relevancia global se ha documentado la resistencia de *Botrytis cinerea* a los fungicidas tipo benzimidazoles

como benomil y carbendazima, también a carboxina, benodanilo, flutolanilo, entre otros, del grupo de los inhibidores de la succinato deshidrogenasa, y a ciprodinilo, mepanipirima y pirimetanilo, del grupo del grupo de las aninilinopirimidinas (revisado en Brauer et al., 2019). Para esta especie de fitopatógeno, la resistencia a los benzimidazoles se debe a mutaciones puntuales en el gen de la β-tubulina, en el segundo caso a mutaciones en el gen de la de la succinato deshidrogenasa. En el caso de las aninilinopirimidinas, no se conoce con precisión el mecanismo de resistencia, aunque se ha sugerido que puede deberse a la sobreexpresión de bombas de eflujo tipo ABC o a la modificación del sitio blanco (revisado en Brauer et al., 2019). Así, tanto la necesidad de asegurar la producción agrícola, como la baja en la eficacia de los agroquímicos empleados para ciertas plagas y enfermedades, ha llevado a la búsqueda de alternativas para el combate de plagas y fitopatógenos, que no sean tóxicas para los ecosistemas y el ser humano. Una de esas alternativas es el "control biológico" o "biocontrol". Entre las definiciones de "biocontrol" o "control biológico" podemos mencionar algunas relativamente simples, como "el control de un organismo por parte de otro" (Narayanasamy, 2013), o bien otrás más complejas que lo mencionan como "el uso de organismos naturales o modificados, genes o productos de genes para reducir los efectos de organismos no deseados (patógenos) y favorecer organismos deseables como cultivos, árboles, animales, insectos y microorganismos" (Narayanasamy, 2013). Existe una gran variedad de organismos biocontroladores que incluyen principalmente a bacterias, hongos, insectos y otros invertebrados, los cuales se comercializan para ser aplicados en diversos cultivos de relevancia agronómica a nivel global, generando en conjunto un nicho de mercado importante (van Lenteren et al., 2018). Entre las características que hacen atractivo el uso de organismos para el control biológico se incluyen la baja toxicidad hacia la salud humana y vida silvestre, la reducción en la cantidad de residuos tóxicos derivados de los pesticidas, el éxito para el combate de plagas y patógenos que han desarrollado resistencia a los agroquímicos convencionales, entre otros (van Lenteren et al., 2018).

Entre los hongos, uno de los géneros más empleados para el biocontrol de microorganismos fitopatógenos es *Trichoderma* (Guzmán-Guzmán et al., 2019; Sood et al., 2020), aunque especies de otros géneros como *Ampelomyces quisqualis*, *Coniothyrium minitans* y *Gliocladium virens* se han incorporado con éxito en productos de biocontrol (De Silva et al., 2019). Aunque los hongos basidiomicetes no han sido ampliamente evaluados en cuanto a su capacidad de

antagonismo y biocontrol de hongos fitopatógenos, se ha documentado el antagonismo *in vitro* de especies de basidiomicetes hacia microorganismos fitopatógenos mediante el empleo de diversos mecanismos (Gholami et al. 2019; White and Traquair 2006). Además, una gran variedad de extractos y metabolitos producidos por hongos basidiomicetes poseen actividad antimicrobiana capaz de inhibir el crecimiento de microorganismos patógenos de relevancia agrícola (Shen et al. 2017). Es por esto por lo que en el presente trabajo se busca explorar la actividad antagónica e inhibitoria de cepas de basidiomicetes silvestres hacia hongos y oomicetes fitopatógenos.

I.2 El Reino Fungi, diversidad y relevancia ecológica y biotecnológica

El reino Fungi es un grupo taxonómicamente complejo, en el que se considera que existen entre 2.2 y 3.8 millones de especies (Hawksworth y Lücking, 2017). Las especies dentro de este reino presentan diversos estilos de vida, incluyendo parasitismo, saprofitismo y mutualismo con especies de otros grupos biológicos, tanto en ambientes terrestres como acuáticos (Naranjo-Ortiz y Gabaldón, 2019). Los hongos también presentan una notable variedad morfológica macroscópica y microscópica y de diferenciación celular en su ciclo de vida, además de variaciones en la estructura genómica y sus procesos metabólicos (Naranjo-Ortiz y Gabaldón, 2019). Dentro del reino de los hogos destacan los *phyla* Ascomycota y Basidiomycota, por ser los grupos más estudiados y mejor conocidos, los cuales incluyen a 83,837 y 48,405 especies, respectivamente (James et al., 2020). Ambos grupos conforman, junto con Entorrhizomycota, un grupo monofilético robusto designado como el subreino Dikarya, que se caracteriza por formar micelios dicarióticos, en el que se encuentra el 97% de las especies fúngicas descritas en la actualidad (Hibbett et al., 2018; James et al., 2020).

El phylum Basidiomycota representa el 35% de los hogos descritos, y comprende a los subphyla Pucciniomycotina, Ustilaginomycotina, Agaricomycotina y Wallemiomycotina (Tedersoo et al., 2018). Dentro de Agaricomycotina se encuentran especies de gran relevancia científica, ecológica, socioeconómica y biotecnológica. Por ejemplo, *Coprinopsis cinerea* y *Schizophyllum commune* han sido útiles como modelos de investigación eucariótica en desarrollo, sistemas de reproducción sexual y diferenciación, debido a la factibilidad y facilidad para obtener su ciclo de vida completo en el laboratorio (de Mattos-Shipley et al., 2016). Por otra parte, *Agaricus bisporus* y *Pleurotus ostreatus*, especies identificadas coloquialmente como

champiñón y seta, son basidiomicetes de relevancia económica y alimenticia, ya que constituyen el porcentaje mayoritario de las 9 926 966 ton de hongos comestibles que se consumen anualmente, representando un alimento saludable como excelente fuente de proteínas, vitaminas, fibras y minerales, pero con bajo contenido en calorías y grasas, y libres de colesterol (de Mattos-Shipley et al., 2016). De especie relevancia en biotecnología, es la producción de metabolitos con actividad biológica por distintos grupos dentro de Basidiomycota, particularmente útiles para el tratamiento de enfermedades infecciosas y crónico-degenerativas, entre los taxa que destacan por dicha característica y que han sido más estudiados al respecto se encuentran las especies del género *Ganoderma* (de Mattos-Shipley et al., 2016). Por último, tanto su actividad saprofítica como sus asociaciones simbióticas, desde mutualistas hasta parasíticas, con especies vegetales y otros grupos biológicos, los basidiomicetes juegan un papel ecológico relevante en los ciclos de los ecosistemas forestales y en la producción agrícola (de Mattos-Shipley et al., 2016). Entre estas últimas se puede nombrar a especies como *Laccaria bicolor*, que forma micorrizas con especies arbóreas, y *Leucoagaricus gongylophorus* simbionte de distintos grupos de hormigas, mientras que en el grupo de fitopatógenos se puede mencionar a *Armillaria mellea*, patógeno de un amplio grupo de especies arbóreas de relevancia forestal, y a *Moniliophthora perniciosa*, que causa pérdidas importantes en la producción de chocolate al atacar la planta y el fruto del árbol *Theobroma cacao* (de Mattos-Shipley et al., 2016).

I.3.1 El género *Ganoderma*

Como se especificó anteriormente, el género *Ganoderma* se ubica dentro del subphylum Agaricomycotina, dentro de la clase de Agaricomycetes en el orden de los Polyporales y la familia Polyporaceae (synonym. Ganodermataceae (Donk) Donk 1948), siendo uno de los géneros más estudiados dentro de dicha familia, en el que todavía se presentan dudas taxonómicas y filogenéticas, debido a su complejidad y variabilidad (Richter et al., 2015; Justo et al., 2017). El número de especies incluidas dentro del género se estima en 214, aunque existen 420 registros con estatus legitimado en Mycobank (http://www.mycobank.org/), y se han propuesto otros números de acuerdo con los criterios de discriminación del género y la delimitación de especies (Richter et al., 2015; Papp, 2019). El género *Ganoderma* es cosmopolita, con presencia en ecosistemas templados y tropicales, aunque todavía está pendiente un trabajo biogeográfico de los distintos complejos, agregados y especies dentro del

género que permitan una mejor comprensión de su ecología y distribución global en distintos ecosistemas (Richter et al., 2015; Papp, 2019). Considerando las controversias taxonómicas anteriormente mencionadas, en México se ha documentado la presencia de 15 especies del género *Ganoderma* subgénero *Ganoderma*, e incluyen a *G. colossus*, *G. curtisii*, *G. mexicanum*, *G. oerstedii*, *G. oregonense*, *G. perturbatum*, *G. resinaceum*, *G. sessile*, *G. sessiliforme*, *G. subincrustatum*, *G. weberianum* y *G. zonatum* (Torres-Torres et al., 2015). Las distintas especies se han documentado en regiones tropicales y templadas, con una predominancia de registros para el centro y sureste del país (Torres-Torres et al., 2015).

Las especies del género *Ganoderma* se han descrito como fitopatógenos de diversas especies arbóreas, particularmente causando pudrición de raíz entre las que se encuentran *G. philippii* causante de dicha enfermedad en *Acacia mangium* y *Eucalyptus* sp. (Coetzee et al., 2011), *G. steyaertanum* en *A. mangium* y *Acacia auriculiformis* (Hidayati et al., 2014), *G. enigmaticum*, *G. destructans* y *G. austroafricanum* en *Jacaranda mimosifolia* (Coetzee et al., 2015), y *G. adspersum* en *Pinus pinea* (De Simone y Annesi, 2012). Particularmente en India, se ha reportado la presencia de 13 especies de *Ganoderma* causando enfermedades en 144 especies arbóreas distintas, incluyendo a los géneros *Acacia*, *Cassia*, *Cedrus*, *Ficus*, *Picea*, *Pinus*, *Popolus* y *Quercus*, entre otros (Sankaran et al., 2005). No obstante, se ha sugerido que algunas especies como *G. sessile* pueden ser saprófitos facultativos (Loyd et al., 2018) o ser endófitos en troncos de árboles vivos (Martin et al., 2015).

Entre los metabolitos con actividad biológica descritos en las especies del género *Ganoderma* se encuentran polisacáridos, particularmente β- 1, 3 glucanas intracelulares y extracelulares, triterpenoides designados como ácidos ganodéricos y ganoderol, proteínas inmunomoduladoras, esterol y nucleótidos (Hapuarachchi et al., 2017; Hsu y Cheng, 2018). Entre las actividades farmacológicas de los extractos y metabolitos descritas para *Ganoderma* spp. incluyen la capacidad antitumoral, inmonumoduladora, hepatoprotectora, antioxidante y antimicrobiana (Wang et al., 2020). Por la capacidad de las especies del género *Ganoderma* para producir metabolitos secundarios, las cepas silvestres de las especies de dicho taxón pueden ser utilizadas como una herramienta en el desarrollo de nuevos compuestos con actividad antimicrobiana (Zjawiony, 2004; Agyare y Agana, 2019). Un aspecto relevante de los estudios sobre extractos y metabolitos bioactivos del género *Ganoderma* es que la gran mayoría de los estudios se han realizado en la especie *G. lucidum* (Ahmad, 2018), particularmente en aislados geográficos

provenientes de China (Hapuarachchi et al., 2018). Los estudios en los que se documenta la actividad antibacteriana en otras especies del género son escasos, e incluyen a los extractos etanólicos del basidiocarpo de *G. atrum* (Li et al., 2012), extractos orgánicos del basidiocarpo y micelio *G. carnosum* (Yamaç y Bilgili, 2006), así como a los exopolisacáridos de *G. applanatum* (Osińska-Jaroszuk et al., 2014). No obstante, existen estudios que documentan nula o baja actividad antimicrobiana, como los extractos acuosos y metanólicos de los basidiocarpos de *G. applanatum* y *G. resinaceum* (Zengin et al., 2005). Además, los metabolitos y las actividades farmacológicas caracterizadas se han realizado mayoritariamente en extractos de basidiocarpo, siendo pocos los estudios que evalúan la capacidad del micelio vegetativo para producir metabolitos.

I.3.2 El género *Irpex*

El género *Irpex*, al igual que el caso de *Ganoderma*, se encuentra dentro del orden de los Poliporales, y la clasificación más reciente lo ubica dentro de la familia Irpicaceae (He et al., 2019). La especie tipo de este género, la cual permaneció como la única dentro de dicho taxón por largo tiempo, es *I. lacteus*, y aunque inicialmente se consideró a *I. canescens* como una especie distinta, fue descartada como una fase de crecimiento de la primera especie (Maas Geesteranus, 1974). Posteriormente se añadió a *I. hydnoides* como una nueva especie del género, con base en evidencia morfológica del basidiocarpo, del crecimiento colonial en medio PDA y la reconstrucción filogenética empleando la región ITS (Lim y Jung, 2003). Más tarde, con base en evidencia morfológica, macro y microscópica, se sugirió la nueva especie *I. cremicolor* (Miettinen et al., 2007), y agregando filogenia molecular a *I. hacksungii* (Lee et al., 2008). También, se ha sugerido trasladar la mayoría de las especies del género estrechamente relacionado *Steccherinum* al género *Irpex* (Kotiranta y Saarenoksa, 2002), sin embargo, es una propuesta en la que no todos los autores coinciden (Gorjón, 2020), y no es respaldada por la evidencia filogenética, ya que las especies de ambos géneros no se agrupan en el mismo clado terminal (Miettinen et al., 2012). La especie tipo *I. lacteus* se encuentra en zonas templadas del hemisferio norte, a menudo se encuentra en América del Norte y Europa (Novotný et al., 2009). En México, de acuerdo con las bases de datos de la CONABIO y GBIF se encuentra distribuido en los estados de Sonora, Guanajuato, Hidalgo, Querétaro, Morelos, Veracruz, Nuevo León, Jalisco, San Luis Potosí y Chiapas.

A nivel biotecnológico, *I. lacteus* se ha evaluado particularmente su capacidad para producir las enzimas ligninolíticas extracelulares características de los hongos de pudrición blanca, incluidas la lacasa (Cajthaml et al., 2008; Svobodová et al., 2008), la lignin-peroxidasa (LiP, Rothschild et al., 2002), la manganeso-peroxidasa (MnP, Shin et al., 2005; Baborová et al., 2006; Qin et al., 2014; Li et al., 2019), y la más recientemente descrita peroxidasa decolorizaste, (DyP, Salvachúa et al., 2013). Esta capacidad catalítica ha sido evaluada con relación a la degradación de la lignina de rastrojo como alimento animal o para la producción de biocombustibles (Xu et al. 2010; Yang et al., 2014; Salvachúa et al., 2013; Qin et al., 2018), y la degradación de colorantes y distintos compuestos xenobióticos recalcitrantes (Hwang y Song, 2000; Baborová et al., 2006; Choi et al., 2013; Qin et al., 2014).

En los últimos años, además de las aplicaciones biotecnológicas anteriormente mencionadas con relación a la capacidad ligninolítica de *I. lacteus*, se ha evaluado con mayor frecuencia su capacidad para producir metabolitos con actividad farmacológica. No obstante, uno de los primeros metabolitos caracterizados hace más de 35 años fueron tres compuestos con actividad nematicida, dos de ellos derivados del furaldehído y otro el 3-p-anisoloxipropionato (Hayashi et al., 1981). El segundo metabolito identificado en esta especie fue la frecuentina, un derivado de aldehído descrito previamente como antifúngico, inhibiendo eficazmente la germinación de esporas de *Botrytis allii*, *Penicillium gladioli*, *Stachybotrys atra* y *Mucor mucedo* (Curtis et al., 1951). Posteriormente se ha documentado que *I. lacteus* produce los terpenos antifúngicos conocidosol B, 5-demetil conocenol C, irpenigirina B, así como el 4- (4-dihidroximetilfenoxi) benzaldehído, solo por fermentación en sustrato sólido, pero no en medio líquido (Wu et al., 2019). También el compuesto volátil 5-pentenil-2-furaldehído, ha mostrado actividad antifúngica contra los fitopatógenos *Fusarium oxysporum* f. sp. *lycopersici*, *Colletotrichum fragariae* y *Botrytis cinerea* (Koitabashi et al., 2004). Finalmente, el microdiplodiasol producido por esta especie es un derivado de la xantona que inhibe el crecimiento de la especie fúngica *Microbotryum violaceum* (Siddiqui et al., 2011). Más recientemente se han descrito un sesquiterpeno y tres derivados del furano, algunos de los cuales presentan actividad antimicrobiana moderada y buena capacidad antioxidante (Duan et al., 2019).

El Laboratorio de Conservación y Biotecnología Microbiana del Centro Multidisciplinario de Estudios en Biotecnología de la Facultad de Medicina Veterinaria de la UMSNH cuenta con las cepas CMU-0113 de *Ganoderma* sp. y CMU-0113 de *Irpex lacteus*, las cuales forman parte del Cepario Michoacano Universitario (CMU) y fueron aisladas de una muestra de suelo en el año 2009, en el estado de Michoacán de Ocampo. De dichas cepas se desconoce la capacidad para producir metabolitos con actividad antibacteriana, lo que se pretende evaluar en el presente proyecto.

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II. JUSTIFICACIÓN

Por todo lo anterior, y dado que la aparición de cepas de oomicetes y hongos con resistencia a fármacos de uso convencional representa un problema de salud pública y producción agrícola que requiere de estrategias de prevención y combate es necesario implementar procesos biotecnológicos para la producción de nuevos compuestos con actividad farmacológica. La producción de metabolitos secundarios fúngicos es una alternativa viable en la obtención de nuevos fármacos. Los basidiomicetos representan una fuente natural potencial en la producción de metabolitos secundarios, específicamente las cepas de hongos silvestres del estado de Michoacán son una fuente importante de metabolitos con actividad farmacológica, que posibilitan el desarrollo biotecnológico regional para problemas de relevancia global con especificidades a nivel local.

Todos los metabolitos secundarios que producen los macromicetos y que les dan características nutraceúticas especiales han sido en su mayoría aislados del carpóforo y en la actualidad algunos de estos compuestos se extraen para ser empleados en la producción de medicamentos comerciales (Smith et al., 2002). Investigaciones preliminares realizadas sobre basidiomicetos (Miles y Chang, 2004) han puesto de manifiesto que la proporción de estos compuestos varía tanto con el estadio fisiológico del hongo como con el medio en que es cultivado. Aún más, tanto en hongos en general (Quesada-Moraga y Vey, 2003), como en basidiomicetes y *Ganoderma* spp. en particular (Saltarelli et al., 2015) se han reportado variaciones intra-específicas en la producción de metabolitos secundarios cultivando el micelio vegetativo de distintas cepas en las mismas condiciones. Por lo que aun en especies fúngicas relativamente bien estudiadas en relación a la producción de metabolitos secundarios, el análisis de nuevos aislados geográficos puede resultar en el hallazgo de metabolitos previamente no reportados. Si bien es cierto que la obtención de cuerpos fructíferos es sencilla debido a que se pueden usar diferentes sustratos baratos y accesibles, el cultivo tradicional no permite obtener los bioactivos en breves periodos de tiempo (días) y con procesos de purificación sencillos (Stamets, 2011). Es aquí donde el desarrollo de la biotecnología, por la facilidad en el manejo de sus variables, ha permitido realizar el cultivo en medio líquido del micelio de basidiomicetos con aumento en la producción de sus metabolitos, lo que ha impulsado considerablemente la obtención y determinación estructural de compuestos con potencial como medicamentos.

III. HIPÓTESIS

Las cepas de basidiomicetes de *Irpex lacteus* (CMU-0113) y *Ganoderma* sp. (CMU-8413) inhiben el crecimiento fúngico y de oomicetos in vitro, presentando diferencias significativas en el metaboloma extracelular.

IV. OBJETIVOS

IV.1 Objetivo general

Analizar la actividad contra hongos y oomicetos fitopatógenos del micelio y los concentrados extracelulares de las cepas CMU-0113 de *Ganoderma* sp. y CMU-8413 de *Irpex lacteus*.

IV.2 Objetivos específicos

1. Evaluar la actividad antifúngica/antioomicetos del micelio vegetativo de la cepa CMU-0113 de *Ganoderma* sp. y CMU-8413 de *I. lacteus*.

2. Caracterizar la diversidad de metabolitos presentes en los concentrados extracelulares producidos por las cepas de estudio en cultivo axénico.

3. Realizar un análisis bioinformático de los genes y las enzimas asociadas a la producción de metabolitos secundarios mayoritarios en *Ganoderma* spp.

V. RESULTADOS

V.1 Capítulo 1:

Growth Inhibition of Phytopathogenic Fungi and Oomycetes by Basidiomycete *Irpex lacteus* and Identification of its Antimicrobial Extracellular Metabolites

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SHORT COMMUNICATION

Growth Inhibition of Phytopathogenic Fungi and Oomycetes by Basidiomycete *Irpex lacteus* and Identificationof its Antimicrobial Extracellular Metabolites

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Abstract

In dual culture confrontation assays, basidiomycete *Irpex lacteus* efficiently antagonized *Fusarium* spp., *Colletotrichum* spp., and *Phytophthora* spp. phytopathogenic strains, with growth inhibition percentages between 16.7–46.3%. Antibiosis assays evaluating the inhibitory effect of soluble extracellular metabolites indicated *I. lacteus* strain inhibited phytopathogens growth between 32.0–86.7%. Metabolites in the extracellular broth filtrate, identified by UPLC-QTOF mass spectrometer, included nine terpenes, two aldehydes, and derivatives of a polyketide, a quinazoline, and a xanthone, several of which had antifungal activity. *I. lacteus* strain and its extracellular metabolites might be valuable tools for phytopathogenic fungi and oomycete biocontrol of agricultural relevance.

K e y w o r d s: antagonism, antifungal, extracellular metabolites, mycelium, terpenes

Phytopathogenic fungi and oomycetes have caused significant losses in several crop production around the world (Dean et al. 2012; Kamoun et al. 2015). Disease control caused by these pathogens depends to a large extent on agrochemicals, which use has increased worldwide (Carvalho 2017); however, they have accumulated in the trophic chains, affecting wildlife and livestock and causing public health problems (Bourguet and Guillemaud 2016). On the other hand, phytopathogenic microorganisms have increased their resistance to several agrochemicals used to fight them (Sparks and Lorsbach 2017), so they have become less effective. Therefore, the use of alternative plant protection biocontrol methods has been intensively explored during the last decades. Biocontrol methods include both preparations containing living microorganisms and bioactive metabolites obtained from organic or aqueous extracts of different taxa, which may be specific to those that need to be controlled and have low toxicityto wild and human life (Loiseleur 2017).

Previous studies have documented efficient *in vitro* antagonism of basidiomycete species against phytopathogenic microorganisms, through the use of more than one mechanism (White and Traquair 2006; Gholamiet al. 2019). A wide variety of extracts and metabolites obtained from basidiomycete fungi have shown antimicrobial activity, sufficient for growth inhibition of pathogenic microorganisms of medical and agricultural relevance (Shen et al. 2017). Due to the intrinsic ecophysiological role, metabolite production with antimicrobial activity by vegetative mycelium is relevant for species competition; hence, not only antimicrobial activity of biomass extracts but also extracellular filtrates obtained from liquid cultures of vegetative mycelium has been evaluated (Shen et al. 2017). Here we analyzed a wild *I. lacteus* strain antagonist activity against worldwide relevant phytopathogenic fungi and oomycete species. We also conducted a chemical characterization of extracellular filtrates of such strain aiming to identify putative metabolites with antifungal/antimicrobial activity. The

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I. lacteus strain CMU-8413 was isolated from basidiocarps collected in September 2013 from the communityof Atécuaro, Michoacán, Mexico. Both data on collectionand phylogenetic identification of the strain have been previously reported (Damián-Robles et al. 2017). The strain has been deposited in the Laboratorio de Conservación Microbiana y Biotecnología del Centro Multidisciplinario de Estudios en Biotecnología de la Facultad de Medicina Veterinaria y Zootecnia, de la Universidad Michoacana de San Nicolás de Hidalgo, and it may be available for research purposes upon request. The phytopathogenic strains tested correspond to fungi *Fusarium pseudocircinatum*, *Fusarium mexicanum*, *Colletotrichum coccodes*, *Colletotrichum gloeosporioides*, and oomycetes *Phytophthora capsici* and *Phytophthora cinnamomi*. All these phytopathogens have been isolated from crop fields in Michoacán, and they have been properly identified and kindly provided by Dr. Sylvia P. Fernández-Pavía, of the Universidad Michoacana.

Antagonism in dual culture and antibiosis assays were studied according to Steyaert et al. (2016) in three independent replicas for each phytopathogen. The incubation time was adjusted as described below,and incubation temperature to 28°C. The inoculum of *I. lacteus* and phytopathogens for these assays con- sisted of a 6 mm plug obtained from the edge of an actively growing colony in PDA at 28°C. For antago- nism assays, phytopathogens were inoculated at one extreme of a 90 mm Petri plate containing potato dextrose agar (PDA) medium, and they were incubated until the colony reached a 2 cm radius. At this stage, the PDA Petri plate opposite extreme was inoculated in the same way with the *I. lacteus* strain, and then incubation was resumed. The fungi colony diameter was measured every 24 h. A respective control of axenic cultures for each phytopathogenic strain was incubated in the same condition. For antibiosis assays, Petri plates containing PDA were covered with sterilized cellophane sheets, and they were inoculated with *I. lacteus* strain in the center. After a 4-day incubation, the cellophane sheets with the mycelium were aseptically removed. This same PDA plate, on which the *I. lacteus* strain previously grew, was used for phytopathogens inoculation and incubation in independent assays, and colony diameter was measured every 24 h. Each phytopathogenic strain was inoculatedin a fresh PDA medium and was incubated under the same conditions as a control.

For antagonism and antibiosis, assays were finishedwhen the mycelium of each phytopathogen in the con- trol plate had covered 2/3 of the surface area; then, the colony diameter was measured expressing the growth inhibition percentage according to the formula:

$$\% \text{ inhibition} = [(D1 - D2)/D1 \times 100]$$

where D1 = the phytopathogen colony diameter growing in PDA (fresh/axenic), and D2 = the phytopathogen colony diameter growing in dual culture or in the medium that contained *I. lacteus* which had been previously incubated.

In antagonism in dual culture assays, *I. lacteus* showed significant growth inhibition against *F. pseudo-circinatum* (46.3%), *F. mexicanum* (16.7%), *C. coccodes* (22.8%), *P. cinnamomi* (35.0%) and *P. capsici* (22.9%) (Fig. 1), and it was not able to antagonize *C. gloeospo-rioides*. Using the same test, White and Traquair (2006) found that *I. lacteus* showed efficient growth inhibition of *Botrytis cinerea*, and this previous workis the only antagonism study between *I. lacteus* and phytopathogenic fungi, in addition to results reported here. Antibiosis assays showed even better *I. lacteus* growth inhibitory activity against the six phytopatho- genic strains tested, with growth inhibition percent-ages fluctuating between 32.0 and 86.7%. *C. coccodes* was the most susceptible species (Fig. 1). The antibiosisassay is used to determine if a fungal strain produces non-volatile metabolites that diffuse into the medium and affect phytopathogenic fungi growth (Steyaert et al. 2016). As far as we could document, there has beenno previously published work on antibiosis assays that used basidiomycetes to test their *in vitro* activity against phytopathogenic fungi/oomycetes.

Antifungal activity of aqueous and organic extracts from basidiocarp and vegetative mycelium of *I. lacteus*(Shen et al. 2017) had been documented. However, reports evaluating the antifungal activity of extracellular broth filtrates from *I. lacteus* are scarce; consequently, in this work, it was of particular interest to assess such effect and identifying metabolites secreted by studiedstrain. The studied strain growth kinetic was conductedin 250 ml Erlenmeyer flasks with 50 ml of potato dex- trose broth (PDB) medium, inoculated with six inocula obtained as previously described. The inoculated flasks were incubated at 125 rpm of orbital shaking and 28°C for 14 days. The mycelium dry weight was determined every 24 hours, and the CMU-8413 strain reached the stationary phase after a seven-day incubation (data not shown). Extracellular filtrates were recovered three days after the studied strain reached the stationary phase at the end of 10 days of incubation. Broth culture at the stationary phase was filtered through Whatman No. 1 paper. The filtrate broth was recovered and concentrated by evaporation to dryness in a rotary evaporator at 70°C, without adding any solvent. Concentrates obtained were stored for no more than one week in1 ml vials at –4°C until biological assays were carried out. Compounds identification in culture filtrate of independent samples for each assay was performed by UPLC (Acquity™ Class I, Waters Corporation) coupled to an orthogonal QTOF mass spectrometer (Synapt™ G1, Waters Corporation), as described elsewhere (Varela-Rodríguez et al. 2019). MS data were

Fig 1. Phytopathogens growth inhibition in dual culture antagonism and antibiosis assays by *I. lacteus* (CMU-8413). In dual culture antagonism assays, *I. lacteus* was inoculated at the left. Tested phytopathogens were *Fusarium pseudocircinatum*, *Fusarium mexicanum*, *Colletotrichum coccodes*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, and *Phytophthora cinnamomi*. Assays were conducted in potato dextrose agar (PDA) medium at 28°C. Growth inhibition percentages are the mean of three independent assays and standard deviation (S.D.) is shown in parenthesis. Statistically significant ($p < 0.01$) growth inhibition values when compared with their respective control are indicated with an asterisk. Significance was determined by Student's *t*-tests, independent by groups, and they were carried out using STATISTICA data analysis software system (StatSoft, Inc. 2007, version 7. http://www.statsoft.com).

continuously acquired and processed with MassLynx® (version 4.1, Waters Corporation), and metabolites were putatively identified with Progenesis® QI for small molecules (Nonlinear Dynamics version 2.3, Waters Corporation) using Chemspider and Progenesis MetaScope as identification methods. The Progenesis QI software for small molecules considers precursormass accuracy < than 5 ppm, fragmentation pattern, and isotopic similarities, each accounting for 20%. The maximum score is 60% (20 + 20 + 20), pre-identified metabolites have at least 50% of the total score.

A total of 14 extracellular metabolites (Table I)belonging to five chemical groups were found in the *I. lacteus* broth filtrate. The largest chemical group found was terpenes with nine metabolites, followed by aldehydes with two metabolites. The remaining metabolites found belong to polyketides, quinazolines, and xanthone derivatives, with one metabolite each. All metabolites found in *I. lacteus* extracellular broths were previously described in other basidiomycete or ascomycete species (Table II). Eight have been described as extracellular in broth media, like here. Terpenes produced by *I. lacteus* showed antifungal activity against *Nigrospora oryzae*, *C. gloeosporioides,* and *Didymella glomerata* (Wu et al. 2019). It has been previously documented among aldehydes that *I. lacteus* produces 5-pentenyl- 2-furaldehyde, a potent antifungal volatile compound tested against the phytopathogens *Fusarium oxysporum*

f. sp. *lycopersici*, *Colletotrichum fragariae,* and *B. cinerea* (Koitabashi et al. 2004). Also, a frequentin identified here in the *I. lacteus* broth is an aldehyde derivative that has been previously described as antifungal efficiently inhibiting spore germination in *Botrytis allii*, *Penicillium gladioli*, *Stachybotrys atra,* and *Mucor mucedo* (Curtis et al. 1951). Finally, microdiplodiasol is a xanthone derivative inhibiting the growth of fungal species *Microbotryum violaceum* (Siddiqui et al. 2011). Both, previous studies and extracellular metabolites secreted to the broth by the CMU-8413 *I. lacteus* strain allowedus to anticipate its efficient antagonism and growth inhibition of phytopathogenic fungi and oomycetes.

Phytopathogens growth inhibition assays con- ducted in solid PDA medium were used as indicative of extracellular inhibitory metabolites by CMU-8413

Table I
Extracellular metabolites produced by *Irpex lacteus* (strain CMU-8413) at stationary phase.

Compoundname	Molecular formula	Observed m/z	Adduct	Main fragment ions m/z	Compound class
Apotrichodiol	$C_{15}H_{24}O_3$	251.1652	M-H	233.1547	Sesquiterpene
Apotrichothecene	$C_{15}H_{24}O_2$	201.1637	M+H-$2H_2O$	157.1481, 186.1403, 173.1348, 159.1185, 145.1029, 128.0639, 115.0558, 105.0711	Sesquiterpenoid epoxide
Blennin D	$C_{15}H_{22}O_4$	265.1436	M-H	237.1386, 221.1418, 205.1473, 191.1441, 187.1406, 175.1412	Sesquiterpene
Collybial	$C_{15}H_{20}O_2$	215.1424	M+H-H_2O/ M+H	187.1512, 173.1325, 159.0804, 157.1008, 142.0792, 128.0869, 115.0534	Sesquiterpene
Cyclocalopin A	$C_{15}H_{20}O_6$	295.1168	M-H	251.1160, 233.1149, 221.1123, 215.0924, 203.1027, 189.1142, 173.0818, 167.0634, 157.0526	Sesquiterpene
Dehydrooreadone	$C_{14}H_{18}O_3$	279.1218	M+FA-H	261.0987, 233.1046, 219.0902, 201.0804, 189.1178, 185.0502, 183.0663, 173.0874	Sesquiterpene
Dictyoquinazol A	$C_{17}H_{16}N_2O_4$	311.1090	M-H	267.1079, 237.0366, 205.1134, 193.1114, 187.1005, 175.1036, 159.0740, 151.0638, 149.0482	Quinazoline
Dihydromarasmone	$C_{15}H_{20}O_5$	279.1216	M-H	235.0976, 219.1390, 217.1131, 207.1277, 191.1342, 173.1250, 163.0871	Sesquiterpene
Frequentin	$C_{14}H_{20}O_4$	233.1181	M-H_2O-H	205.1234, 196.8934, 189.1195, 173.0864	Cyclohexanecarbaldehyde
Ganodermic acid Jb	$C_{30}H_{46}O_4$	453.3436	M+H-H_20	322.2571, 208.3768, 119.0945	Triterpene
Geosmin	$C_{12}H_{22}O$	203.1431	M+Na-2H	201.1177, 188.1100, 187.1010, 175.1426, 147.0792	Sesquiterpene
Microdiplodiasol	$C_{15}H_{18}O_7$	309.0948	M-H	265.0858, 203.0963, 193.1139, 187.0612, 175.1035	Xanthone derivative
Pandangolide 1	$C_{12}H_{20}O_5$	243.1242	M-H	203.6930, 181.1234	Polyketide
Piperdial	$C_{15}H_{22}O_3$	251.1617	M+H	233.1537, 215.1455, 205.1587, 191.1067, 187.1503, 177.0810, 159.1189, 145.1029	Unsaturated dialdehyde

Table II
The previous reports on extracellular metabolites produced by *Irpex lacteus* (strain CMU-8413) at stationary phase.

Metabolite	Fungal species	Source[a]	Bioactivity/Comment[a]	Reference
Apotrichodiol	*Fusarium* spp.	EM	mycotoxin	(Lebrun et al. 2015)
Apotrichothecene	*Fusarium* spp.	EM	mycotoxin	(Lebrun et al. 2015)
Blennin D	*Lentinellus cochleatus*	BE	inhibitor of leukotriene biosynthesis	(Wunder et al. 1996)
Collybial	*Collybia confluens*	EM	antibacterial, antiviral	(Simon et al. 1995)
Cyclocalopin A	*Caloboletus radicans*	BE	antioxidant, antibacterial	(Tareq et al. 2018)
Dehydrooreadone	*Marasmius oreades*	EM	NT	(Ayer and Craw 1989)
Dictyoquinazol A	*Dictyophora indusiata*/other basidiomycetes	BE	neuroprotective	(Lee et al. 2002)
Dihydromarasmone	*Marasmius oreades*	EM	antimicrobial	(Ayer and Craw 1989)
Frequentin	*Penicillium frequentans*/*Penicillium* spp.	EM	antifungal, antibacterial	(Curtis et al. 1951)
Ganodermic acid Jb	*Ganoderma lucidum*	ME	NT	(Shiao et al. 1988)
Geosmin	*Cortinarius herculeus*/*Cystoderma* spp.	BE	musty-earthy odor	(Breheret et al. 1999)
Microdiplodiasol	*Microdiplodia* sp.	EM	antifungal antibacterial	(Siddiqui et al. 2011)
Pandangolide 1	*Cladosporium marine*	EM	NA	(Gesner et al. 2005)
Piperdial	*Lactarius* spp./*Russula queletii*	BE	produced by damage	(Sterner et al. 1985)

BE – basidiocarp extract;
EM – extracellular metabolite produced by mycelium growing in broth;ME – mycelium extract; NT – not tested as far as we know;
NA – not biological activity detected in conducted assays

I. lacteus strain. Based on such results, the identification of the extracellular metabolites was conducted in broth culture to ensure metabolites production in sufficient quantity to be identified by MS. However,it should be noted that the antagonistic and antibiosis assays performed in a solid medium might induce specific metabolite production, which was not produced in broth. In this regard, it has been previously documented that *I. lacteus* produces the antifungal terpenes conocenol B, 5-demethyl conocenol C, irpenigirin B, as well as 4-(4-dihydroxymethylphenoxy)benzaldehyde only by fermentation in the solid substrate but not in broth culture (Wu et al. 2019). Furthermore, while *I. lacteus* only produced the first compound in co-culture with the phytopathogen *N. oryzae*, the remaining three metabolites were produced both in axenic culture andin co-culture. Therefore, extracellular metabolites identified here were not necessarily the same as induced by antagonism in dual culture and antibiosis assays. Co-culture induction of specific metabolites may explain high percentages of fungal/oomycete growth inhibition found here in antagonism/antibiosis assays; however, these findings require further study. Hence, extracellular metabolites produced by the CMU-8413 strain described in this investigation may be considered basal, given that no induction conditions to increase its secretion were evaluated. Based on culture conditions described here, it may be suggested that gene clusters responsible for the synthesis of the identified extracellular metabolites were constitutively expressed or they were not subject to carbon catabolite repression (CCR). CCR and transcription factors associated with secondary metabolism were molecular and metabolic issues scarcely studied in basidiomycetes (Adnan et al. 2018; García-Estrada et al. 2018). Necessary physiological and metabolic studies, like those conducted here, could helpto know what kind of metabolites may not be subject tothis metabolic control.

It should be noted that extracellular metabolites produced by the CMU-8413 strain have a wide variety of pharmacological activities, besides antimicrobials previously described (Table II). For instance, apotrichodiol and apotrichothecene are neurotoxic mycotoxins (Lebrun et al. 2015), whereas dictyoquinazol A has been described as neuroprotective (Lee et al. 2002) and blennins are inhibitors of leukotriene biosynthesis (Wunder et al. 1996). So, it will be necessary to select favorable culture conditions to develop an enrichment protocol or use heterologous expression systems (Qiao et al. 2019) to obtain the desirable metabolites and avoid toxic molecules' synthesis. Further studies incubating the CMU-8413 strain in different induction conditions could show its potential to secrete a chemical diversity of metabolites useful for agriculture or other biotechnological applications.

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Conflict of interest

The authors do not report any financial or personal connectionswith other persons or organizations, which might negatively affectthe contents of this publication and/or claim authorship rights to this publication.

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V.2 Capítulo 2:

Evaluation of *Ganoderma curtisii* antagonism and its extracellular broth filtrates for the growth inhibition of phytopathogenic fungi and oomycetes

Evaluation of *Ganoderma curtisii* antagonism and its extracellular broth filtrates for the growth inhibition of phytopathogenic fungi and oomycetes

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Abstract

The biocontrol of phytopathogenic microorganisms that affects crops of agronomic relevance constitutes an environmentally friendly alternative of agrochemicals that contaminate soil and water bodies, causing public health problems and have been least efficiency due to the emergence of resistant strains. Basidiomycete fungi and its secondary metabolites are good alternatives as biocontrol tools, but scarcely evaluated. Here we analyzed the potential of the basidiomycete strain CMU-0113 for its use in the biological control of phytopathogenic microorganisms. A robust phylogenetic analysis assigned the CMU-0113 strain to *Ganoderma curtisii* species. In dual culture assays, the strain CMU-0113 inhibits the growth of the fungal species *Fusarium mexicanum, Fusarium pseudocircinatum*, *Colletotrichum coccodes, Colletotrichum gloeosporioides*, and the oomycetes *Phytophthora capsici* and *Phytophthora cinnamomi* between 28.9 and 53.6%. Antibiosis assays in solid medium showed growth inhibition against *Fusarium* spp. and *Colletotrichum* spp. between 10.1 and 22.2% but did not inhibits the *Phytophthora* spp. strains. The extracellular broth filtrates of the strain CMU-0113 showed mycelial growth inhibition only against *F. mexicanum* (21.5%) in the disk diffusion assay. The putative identification by UPLC-QTOF-MS of 23 extracellular metabolites in the broth filtrate includes nine terpenes, extracellular metabolites found in the grow broth of the strain CMU-0113 included nine terpenes, three polyketides, two phenol derivatives, two lipids, and one of each seven other chemical groups. Of these, 2-aminoquinoline, enokipodines B and D, strobilurin A, as well as indolelactic and porric B acids are antifungal. The results indicate that the *G. curtisii* CMU-0113 strain and its extracellular metabolites might be valuable tools for the biocontrol of phytopathogenic fungi and oomycete of agricultural relevance.

Key words antagonism, antifungal, extracellular metabolites, mycelium, phytopathogens

Introduction

The rise in crops production since the second half of the last century has been accompanied with an increased use of chemical pesticides (Ramankutty et al., 2018; Sharma et al., 2020). Although agrochemicals have constituted valuable tools to control phytopathogenic microorganism, its environmental and health toxic effects have resulted in regulations in their application and in the search for environmentally friendly alternatives (Bourguet and Guillemaud, 2016; Sharma

et al., 2020). Biocontrol using a wide variety of plants, insect and microorganisms and its metabolites has been generated commercial products available to efficiently phytopathogens control (van Lenteren et al., 2018). Among phytopathogens, several fungal and oomycete species are considered has one of the main threats to crop production (Dean et al., 2012; Kamoun et al., 2015), and its main biocontrollers are other fungal species (van Lenteren et al., 2018). Despite its remarkable capability to produce extracellular enzymes and metabolites associated to control of phytopathogens (Schmidt-Dannert, 2016; Badalyan et al., 2002, 2008; De Silva et al., 2013), basidiomycete fungi have been scarcely evaluated on this regard, in contrast to ascomycete species (van Lenteren et al., 2018; Sivanandhan et al., 2017). Among basidiomycetes, *Ganoderma* genus stands out to produce a great variety of metabolites with antimicrobial activity (Baby et al., 2015; Sivanandhan et al., 2017), but secondary metabolites analysis has been conducted mainly in the basidiocarp than in mycelium, and its specific activity against phytopathogenic fungi and oomycetes has been scarcely evaluated. Furthermore, studies on extracellular metabolites active against phytopathogenic fungi/oomycetes produced by *Ganoderma* spp. mycelium in broth culture are evenly more restricted than those conducted of basidiocarp and mycelium extracts (Basnet et al., 2017; Ćilerdžić et al., 2016a, 2016b).

In a previous work, we reported the isolation and putative identification of a *Ganoderma* sp. strain from central Mexico (Mukhtar et al., 2019), being of interest both to corroborate its taxonomic assignation and to explore the potential to produce extracellular secondary metabolites. In view of the growing demand for molecules with potential agricultural applications that could help fight pesticide resistant microorganisms, this work evaluates the potential of this *Ganoderma* sp. strain to be an efficient biocontrol agent against phytopathogenic fungi and oomycete species of worldwide relevance. We conducted typical antagonism in dual culture assays between the studied strain and different phytopathogenic fungi/oomycete strains and tested e the growth inhibition effect of extracellular broth filtrates of the *Ganoderma* sp. mycelium against same phytopathogens. Also, we characterized the putative compounds composition in the extracellular filtrates of the studied strain, with the aim to identify secondary metabolites with antifungal/antimicrobial activity. Complementarily, a robust phylogenetic identification analysis to identify the *Ganoderma* sp. strain was carried out. Obtained results are contrasted with previous works analyzing other *Ganoderma* species and its metabolites for antagonism and biocontrol of phytopathogenic fungi/oomycetes.

Materials & Methods

Studied strain

The strain of *Ganoderma* spp. (CMU-0113) was isolated from basidiocarps collected in the community of Atécuaro, Michoacán, Mexico, in September 2013, and details on its isolation was previously reported (Mukhtar et al., 2019). The strain was deposited in the Microbial Conservation and Biotechnology Laboratory of the Multidisciplinary Center for Biotechnology Studies of the Faculty of Veterinary Medicine and Zootechnics, at the University of Michoacán. Test phytopathogenic strains correspond to the species of fungi *Fusarium pseudocircinatum*, *Fusarium mexicanum*, *Colletotrichum coccodes*, *Colletotrichum gloeosporioides*, as well as the oomycetes *Phytophthora capsici* and *Phytophthora cinnamomi*. These strains were gently provided by Dr. Sylvia P. Fernández-Pavia of the Plant Pathology Laboratory of the Institute of Agricultural and Forestry Research of the University of Michoacán.

Media

Potato dextrose agar (PDA) solid medium (Difco, USA) was used for the maintenance of the study strain, biomass generation for DNA extraction, inocula obtaining, and to perform the assays of antagonism in dual culture. The potato dextrose broth (PDB) liquid medium (Difco, USA) was used to perform growth kinetics and to obtain extracellular filtrates from the studied strain. Both media were prepared according to the supplier's instructions and sterilized for 15 minutes at 15 lb/in^2.

Inocula production

Both *Ganoderma* sp. studied strain and phytopathogenic strains were preserved in PDA medium at 4 ° C and reseeding every 15 days. Inocula were taken from the margin of a colony from a PDA preserving plate using a 6 mm internal diameter punch. These inocula were placed in the center of 90 mm Petri plates with PDA medium and incubated at 28 ° C until the mycelium covered 2/3 of the medium surface. Inocula obtained as previously described from the colony margin of these actively growing mycelium were used to initiate solid or liquid cultures.

Phylogenetic analysis

Genomic DNA from mycelium of the CMU-0113 strain was obtained as previously described (Damián-Robles et al., 2017). The primer pair ITS-1/ITS-4 (White et al., 1990) was used for amplification of the Internal Transcribed Spacer regions 1 and 2 (ITS1-5.8S- ITS2) of the rRNA gene cluster, and the primer pairs G-RPB2-F1/G-RPB2-R1 and G-TEF1-F1/G-TEF1-R1 (Cao et al., 2012) for the amplification of partial sequences of the *rpb2* and *tef1*-α genes, respectively. The amplification products were purified and sequenced by Elim Biopharm (Hayward, CA, USA).

The three DNA sequences obtained were submitted to the NCBI GenBank database using the Blast algorithm to search for similar sequences. Based on the Blast results, additional sequences of the same genetic regions of other *Ganoderma* species were retrieved from GenBank. Only complete sequences from well-identified *Ganoderma* species were selected for phylogenetic analysis. To conduct a robust phylogenetic analysis of the CMU-0113 strain, four phylogenetic trees were generated. The first three trees were obtained using the ITS region and the partial *rpb2* and *tef1-α* gene sequences retrieved from GenBank, and the second tree was reconstructed with the concatenated ITS-*rpb2*-*tef1*-α sequences. Because all trees generated similar topologies and clusters equal to the CMU-0113, only the ITS and concatenated tree are showed. The sequence of the ITS region is considering robust barcode for basidiomycetes and *Ganoderma* genus (Gunnels et al., 2020; Liao et al., 2015) and is available for most of *Ganoderma* species analyzed in the literature and even additional sequences for species not associated to any publication are found in Genbank. However, the sequences of the *rpb2* and *tef1*-α genes are not widely available as the ITS region for all *Ganoderma* species described in a phylogenetic analysis.

Thus, not all species used for the ITS phylogenetic analysis were used to reconstruct the tree with the concatenated sequences. In this last case, only selected species for which the three good quality sequences are available were used.

Sequences were aligned with SATé (Liu et al., 2009) using MAFFT (Katoh et al., 2005) as the external sequence alignment tool and RaxML (Stamatakis, 2006) as the tree estimator. The alignments were improved by hand at both extremes and were used for Maximum Likelihood (ML) and Bayesian Inference (BI) analyses to reconstruct the phylogenetic relationships. The fasta archive of the aligned sequences obtained by MAFFT were used to

search for the evolutive model selection (Kalyaanamoorthy et al., 2017) and obtained best model used for ML phylogenetic reconstruction with the ultrafast bootstrap approximation to assess branch support (Hoang et al., 2018), all this using the IQ-TREE server (Nguyen et al., 2015; Trifinopoulos et al., 2016). The best evolutive model for ITS and partial *rpb2* and *tef1-α* gene sequences were TNe+I+G4, K2P+I, and TIM3e+I, respectively, chosen according to Bayesian Information Criterion. For the concatenated tree reconstruction, the edge-proportional partition model was used (Chernomor et al., 2016). Bayesian inference (BI) was conducted in MrBayes 3.2.7 (Ronquist et al., 2012), which performed a sampling to determine the most appropriate model for nucleotide substitution during the run, so for the ITS phylogeny it was not necessary to estimate a priori such parameter. In the concatenated sequences the partitioned analysis was run using the previously obtained best evolutive models. For each sequence and concatenated sequences, four MCMC chains were run simultaneously starting from random trees for 10,000,000 generations. Trees were sampled every 1,000th generation for a total of 10,000 trees, and the first 2,500 trees were discarded as the burn-in phase. Posterior probabilities were determined from a majority-rule consensus tree generated with the remaining 7,500 trees. Because no incongruences were observed between MP tree and Bayesian inference, the different matrices were combined for the final phylogenetic tree. Trees were edited and visualized with in the iTOL web server (Letunic and Bork, 2019).

Antagonism in dual culture and antibiosis assays

Antagonism in dual culture and antibiosis assays were performed according to Steyaert et al., (2016), adjusting incubation times and culture conditions as described below. For antagonism assays, the phytopathogens were inoculated at one extreme of a 90 mm Petri plate with PDA medium, using a 6 mm plug inoculum obtained as previously described, and incubated at 28 °C until colony grew at 1-2 cm radius, depending on the growth rate of each phytopathogen. Once this stage was reached, *Ganoderma* sp. strain was inoculated on the opposite extreme of the Petri plate with a similar inoculum, in independent assays for each phytopathogen. Confronted strains were inoculated at 6 cm from each other and incubated in darkness at 28 °C. A corresponding control for each phytopathogenic strain, inoculated alone in PDA medium, was incubated in same condition.

For the antibiosis assays, the PDA medium of a 90 mm diameter Petri plate was covered with a sterilized cellophane sheet and inoculated in the center using a 6 mm plug inoculum obtained as previously described. After incubating at 28 °C for four days, time spent for the strain studied to cover 2/3 parts of the medium surface, the cellophane sheet with the mycelium were aseptically removed. This same PDA plate on which the *Ganoderma* sp. strain previously grew was used to inoculate the phytopathogens tested, in independent assays, and incubated at 28 °C. A corresponding control for each phytopathogenic strain, inoculated in fresh PDA medium, was incubated under the same condition.

For antagonism and antibiosis, the assays concluded when the mycelium of the control plate had covered 2/3 parts of the medium surface, and then the colony diameter of each phytopathogen was measured expressing the percentage of inhibition according to the formula: % inhibition = [(D1-D2)/D1 x 100], where D1 = diameter of the phytopathogenic colony growing in PDA (fresh/alone), and D2 = diameter of the phytopathogenic colony growing in dual culture or in the medium in which it had previously been incubated with *Ganoderma* sp. (Worasatit et al., 1994).

Mycelium growth kinetics

The growth kinetic of the study strain was conducted in 250 mL Erlenmeyer flasks with 50 mL of PDB medium, inoculated with six inocula obtained as previously described. The inoculated flasks were incubated at 125 rpm of orbital shaking and 28 °C for 14 days. The dry weight of the mycelium was determined every 24 hours.

Extracellular filtrate concentration

Broth culture at stationary phase was filtered through Whatman No. 1 paper, the mycelium was discarded, and the filtrate broth recovered and concentrated by evaporation to dryness in a rotary evaporator at 70 °C, without adding any solvent. The concentrates obtained were stored in vials of 1 mL at - 4 °C until the biological assays were carried out, for no more than one week.

Inhibition assay by agar dilution

This assay was performed according to Mihara et al., (2005) with minor modifications. The concentrated filtrate of the studied strain obtained as previously described was added at final

concentration of 2% (v/v) to PDA medium at 40 °C, before solidifying, and then poured into a 90 mm Petri plate. Once solidified, this supplemented medium was inoculated with an inoculum of each of the test phytopathogens in independent assays, incubating at 28 ° C, and measuring the colonial diameter every 24 h. PDA plates not supplemented with the filtrate of the study basidiomycete were used as a control.

Inhibition assay by disk diffusion

The disk diffusion method was conducted according to Gurgel et al., (2005) with some modifications. Phytopathogenic microorganisms of interest were inoculated in the center of a Petri plate with PDA medium, as previously described. Once the phytopathogen was inoculated, 0.5 cm diameter filter discs impregnated with 250 µL (10 mg of extract) of the extracellular filtrate concentrate was placed around the inoculum at 2 cm of distance. The plates were incubated at 28 ° C, and after 18 to 24 hours the presence or absence of inhibition halos around each of the discs was documented.

Identification of extracellular metabolites

Putative identification of compounds in culture filtrate was performed by UPLC (Acquity™ Class I, Waters Corporation) coupled to an orthogonal QTOF mass spectrometer (Synapt™ G1, Waters Corporation). The instrument was equipped with an Acquity™ HSS T3 (2.1 x 100 mm, 1.8 µm, Waters Corporation) column; the mobile phase used was Milli-Q purified water (solvent A) (Simplicity UV, Millipore) and acetonitrile (solvent B, J.T. Baker), both acidified with 0.1% formic acid, v/v) and were MS-grade. The solvents were degassed by sonication in an ultrasonic bath (Branson 1800, Emerson™). The compounds were eluted via a gradient separation as follows: 0 min, 2%B; 0.5 min, 2% B; 20 min, 100% B; 21.5 min, 100% B (column washing); 21.75 min, 2% B; 25 min (column re-equilibration). Samples (100 mg/mL) were filtered with a 0.20-µm PTFE syringe filter (Captiva Econo Filter, Agilent®) and maintained at 4 °C during the assay. The chromatographic conditions were performed as follows: the flow rate was set at 0.4 mL/min throughout the gradient from the UPLC system into the MS detector; the injection volume was 10 µL; and the column temperature was maintained at 40 °C. The samples were analyzed by electrospray ionization source (ESI) in negative and positive ionization mode. Spectra were acquired over a mass range from 50 to 1500 Da using the MSE acquisition mode.

The precursor ion collision energy was set to 6 eV (trap section) and 20 to 40 eV in the transfer section. The optimum values of the ESI-MS parameters were obtained as follows: capillary voltage, 3.0 kV; sampling cone, 40.0 V; extraction cone, 4.0 V; source and desolvation temperature, 120 °C and 350 °C; cone and desolvation gas flow, 20.0 L/h and 500 L/h, respectively. MS data were acquired in continuum mode and processed with MassLynx® (version 4.1, Waters Corporation), and the compounds were putatively identified with Progenesis® QI for small molecules (Nonlinear Dynamics version 2.3, Waters Corporation) using Chemspider and Progenesis MetaScope as identification methods. The search parameters were as follows: precursor tolerance of 30 ppm, theoretical fragmentation, and fragment tolerance of 30 ppm, with an isotope similarity filter of 90%. The databases consulted were AraCyc, PlantCyc, KEGG and HMDB. Features were pre-identified using Progenesis QI for small molecules, the software consider precursor mass accuracy < than 5 ppm, fragmentation pattern and isotopic similarities, each one account for 20 %, where the maximum score is 60 % (20+20+20), pre-identified features have at least 50% of the total score.

Statistical analysis

All previously described assays were conducted in three independent assays by triplicate. The significance was determined by Student's t-test independent by groups with a p-value less than 0.05 considered to be statistically significant. Student's t-test were performed using the data analysis software system STATISTICA (StatSoft, Inc. 2007, version 7. http://www.statsoft.com). Statistically significant ($P<0.05$) growth inhibition values when compared with their respective control are indicated with an asterisk.

Results

Phylogenetic analysis

Both the ML and Bayesian phylogenetic reconstruction performed with the three genes independently and the concatenated sequences displaces with high bootstrap confidence the CMU-0113 strain in the terminal clade that includes two different geographical isolates of *Ganoderma curtisii* (Figure 1). Such results strongly suggest that the study strain belongs to *G. curtisii* species.

Antagonism in dual culture and inhibition by antibiosis

In the antagonism in dual culture assays, the CMU-0113 strain inhibits the growth of phytopathogens between 28.9% for *F. mexicanum* and 53.6% for *P. cinnamomi* (Figure 2). The antibiosis assay did not show an inhibitory growth effect towards *Phytophthora* strains by the secreted metabolites of the CMU-0113 strain, but it did show a significant inhibitory effect ($p<0.05$) against *F. pseudocircinatum* (13.08%), *F. mexicanum* (22.22%), *C. coccodes* (10.12%) and *C. gloeosporioides* (19.87%). Representative colony growth and inhibition of both assays for each phytopathogen is showed in Figure 2.

Growth kinetics

In the growth kinetics of the CMU-0113 strain, mycelium reaches the mid-log phase at day seven and the stationary phase after 12 of the incubation (Figure 3). The total biomass yield at the end of 14 days of incubation was 99.53 mg (±6.38) of dry weight. The yield of the extracellular concentrate at same incubation time was 142.67 μg/μL (±5.1). Based in such result, the day 14 of incubation has been selected to recover the extracellular filtrates for further inhibition assays, time in which the strain had three days within the stationary phase.

Inhibition by agar dilution and disk diffusion assays

The results of the agar dilution assays show a marginal inhibitory effect on the growth of phytopathogens. Interestingly, the extracellular filtrate of the CMU-0113 strain showed growth promotion of *P. capsici*, increasing the diameter of the colony by 23.7% compared to the control (Figure 4). In a similar way, growth inhibition of phytopathogens by the disk diffusion assay of extracellular filtrates of the studied strain gave positive results of inhibition only against *F.*

mexicanum (21.5%). Representative colony growth and inhibition of both assays for each phytopathogen is showed in Figure 4.

Secondary metabolites in culture filtrates

The analysis of the 23 extracellular metabolites found in the grow broth of the strain CMU-0113 included nine terpenes, three polyketides, two phenol derivatives, two lipids, and one of each seven other chemical groups (Table I).

Discussion

Phylogenetic analysis

Previous report only based in Blast search and Neighbor-Joining analysis indicated that CMU-0113 belongs to *G. curtisii* species (Mukhtar et al., 2019). However, both pairwise similarity performed by Blast algorithm and distance-based grouping pattern may wrongly reflect taxonomic entities or phylogenetic relationships (Lücking et al., 2020). Thus, here we used better performance of both grouping pattern criteria and bioinformatic tools, in order to obtain a robust phylogenetic associations and taxonomic identification of the CMU-0113 strain. The phylogenetic analysis strongly suggests that the CMU-0113 strain belongs to the *G. curtisii* (Berk.) Murrill species. This is a worldwide distribution species reported in China, Africa, Europe, and India, as well as in several states of México (Torres-Torres and Guzmán-Dávalos, 2005; Torres-Torres et al., 2015). In North America, this species has been described associated to oak forest in U.S.A., proposed as an opportunistic phytopathogen of old trees or attacking young ones at roots (Loyd et al., 2017, 2018). In Mexico it is also found in temperate pine-oak and subtropical forests with oak (Torres-Torres et al., 2015). Species within the *Ganoderma* genus are described as good producers of secondary metabolites, including those with antimicrobial activity (Baby et al., 2015; Sivanandhan et al. 2017). However, *G. curtisii* mycelium has been poorly studied on this regard. In the same way, there is a scarcely of information on the antagonism and inhibition capability of *Ganoderma* spp. mycelia against phytopathogenic microorganism.

Antagonism

In assays of antagonism in dual culture, *Ganoderma* sp. strain CMU-0113 efficiently antagonized all phytopathogens tested, except *F. pseudocircinatum*. The few studies of antagonism in dual culture that exists between *Ganoderma* species against fungi of other taxonomic groups reported contrasting results. In agreement with the results here obtained, an unidentified strain of *Ganoderma* sp. efficiently antagonized strains of the mycoparasites *Trichoderma harzianum*, *Trichoderma pseudokoningii*, *Trichoderma viride* and *Clonostachys rosea* (Badalyan et al., 2004). The *Ganoderma* strain studied in such work showed the second highest rate of antagonism among 17 basidiomycete species evaluated, only below *Pleurotus ostreatus*. This result suggests that some *Ganoderma* phytopathogenic species are resistant to common biocontrol fungi. Contrastingly, White & Traquair (2006) evaluated 26 strains belonging to 22 species of ligninolytic basidiomycetes, finding that *Ganoderma applanatum* did not able to antagonize it efficiently to *Botrytis cinerea*. Both previous studies conducted the confrontation assays at 24 °C and the present work at 28 °C, but all three works perform the dual cultures in PDA medium, being the incubation temperature, pH, and other culture media relevant issues to further assess the *Ganoderma* spp. capability to antagonize ascomycete fungi and oomycetes, mainly global concern phytopathogens.

The *in vitro* antagonistic capability of a fungal strain can be associated with their production of extracellular hydrolytic enzymes and secondary metabolites production. The CMU-0113 strain here analyzed is robustly identified as *G. curtisii*, a species scarcely evaluated at enzymatic and metabolic level. However, within the same genus, extracellular glucanases and chitinases associated with the process of antagonism and biocontrol (Daguerre et al., 2014) are produced by *Ganoderma lucidum* (Jain et al., 2020). The production of antagonism related enzymes varies according to the incubation conditions and the strains/species involved in the interaction (Köhl et al., 2019), so it will be necessary to evaluate the activity of these enzymes in different interaction conditions, during the antagonism process in the *in vitro G. curtisii* -phytopathogen interactions here studied.

Inhibition by antibiosis

The antibiosis assay is used to determine if a fungal strain produces non-volatile metabolites that diffuse into the medium, affecting the growth of phytopathogenic fungi (Steyaert et al.,

2016). In the assays here conducted, the strain CMU-0113 significantly inhibited the *Fusarium* spp. and *Colletotrichum* spp. strains. As far as we could document, there are not previous published work of this type of assays using *Ganoderma* strains to test its *in vitro* activity against phytopathogenic fungi/oomycetes. However, there are previous studies in *Ganoderma* spp. that can aid to explain the inhibitory capacity observed in the antibiosis assays for the CMU-0113 strain here used. It has been documented that the culture in PDA solid medium increases the production of ganoderic acids by the mycelium of *G. lucidum*, when compared to the culture in PDB liquid medium (You et al., 2012). This species secretes such metabolites to the growth medium (Zhang et al., 2014), and some of them have antifungal activity (Smania et al., 2003). Besides *G. lucidum*, several *Ganoderma* species can produce ganoderic acids (Baby et al., 2015), metabolites not identified in the strain here studied. Thus, further work is necessary in order to evaluate if the *Ganoderma* sp. strain here studied is able to secrete ganoderic acids, and the culture conditions to improve its secretion, and if this is one of the factors associated with the inhibition of phytopathogens in the antibiosis assays here performed.

Growth of the CMU-0113 strain

The regulation of secondary metabolism pathways in fungi is a complex process that involves cross talk among global and specific mechanisms, in response to both external stimuli and development stages (Keller, 2019). The change in the production of secondary metabolites in the transition from exponential growth to the stationary growth phase, also called idiophase, is a well-documented physiological characteristic in industrial microbiology (Demain et al., 2005). As example, the production of extracellular polyphenols and protocatechuic acid in broth culture by *G. lucidum* increases with time incubation, reaching its maximum at day 12th, whereas isoflavone contents reaches its lowest concentration at same time (Chien et al., 2011). The concentration increase of the former metabolites correlates with the maximal antioxidant activity of the broth filtrate. Therefore, to perform the inhibition assays by disk diffusion and agar dilution, the growth kinetics on the PDB medium of study strain was constructed, to know its entry into the stationary phase to recover extracellular filtrates. However, the extracellular broth concentrates showed limited inhibitory activity against phytopathogens tested (see below). Despite the relevance to evaluate physiological states in which metabolic changes will occur, to the best of our knowledge, there are not previous basic physiological studies that document the

kinetic growth of *G. curtisii* in broth culture. The yield for both biomass and extracellular metabolites here obtained for CMU-0113 strain is comparative low when compared with other *Ganoderma* spp. species (Table II). Although comparisons in this regard with previous works must be taken cautiously because differences in growth media and culture conditions. This highlights the need to perform further physiological studies in CMU-0113 strain to compare growth kinetics in different broth media and culture conditions, and its correlation with extracellular metabolites production.

Effect of extracellular filtrates of the CMU-0113 strain on phytopathogens

In the agar dilution assays, the filtrate of the CMU-0113 strain promoted the growth of *P. capsici*, but in the disk diffusion assay significantly inhibited the mycelial growth of *F. mexicanum*. The antifungal activity of aqueous and organic extracts from basidiocarp and vegetative mycelium of different species of *Ganoderma* has been documented (Basnet et al., 2017). However, previous works on *Ganoderma* spp. to evaluate the inhibitory activity of extracellular filtrates and their extracts against phytopathogenic fungi and oomycetes are scarce. When comparing the data here generated with the few similar studies carried out with extracellular filtrates conducted in *Ganoderma* species, heterogeneous results are observed. In a study that analyzes the organic extracts of the mycelia from 87 basidiomycete species, a *Ganoderma* sp. strain were not included amongst the 15 with the highest antimicrobial activity (Rosa et al., 2003). Such work used as tested strains yeast species, which are relevant human pathogens. Similarly, it has been reported that organic extracts from the extracellular filtrate of *G. applanatum* and *Ganoderma lobatum* do not exhibit antifungal activity towards the human pathogens *C. albicans* and *Sporothrix schenkii* (Gonzalez-Barranco et al., 2010). In contrast, the ethyl acetate extract of the extracellular filtrate of *Ganodema* sp. inhibited the mycelial growth of the fungus *Curvularia clavata* and the oomycete *Phytophthora nicotianae* in 48.8 and 53.3%, respectively (Palacios-Atencio et al., 2011). In the same way, the extracellular filtrates of the mycelium growing in broth medium of strains of *G. applanatum, G. lucidum* and *Ganoderma carnosum* significantly inhibited the mycelial growth of *Aspergillus fumigatus, Aspergillus niger, Candida albicans, Penicillium cyclopium* and *Paecilomyces variotii* (Ćilerdžić et al., 2016a). This last report documents that those filtrates of *Ganoderma* spp. with antifungal

activity were rich in phenols and flavonoids, although they appear to be specific combinations of these substances responsible for the activity.

The extracellular filtrate of the CMU-0113 strain promoted the growth of some of the phytopathogens here tested, a response that may be related to hormesis. Hormesis is defined as a biphasic dose response phenomenon, in which a chemical compound stimulates a physiological or biochemical process at a low concentration but inhibits it at a high concentration (Pradhan et al., 2017). This response has been documented in the activity of conventional fungicides (Pradhan et al., 2017), but Cantrell et al., (2008) also observed it on *C. gloeosporioides* as response to the ethyl acetate extract from the fruiting body of *Gomphus floccosus*. Describing the hormesis in an extract/filtrate or metabolite that can be used in biocontrol is relevant, since it allows to determine the doses at which the application in the field will cause the desired inhibition effect, avoiding promoting the growth of the target phytopathogen (Pradhan et al., 2017). Subsequent studies of the CMU-0113 strain here analyzed may determine at what concentrations of the broth filtrates are inhibitory of the growth of the phytopathogens of interest, which in consequence might demonstrate the hormesis.

Antifungal metabolites identified in extracellular filtrate of CMU-0113 strain

Among the preidentified extracellular metabolites detected in the broth culture of the strain CMU-0113, several have been previously reported as antifungals produced by other fungi, mainly basidiomycetes. Such metabolites include 2-aminoquinoline, enokipodines B and D, strobilurin A, as well as indolelactic and porric B acids (Table III). Additionally, closely structural derivates of lactarazulene also shows antifungal activity. These compounds or structural relatives can be responsible for the antifungal activity showed by the *G. curtisii* strain in the antagonism, antibiosis, and disk diffusion assays. Obtained results suggest that such kind of metabolites do not inhibit the growth of oomycetes. It should emphasize that previous works in other basidiomycete species documents that the antagonistic and antibiosis assays performed in a solid medium may induce specific metabolite production not produced in broth culture (Wu et al., 2019). Therefore, extracellular metabolites identified in the broth culture of CMU-0113 strain were not necessarily the same induced in antagonism in dual culture and antibiosis assays. Coculture induction of specific metabolites in solid medium may contribute to explain high

growth inhibition percentages of fungal/oomycete found here in these antagonism assays; these probable differences among solid and submerged fermentation will require further study.

Besides its antifungal compounds preidentified, the strain CMU-0113 produces compounds with different biological activities, but not previously described as extracellular mycelial metabolites (Table III). As example, cinnatriacetin A, isolated from *Fistulina hepatica* is active against Gram- positive bacteria (Tsuge et al., 1999), and the D8'-merulinic acid C isolated from the fruiting bodies of *Merulius tremellosus* and *Phlebia radiata* with antibacterial activity but not active against fungi (Giannetti et al., 1978). In the same way, collybial was characterized from mycelial extract of *Collybia confluens* as antibacterial and antiviral (Simon et al., 1995). The nucleoside clitocine isolated from basidiocarp of *Clitocybe inversa* showed insecticidal activity (Kubo et al., 1986). Cytotoxic activity against cancer cell lines, anti-germinative, neuroprotective and antihemolytic are also observed in the identified metabolites. Finally, 3-hydroxytrichothecene and diplosporin are mycotoxins, which awareness on the relevance to found culture conditions or use molecular tools to inhibit its production whereas promoting the desirable ones, when looking for scaling biotechnological process. Such structural and bioactivity diversities indicates that extracellular metabolites produced by *G. curtisii* might have a way variety of biotechnological and biomedical applications. Extracellular metabolites produced by CMU-0113 strain here described can be considered basal, because no induction conditions to increase its secretion were assessed. The culture conditions here used suggest that gene clusters responsible for the synthesis of the identified extracellular metabolites are constitutively expressed or not subject to carbon catabolite repression (CCR). CCR and transcription factors associated with secondary metabolism were molecular and metabolic issues scarcely studied in basidiomycetes (Adnan et al., 2018; García-Estrada et al., 2018). Further physiological and metabolic studies could help to know what kind of metabolites may not be subject to this metabolic control.

Nine out of the 23 extracellular secondary metabolites produced by the CMU-0113 strain are terpenes. Terpenes are widely documented in *Ganoderma* spp. (Hapuarachchi et al., 2017), but most studies have been conducted in basidiocarp extracts, with only few studies on mycelia an even few on extracellular terpenes in broth media. Particularly in *G. curtisii* there were not previous studies on extracellular metabolites produced by mycelium growth in submerged culture. Detecting and characterization of terpenes produced by *Ganoderma* spp. mycelium is

relevant because some cases these are different from those found in basidiocarp, and former present antimicrobial bioactivity not found in the latter (Baby et al., 2015; Isaka et al., 2016, 2020). Recently, 29 terpenes were identified in ethanolic extracts of the *G. curtisii* basidiocarp, all of them lanostane triterpenoides (Table IV) (Jiao et al., 2016). At least 19 of such compounds showed high to moderate inhibition of nitric oxide production in BV-2 microglia cells without cytotoxicity against it, thus being promise candidates to neurodegenerative disorders. However, such compounds have not yet evaluated as antimicrobials (Table IV). Other six lanostane terpenoids have been described in the methanolic extracts of *G. curtisii* basidiocarps, which are useful chemotaxonomic markers (Welti et al., 2015). Lanostane triterpenoids obtained from *Ganoderma* spp. fruiting bodies and extracellular filtrates showed antiparasitic (Wahba et al., 2019; Isaka et al., 2020) and antibacterial activities (Isaka et al., 2016), but we cannot find references documented inhibitory activities against fungi or oomycetes.

Conclusions

The results of the present work suggest that the CMU-0113 strain secrete a greater diversity of antimicrobial metabolites, or more active ones, in solid medium than in broth medium. Also, CMU-0113 strain can secrete metabolites under basal and induced growth conditions, as showed by obtained results of antibiosis and dual culture antagonism assays, respectively. The studied strain has anti-fungal/oomycetes activity against phytopathogens of agricultural relevance worldwide. The biological activities and metabolites here described should be considered as basal, since besides the challenge in dual culture antagonism assays, no induction conditions that can increase the secretion of metabolites with antifungal activity of extracellular filtrates of the studied strain were evaluated. CMU-0113 strain is undoubtedly a source of bioactive metabolites that are yet to show their chemical diversity and antimicrobial activity.

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Figure captions

Fig. 1 Phylogenetic reconstruction of the CMU-0113 strain. The trees were generated according to the Maximum Parsimony (MP) criteria and Bayesian inference (BI) using the ITS (ITS1-5.8S-ITS2) rDNA region (A) and the concatenated ITS-*rpb*2-*tef1*-α sequences (B). The bootstrap values (1000 replicates)/Bayesian posterior probabilities are shown at each node, and because all this last support values were 1.0 in the concatenated tree, these are not depicted for better tree visualization. For each sequence retrieved from the GenBank, the strain/basidiocarp code, country of sampling, and accession number follows the name of the species. Because the *rpb*2 and *tef1*-α gene sequence are not available for all species, only selected taxa for which three sequences are available were used in the concatenated tree. See Materials and Methods for details.

Fig. 2 Growth inhibition of phytopathogens by *Ganoderma* sp. (CMU-0113) in dual culture antagonism and antibiosis assays. Assays were conducted in PDA medium at 28 °C. In the left top of each assay plate the mean values (±S.D.) of three replicas of three independent assays are depicted. Significant growth differences ($p<0.05$) with their respective control plate are indicated by an asterisk. Tested phytopathogens: *Fusarium pseudocircinatum*, *Fusarium mexicanum*, *Colletotrichum coccodes*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, *Phytophthora cinnamomi*. See Materials and Methods for details.

Fig. 3 Growth kinetics of *Ganoderma* sp. (CMU-0113) mycelium. The strain studied was incubated in PDB medium at 28 ° C and 120 rpm. In each day, the dry weight mycelial biomass growth is the mean values (±S.D.) of three replicas of three independent.

Fig. 4 Growth inhibition of phytopathogens by disk diffusion and agar dilution assays of extracellular filtrate of *Ganoderma* sp. (CMU-0113) from the stationary phase of growth. In disk diffusion assays the disk with extracellular filtrate of the strain CMU-0113 might be observed. The control plates have a disk without filtrate (disk diffusion assays) or no filtrate of the CMU-0113 strain was added to the medium (agar dilution assays). Assays were conducted in PDA medium at 28 °C. In the left top of each assay plate the mean values (±S.D.) of three replicas of three independent assays are depicted. Significant growth differences ($p<0.05$) with their respective control plate are indicated by an asterisk. The tested phytopathogens are the same of the Figure 2. See Materials and Methods for details.

Figure 1

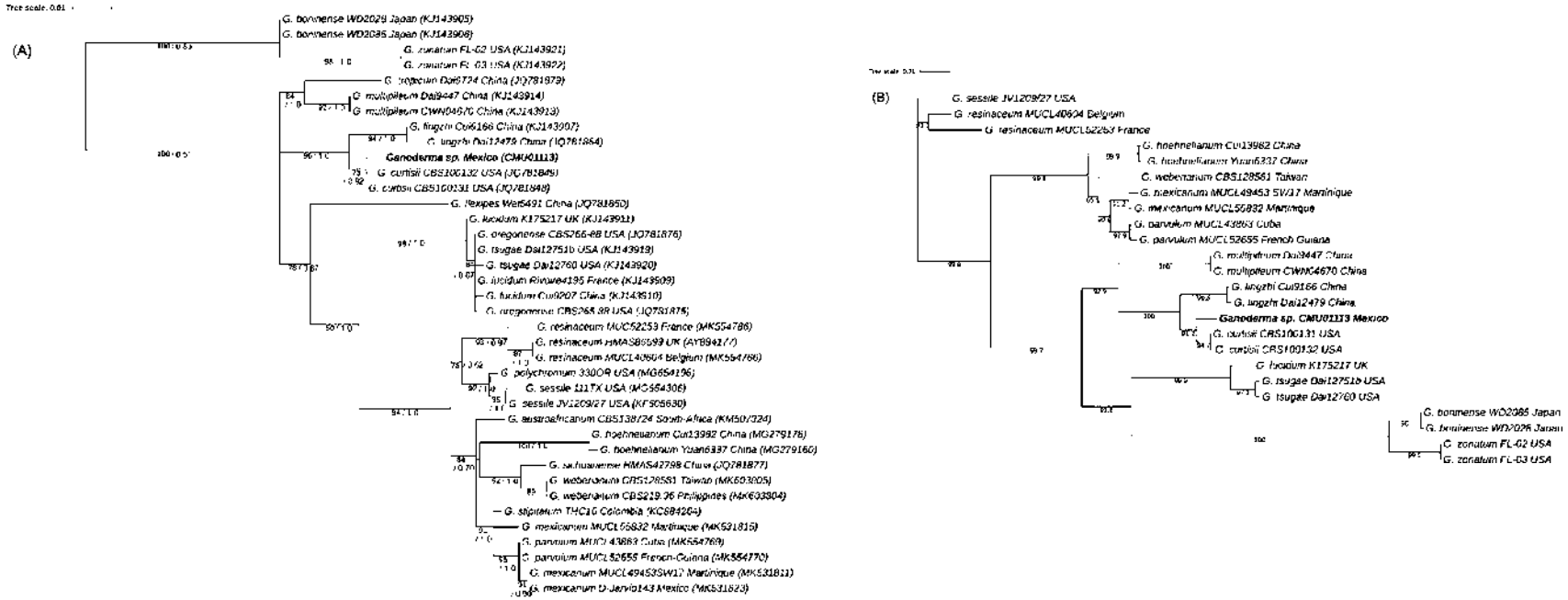
(A)
G. bonmense WD2029 Japan (KJ143905)
G. bonmense WD2086 Japan (KJ143908)
G. zonatum FL-02 USA (KJ143921)
G. zonatum FL-03 USA (KJ143922)
G. tropicum Dai8724 China (JQ781879)
G. multipileum Dai9447 China (KJ143914)
G. multipileum CWN04670 China (KJ143913)
G. lingzhi Cui9166 China (KJ143907)
G. lingzhi Dai12479 China (JQ781884)
Ganoderma sp. Mexico (CMU01113)
G. curtisii CBS100132 USA (JQ781849)
G. curtisii CBS100131 USA (JQ781848)
G. flexipes Wei5491 China (JQ781850)
G. lucidum K175217 UK (KJ143911)
G. oregonense CBS266-88 USA (JQ781876)
G. tsugae Dai12751b USA (KJ143919)
G. tsugae Dai12760 USA (KJ143920)
G. lucidum Rivoire4195 France (KJ143909)
G. lucidum Cui9207 China (KJ143910)
G. oregonense CBS268.88 USA (JQ781875)
G. resinaceum MUCL52253 France (MK554786)
G. resinaceum HMAS86599 UK (AY894177)
G. resinaceum MUCL40604 Belgium (MK554786)
G. polychromum 3300R USA (MG654196)
G. sessile 111TX USA (MG554306)
G. sessile JV1209127 USA (KF906630)
G. australafricanum CBS138724 South Africa (KM507324)
G. hoehnelianum Cui13982 China (MG279176)
G. hoehnelianum Yuan6337 China (MG279160)
G. sichuanense HMAS42798 China (JQ781877)
G. weberianum CBS126561 Taiwan (MK503805)
G. weberianum CBS219.36 Philippines (MK633804)
G. stipitatum THC16 Colombia (KC884264)
G. mexicanum MUCL55832 Martinique (MK531815)
G. parvulum MUCL 43863 Cuba (MK554769)
G. parvulum MUCL52655 French-Guiana (MK554770)
G. mexicanum MUCL49453SW17 Martinique (MK531811)
G. mexicanum D-Jarvis143 Mexico (MK531823)
(B)
G. sessile JV1209127 USA
G. resinaceum MUCL40604 Belgium
G. resinaceum MUCL52253 France
G. hoehnelianum Cui13982 China
G. hoehnelianum Yuan6337 China
G. weberianum CBS126561 Taiwan
G. mexicanum MUCL49453 SW17 Martinique
G. mexicanum MUCL55832 Martinique
G. parvulum MUCL43863 Cuba
G. parvulum MUCL52655 French Guiana
G. multipileum Dai9447 China
G. multipileum CWN04670 China
G. lingzhi Cui9166 China
G. lingzhi Dai12479 China
Ganoderma sp. CMU01113 Mexico
G. curtisii CBS100131 USA
G. curtisii CBS100132 USA
G. lucidum K175217 UK
G. tsugae Dai12751b USA
G. tsugae Dai12760 USA
G. bonmense WD2086 Japan
G. bonmense WD2026 Japan
G. zonatum FL-02 USA
G. zonatum FL-03 USA

Figure 2

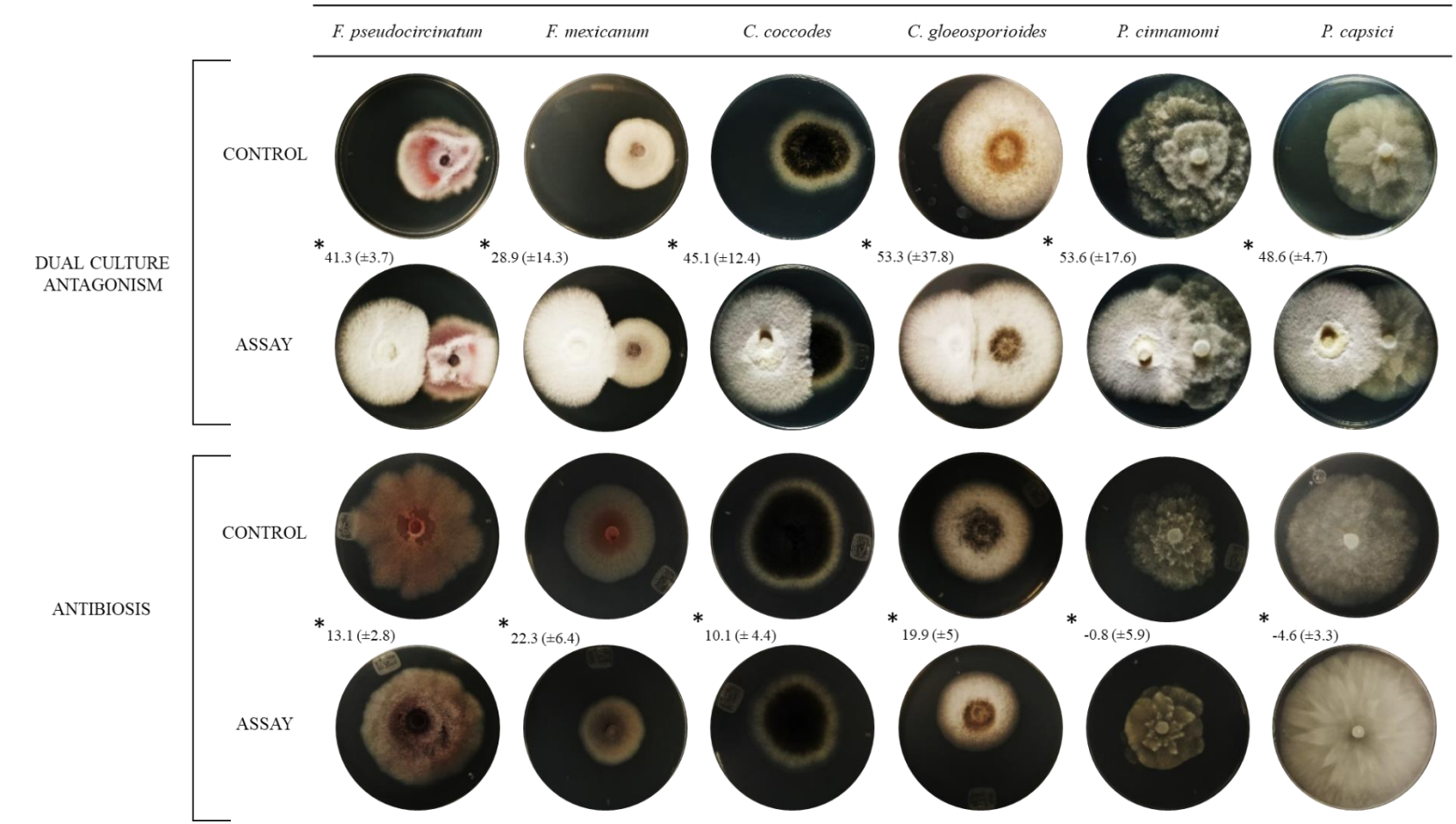
F. pseudocircinatum
F. mexicanum
C. coccodes
C. gloeosporioides
P. cinnamomi
P. capsici
DUAL CULTURE ANTAGONISM
CONTROL
ASSAY
* 41.3 (±3.7)
* 28.9 (±14.3)
* 45.1 (±12.4)
* 53.3 (±37.8)
* 53.6 (±17.6)
* 48.6 (±4.7)
ANTIBIOSIS
CONTROL
ASSAY
* 13.1 (±2.8)
* 22.3 (±6.4)
* 10.1 (± 4.4)
* 19.9 (±5)
* -0.8 (±5.9)
* -4.6 (±3.3)

Figure 3

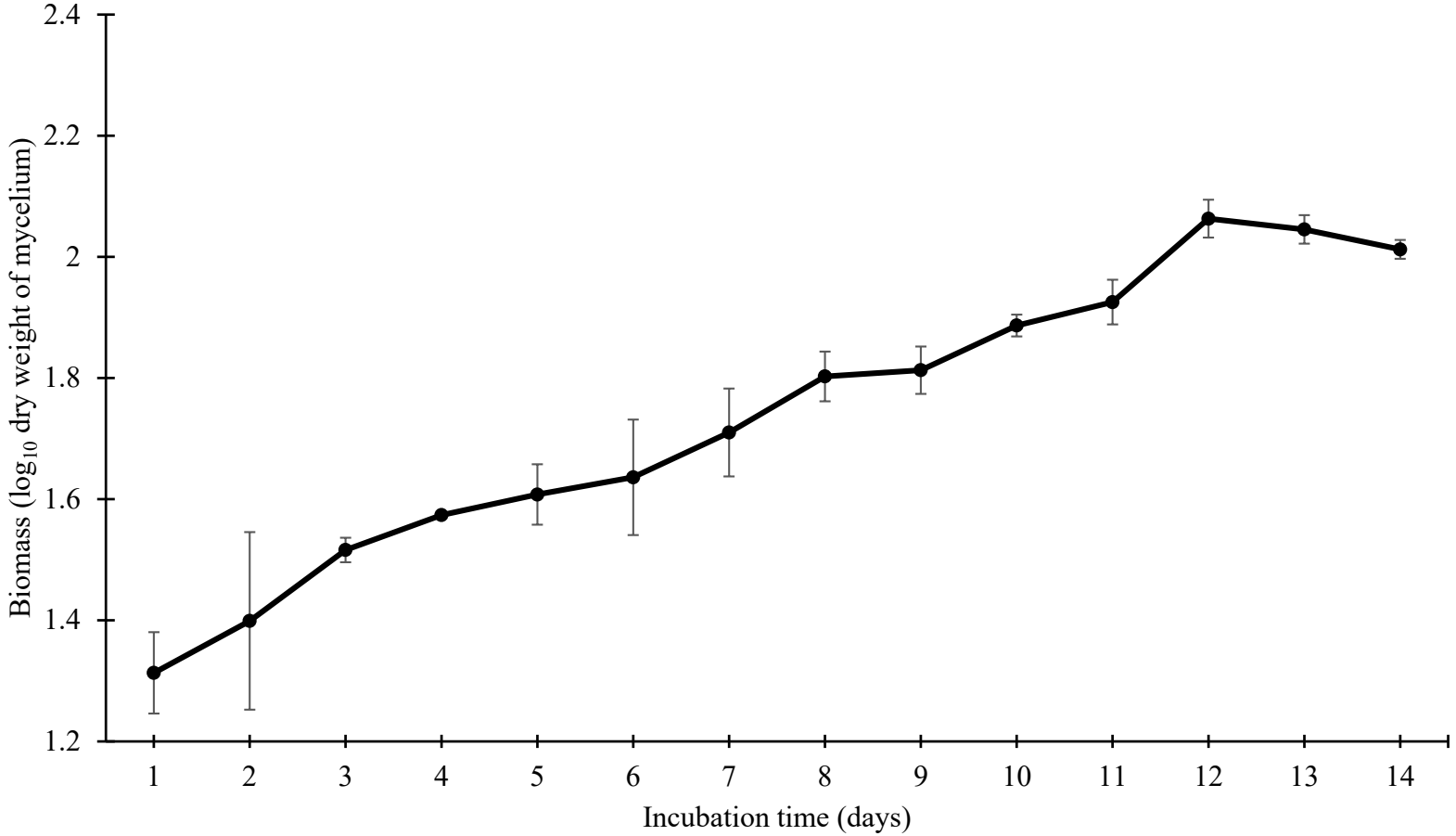
2.4
2.2
2
1.8
1.6
1.4
1.2
Biomass (log10 dry weight of mycelium)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Incubation time (days)

Figure 4

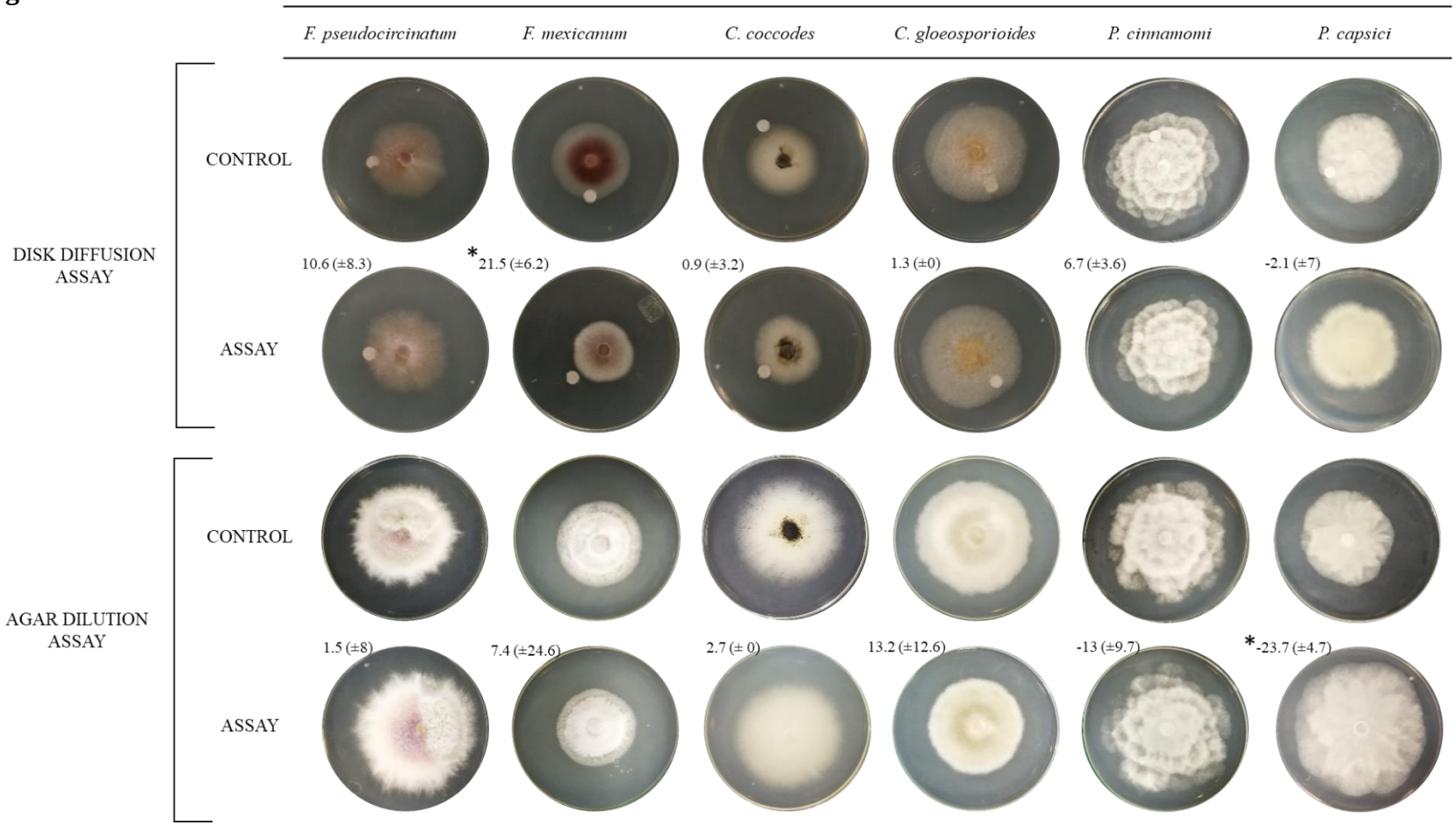
F. pseudocircinatum
F. mexicanum
C. coccodes
C. gloeosporioides
P. cinnamomi
P. capsici
DISK DIFFUSION ASSAY
CONTROL
ASSAY
10.6 (±8.3)
*21.5 (±6.2)
0.9 (±3.2)
1.3 (±0)
6.7 (±3.6)
-2.1 (±7)
AGAR DILUTION ASSAY
CONTROL
ASSAY
1.5 (±8)
7.4 (±24.6)
2.7 (± 0)
13.2 (±12.6)
-13 (±9.7)
*-23.7 (±4.7)

Table I. Extracellular metabolites produced by the strain CMU-0113 of *Ganoderma curtisii* at stationary phase.

Compound name	Molecular Formula	Observed m/z	Adduct	Compound class
1,6-Dimethoxypyrene	$C_{18}H_{14}O_2$	261.0877428	M-H	Pyrene
2-Aminoquinoline	$C_9H_8N_2$	145.0762087	M+H	Aminoquinoline
3-Hydroxytrichothecene	$C_{15}H_{22}O_3$	249.1498331	M-H	Sesquiterpene
Blennin D	$C_{15}H_{22}O_4$	265.1444962	M-H	Sesquiterpene
Cinnatriacetin A	$C_{23}H_{20}O_5$	377.1452584	M+H	Acetylenic derivative
Clitocine	$C_9H_{13}N_5O_6$	252.0720886	M+H-2H_2O	Nucleoside
Collybial	$C_{15}H_{20}O_2$	197.1282533	M+H-2H_2O	Sesquiterpene
D8'-Merulinic acid C	$C_{24}H_{38}O_3$	339.2750406	M+H-2H_2O	Resorcinolic lipid
Dictyoquinazol C	$C_{18}H_{18}N_2O_5$	365.1044552	M+Na	Quinazoline
Diplosporin	$C_{12}H_{16}O_4$	266.1379673	M+ACN+H	Polyketide
Enokipodin B	$C_{15}H_{18}O_3$	227.103165	M-H_2O-H	Sesquiterpene
Enokipodin D	$C_{15}H_{18}O_4$	227.1019245	M+H-2H_2O	Sesquiterpene
Flavidulol A	$C_{17}H_{22}O_2$	241.1542397	M+H-H_2O	Geranyl-phenol
Flavidulol B	$C_{17}H_{22}O_2$	303.1370119	M+2Na-H	Geranyl-phenol
Indolelactic acid	$C_{11}H_{11}NO_3$	188.0700867	M+H-H_2O	Tryptophan derivative
Itaconic acid	$C_5H_6O_4$	111.0085153	M-H_2O-H	Fatty acid
Lactarazulene	$C_{15}H_{16}$	241.1204421	M+FA-H	Sesquiterpene
Mollicellin B	$C_{21}H_{18}O_7$	347.0934719	M+H-2H_2O	Poliketide
Osmundalactone	$C_6H_8O_3$	192.0652383	M+ACN+Na	Sesquiterpene lactone
Pogostol	$C_{15}H_{26}O$	240.2318878	M+NH_4	Terpene
Porric acid B	$C_{15}H_{12}O_6$	269.050899	M-H_2O-H	Dibenzofuran
Strobilurin A	$C_{16}H_{18}O_3$	257.1146975	M-H	Polyketide
Tyromycic acid	$C_{30}H_{44}O_3$	453.3422174	M+H-H_2O, M+H	Triterpene

Table II. Biomass (mycelium dry weight) and extracellular metabolite yields in submerged fermentation of *Ganoderma* spp.[1]

Species	Biomass yield (g/L)	Yield/Metabolite (g/L)[2]	Medium[3]	Incubation time (d)	Reference
G. curtisii	1.99 (±0.13)	142.67 (±5.1)	PDB	14	This study
G. formosanum	7.7 (±0.2)	0.292 (±0.016)/ EPS	ME	9	Hsu et al., 2017
G lucidum	5.22 (±0.31)	1.43 (±0.14)/ EPS	PDB	6	Asadi et al., 2021
	0.80 (±0.02)	0.077 (±0.002)/ EPS	YE	7	Chang et al., 2006
	18.95 (±0.52)	~15.0 mg/g MDW[4]/GA	P	n. s.	Xu et al., 2008
G. pfeifferi	3.63	0.370 (± 0.046)/ EPS	YE	10	Supramani et al., 2019
G. resinaceum	2.33 (±0.18)	0.94 (±0.19)/ EPS	MP/YE	7	Kim et al., 2006

Notes: [1] The data is not the necessarily the highest found in all studies, but the most closely related to medium composition and incubation conditions to the present paper; [2] Showed yields correspond to exopolysaccharides (EPS) and ganoderic acid (GA); [3] The incubation medium for each work is supplemented with different salt, vitamins, and other chemicals, but showed data use glucose as carbon source. PDB, potato dextrose broth; ME, malt extract; MP, Meat peptone; P, peptone; YE, yeast extract; n.s., not specified; [4] the yield data of this work is provided as mg/ g of mycelium dry weight (MDW).

Table III. The previous reports on extracellular metabolites produced by *Ganoderma curtisii* (CMU-0113) at stationary phase.

Metabolite	Fugal species[1]	Source[2]	Bioactivity/Comment	Reference
1,6-Dimethoxypyrene	*Penicillium glabrum*[A]	ME/ EBF	Not tested	Wunder et al., 1997
2-Aminoquinoline	*Lucopaxillus albissimus* var. *paradoxus*[B]	BE	Antibacterial, antifungal, anthelmintic	Pfister, 1988; Schwan et al., 2010
3-Hydroxytrichothecene	*Fusarium sporotrichioides*[A]	ME	Mycotoxin	Corley et al., 1987
Blennin D	*Lentinellus cochleatus*[B]	BE	Inhibitor of leukotriene biosynthesis	Wunder et al., 1996
Cinnatriacetin A	*Fistulina hepatica*[B]	BE	Antibacterial (Gram -)	Tsuge et al., 1999
Clitocine	*Clitocybe inversa (Lepista inversa)*[B]/*Leucopaxillus giganteus*[B]	BE	Insecticidal, anti-proliferative on HeLa cells	Kubo and Kim, 1986; Ren et al., 2008
Collybial	*Collybia confluens*[B]	EBF	Antibacterial, antiviral	Simon et al., 1995
D8'-Merulinic acid C	*Merulius tremellosus*[B]	BE	Antibacterial, antihemolytic	Giannetti et al., 1978; Stasiuk et al., 2004
Dictyoquinazol C	*Dictyophora indusiata*[B]	BE	Neuroprotection against toxins	Lee et al., 2002
Diplosporin	*Diplodia macrospora*[A]	KE	Mycotoxin	Chalmers et al., 1978
Enokipodin B	*Flammulina velutipes*[B]	EBF	Antibacterial, antifungal	Ishikawa et al., 2000
Enokipodin D	*Flammulina velutipes*[B]	EBF	Antibacterial (Gram +), antifungal	Ishikawa et al., 2001
Flavidulol A	*Lactarius flavidulus*[B]	BE	Antibacterial (Gram +)	Takahashi et al., 1988
Flavidulol B	*Lactarius flavidulus*[B]	BE	Antibacterial (Gram +)	Takahashi et al., 1988
Indolelactic acid	*Candida* spp.[A]/*Cryptococcus neoformans*[B]	EBF	Antibacterial, antifungal	Narayanan and Rao, 1976; Gunasekaran, 1980
Itaconic acid	*Aspergillus* spp.[A] *Candida* sp.[A]/ *Ustilago* spp.[B]	EBF	Antibacterial, several derivatives have antifungal activity	Kinoshita, 1931; Haskins et al., 1955; Tabuchi et al., 1981[3]
Lactarazulene	*Lactarius deliciosus*/*L. deterrimus*[B]	BE	Some derivatives modulate cytokine production in natural killer cells (NK92)	Bergendorff and Sterner, 1988
Mollicellin B	*Chaetomium mollicellum*[A]/*Chaetomium brasiliense*[A]	NF/ME	Antibacterial, antimalarial, cytotoxicity against cholangiocarcinoma cell lines	Stark et al., 1978; Khumkomkhet et al., 2009
Osmundalactone	*Tapinella atrotomentosa (*syn. *Paxillus atrotomentosus)*[B]	BE	Antibacterial	Buchanan et al., 1995; Béni et al., 2018
Pogostol	*Biscogniauxia nummularia*[A]/ *Geniculosporium* sp.[A]	EBF/VR	Seed antigerminative activity	Amand et al., 2012; Barra et al., 2014
Porric acid B	Non reported in fungi. *Alternaria* sp.[A] produced related porric acid D and *Didymellaceae* sp.[A] porric acid E	-	Antifungal	Carotenuto et al., 1998; Xu et al., 2012; Tian et al., 2018
Strobilurin A	*Strobilurus tenacellus*[B]/ *Bolinea lutea*[A]	ME/ WBE	Antifungal	Anke et al., 1977; Fredenhagen et al., 1990
Tyromycic acid	*Tyromyces abbidus*[B]/ *Tyromyces fissilis*[B]	BE	Not tested, related compounds show not antioxidant and anti-VIH activity	Gaudemer et al., 1967; Quang et al., 2003

Notes: [1] Superscript letter indicates if the species is Ascomycete (A), Basidiomycete (B) or Lichen (L); [2] BE, basidiocarp extract; EBF, extracellular metabolite obtained from broth filtrate; KE, extract of mycelium growing on yellow maize (*Zea mays* L.) kernels; ME, mycelial extract; NF, information not found; VR, volatiles recovery of the mycelium growing in solid medium; WBE, whole broth (mycelium plus broth) extraction; [3] There is an increasing number of fungal taxa reported to produce this metabolite, here we include mainly historical relevant references (see main text for details).

Table IV. Terpenes and other secondary metabolites previously reported for *Ganoderma curtisii*.

Chemical group	Metabolites[1]	Source[2]	Bioactivity
lanostane triterpenoid		B_{EtOH}	NO inhibition
(Jiao et al., 2016)	3β,12β-dihydroxy-7,11,15,23-tetraoxo-lanost-8,20-dien-26-oic acid		
	3β,15α-dihydroxy-7,11,23-trioxo-lanost-8-dien-26-oic acid		
	3β,7β,15α-trihydroxy-4-(hydroxymethyl)-11,23-dioxo-lanost-8-en-26-oic acid		
	3β,7β,12β-trihydroxy-11,15,23-trioxo-lanost-8,20-dien-26-oic acid		
	7β,12β -dihydroxy-3,11,15,23-tetraoxo-5α-lanost-8-en-26-oic acid		
	12β-acetoxy-3β-hydroxy-7,11,15,23-tetraoxo-lanost-8,20E-diene-26-oic acid		
	12β-acetoxy-3,7,11,15,23-pentaoxo-lanost-8,20-dien-26-oic acid		
	15α-hydroxy-3,11,23-trioxo-lanost-8,20-dien-26-oic acid		
	20-hydroxylganodric acid G		
	Ganoderenic acids A, D, K		
	Ganoderic acids A, B, C2, C6, D, **E**, F, **G**, H, J, **K**		
	Ganodermadiol		
	Ganodermanontriol		
	Ganodermatriol		
	Ganolucidic acid A		
	Methyl ganoderate A		
	Methyl lucidenate L		
Lanostane/lucidenic acid	3,7,15,?-tetrahydroxy-4,4,14-trimethyl-11-oxochol-8-en-24-oic acid	B_{MetOH}	NT
(Welti et al., 2015)	3-hydroxy-4.4.14-trimethyl-7,11,15-trioxochol-8-en-24-oic acid		
	7,15,?-trihydroxy-4,4,14-trimethyl-3,11-dioxochol-8-en-24-oic acid		
	Lucidenic acid N		
	Lucidenic acid A		
	Lucidenic acid F		
Sterol	Ergosterol	B_{Hex}	
(Islas-Santillán et al., 2017)	Ergosterol peroxide		
	Estelasterol		

[1] Metabolites with high (bold), moderate (underlined) and low (non-marked) nitric oxide (NO) production inhibitory effects on BV-2 microglia cells activated by LPS; NT, not tested. [2] B_{EtOH}, ethanolic extract of basidiocarp; B_{MetOH}, ethanolic extract of basidiocarp; B_{Hex}, hexanic extract of basidiocarp. Source bioactivity and chemical group are the same for the same reference.

V.3 Capítulo 3:

Phylogenetic and structural analysis of *Ganoderma* spp. terpene synthases

Phylogenetic and structural analysis of *Ganoderma* spp. terpene synthases

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Abstract

Species of the *Ganoderma* genus have been shown to be a relevant source of secondary metabolites with antimicrobial activity. Among the most frequently described metabolites in *Ganoderma* spp. that inhibit microbial growth are terpenes. To date, only the cloning and experimental characterization of two terpene synthase (TS) genes have been described in each of the species *Ganoderma lucidum* and *Ganoderma lingzhi*. The availability of genomes enables bioinformatic analysis to elucidate the number and possible functions of TSs in *Ganoderma* species. This allows evaluating the possible variations of the terpene synthesis capacity between the species of such taxon, at the same time that allows the generation of hypotheses to guide the work for the experimental characterization of TSs. 39 gene sequences annotated as TSs in the available genomes of *Ganoderma* sp., *Ganoderma sinense*, *Ganoderma boninense* and *G. lucidum* species were obtained from public databases. A robust phylogenetic reconstruction, in combination with a detailed search for functional motifs and docking analysis employing both farnesyl diphosphate (FPP) and geranyl-geranyl pyrophosphate (GGPP), allowed the assignment of putative specific properties to most of the analyzed sequences. Putative UbiA-type Class I TSs with non-canonical catalytic motifs not previously described in *Ganoderma* spp. stands out. Also, phylogenetic analysis and molecular modeling show an evolutive and functional relatedness between a squalene cyclase from the bacteria *Alicyclobacillus acidocaldarius* and enzymes from *Ganoderma* spp., suggesting a horizontal transfer from prokaryotes to such fungal genus. The combination of bioinformatic tools here used provides a framework for the evolutionary and functional analysis of TSs in basidiomycetes.

Palabras clave: *Ganoderma*, terpene synthase, phylogeny, molecular docking.

Introduction

The genus *Ganoderma* (Fungi, Agaricomycotina) includes approximately 214 species, although there are 420 records with legitimate status in Mycobank (http://www.mycobank.org/). However, other estimates of the number of species have been proposed according to genus discrimination and species delimitation criteria (Richter et al., 2015; Papp, 2019). These phylogenetic and taxonomic conflicts reflect the complexity and variability of the *Ganoderma* genus (Richter et al., 2015; Justo et al., 2017). The genus *Ganoderma* is cosmopolitan, with presence in temperate and tropical ecosystems, and its species have been described as tree phytopathogens (Coetzee et al., 2011, 2015; De Simone and Annesi, 2012; Hidayati et al., 2014; Sankaran et al., 2005). However, it has been suggested that some species such as *Ganoderma sessile* may be facultative saprophytes (Loyd et al., 2018) or endophytes in live tree trunks (Martin et al., 2015).

The species within the *Ganoderma* genus have a remarkable capacity to produce secondary metabolites, thus being considered a tool for the development of new compounds with biological activity for the treatment of many human diseases (Zjawiony, 2004; Agyare and Agana, 2019). Among the metabolites with biological activity described in the species of the genus *Ganoderma* are polysaccharides, immunomodulatory proteins, sterol, nucleotides, and triterpenoids (Hapuarachchi et al., 2017; Hsu and Cheng, 2018). This last group constitutes one the main kind of secondary metabolites produced by *Ganoderma* spp. and the most studied are the C30 lanostanes, designed generically as ganoderic acids (Baby et al., 2015). Terpenes have an ample diversity of biological activities, including antitumoral, antihypertensive, antihepatotoxic, neuroprotector, antioxidant and antiviral (Xu et al., 2010; Cör et al., 2018) and some of them are in clinical trial studies for its pharmacological use (Liang et al., 2019). Most of fungal terpenes have structures that are not found in other organisms (Schüffler, 2018; Quin et al., 2014), highlighting the relevance on the study of the pathways for its production.

Among the diversity species within the *Ganoderma* genus, the research on secondary metabolites production has been focused on *G. lucidum*, partially because its ancestral use in folk medicine in China (Ahmad, 2018; Wachtel-Galor et al., 2011). Particularly, the metabolic pathway of triterpenoid ganoderic acids production has been dilucidated. This pathway involves several steps from acetyl-CoA via the mevalonate pathway (Miziorko,

2011), being the squalene synthase (SQS), squalene monooxygenase or squalene epoxidase (SQE), and lanosterol synthase (LNS) the terpene synthases (TSs) involved in the final steps of the synthesis (Shi et al., 2010; Liu et al., 2012). Available genomes of several *Ganoderma* species have shown the presence of genes coding for TSs (Kües et al., 2015), but gene cloning for detailed structural, catalytic, and functional studies is still incipient, and has been only conducted in *G. lucidum* and *G. lingzhi* species (Shang et al., 2010; Zhou et al., 2014; Zhang et al., 2017a, 2017b).

Research on the biosynthesis of terpenes and the enzymes associated with this process in *Ganoderma* spp. can contribute to finding more efficient metabolites for fighting diseases and improving the processes for their production. At same time, such analysis can shade light on the evolutionary pathways of terpene synthesis in fungi. Bioinformatic tools are useful to study protein structure-function relationships in terpene synthases of Basidiomycetes and to elucidate enzyme substrates and products (Quin et al., 2013). Thus, the objective of this work was to conduct a bioinformatic analysis to identify and characterize the TSs of *Ganoderma* spp. which whole genome sequences are available or genes have been cloned.

Material and Methods

Terpene synthases retrieving

We retrieved terpene synthase amino acid sequences from cloned genes and whole genome sequences of the species *Ganoderma* sp., *Ganoderma sinense*, *Ganoderma boninense* and *Ganoderma lucidum*, using National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and JGI-MycoCosm (Grigoriev et al., 2014) databases (Supplementary Table I). Other putative TSs proteins from different fungal taxa, mainly basidiomycetes, were retrieved from the same databases. In addition, *Aspergillus terreus* aristolochene synthase (PDB ID 3BNY), *Streptomyces coelicolor* germacradienol/geosmin synthase (PDB ID 5DZ2), *Streptomyces pristinaespiralis* selinadiene synthase (PDB ID 4OKM) and *Homo sapiens* lanosterol synthase (PDB ID 1W6J) crystallographic structures and sequences were downloaded from Protein Data Bank (PDB) (https://www.rcsb.org/) for its use as reference amino acid sequences both for phylogenetic and structural analysis. Functionally characterized enzymes of cloned genes from different taxa were also included for the phylogenetic analysis.

Phylogenetic analysis

All protein sequences selected were aligned with SATé (Liu et al., 2009) using MAFFT (Katoh et al., 2005) as the external sequence alignment tool and RaxML (Stamatakis, 2006) as the tree estimator, and the obtained alignment was further refined by GUIDENCE2 (Penn et al., 2010; Sela et al., 2015). The obtained alignment was used to search for the evolutive model selection (Kalyaanamoorthy et al., 2017) using IQ-TREE server (Nguyen et al., 2015; Trifinopoulos et al., 2016) and using this same tool the ML phylogenetic reconstruction was conducted with the ultrafast bootstrap approximation of 1,000 replicates to assess branch support (Hoang et al., 2018). The best evolutive model for analyzed proteins was LG+G4, chosen according to Bayesian Information Criterion. Bayesian inference (BI) was conducted in MrBayes 3.2.7 (Ronquist et al., 2012), which performed a sampling to determine the most appropriate model for amino acid substitution during the run. Four MCMC chains were run simultaneously starting from random trees for 10,000,000 generations. Trees were sampled every 1,000th generation for a total of 10,000 trees, and the first 2,500 trees were discarded as the burn-in phase. Posterior probabilities were determined from a majority-rule consensus tree generated with the remaining 7,500 trees. Because no incongruences were observed between MP tree and Bayesian inference, the different matrices were combined for the final phylogenetic tree. The tree was edited and visualized with in the iTOL web server (Letunic and Bork, 2019).

Terpene synthase classification

In order to identify the class of the terpene synthases retrieved from *Ganoderma* spp. genomes, a protein domains analysis was performed using InterPro database (Blum et al., 2021). Additionally, multiple sequence alignments using PROMALS server (Pei et al., 2008) were conducted for selected proteins within each of the clades and motif analysis was conducted for each alignment.

Homology modeling

The phylogenetic analysis and the motif analysis using multiple sequence alignment previously described were used as criteria to select the TSs from *Ganoderma* spp. to generate

three-dimensional protein models in SWISS-MODEL server (Bordoli et al., 2009), using a QMEAN value not less than -4.0 and a GMQE value equal to or greater than 0.55. The *Ganoderma* spp. enzymes and the crystal structures of proteins showed to be the best templates for homology modeling are listed in the Supplementary Table II. The accuracy and quality of the modeled structures were improved by refinement in GalaxyRefine server (Heo et al., 2013). Likewise, models were validated by Z-Score and Ramachandran plots using ProSA-Web server (Wiederstein and Sippl, 2007) and ZEUS software (http://www.al-nasir.com/portfolio/zeus/), respectively. Three-dimensional structures were displayed and analyzed in Chimera 1.13.1 software (Pettersen et al., 2004).

Molecular docking

The substrates used for performing a molecular docking analysis were obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) (Supplementary Table II). The docking analysis was performed using a Root Mean Square Deviation (RMSD)= 4.0 in PatchDock server (Schneidman-Duhovny et al., 2005). Top candidate solutions with the highest geometry score from PatchDock were refined in FireDock web server (Mashiach et al., 2008) for obtaining the binding global energy as well as contribution of the van der Waals forces, atomic contact energy and hydrogen bonds to interaction.

Results and Discussion

More than 370 different types of terpenes have been described until now in nearly 20 *Ganoderma* species (Baby et al., 2015). In contrast, structural and functional analyses of terpene synthases have been conducted only in two species, *G. lucidum* and *G. lingzhi*. Thus, in order to evaluate if the terpene synthesis capability of the *Ganoderma* spp. has similar genetic bases of these two better studied species of the genus, in this work we retrieved the genes coding for terpene synthases for *Ganoderma* spp. whose genomes are available in public databases and conducted a bioinformatic analysis of the respective encoded proteins.
The domain analysis package of the *Ganoderma* spp. TPSs performed with InterPro resulting in 21 enzymes belonging to isoprenoid synthases, five squalene cyclases, two trichodiene synthases and one none predicted protein (data not shown). In addition, protein family analysis displayed 19 terpene cyclases-like 2, three trichodiene synthases, five squalene

cyclases and two no annotated proteins. But detailed domain analysis based in multiple alignments and bioinformatic modelling were performed for each TS of Ganoderma spp. subgroups found by phylogenetic reconstruction, as explained below. Amino acid identity among predicted TPS *Ganoderma* spp. varied from 11.11 to 17 %. Protein length ranged from 181 to 419 amino acids in TPS class I and from 726 to 789 amino acids in TPS class II (data not shown).

The phylogenetic reconstruction of the selected TS generates eight main clades with at least five proteins, with bootstrap values above 60% (Figure 1). Four minor clades groups exclusively basidiomycete or ascomycete TSs, but don´t include a *Ganoderma* spp. enzymes. Of the 39 *Ganoderma* spp. sequences analyzed, 31 cluster in four of the main clades and eight, belonging to *Ganoderma* sp. and *G. sinense*, branches early in the tree, outside of the rest of the clusters.

The alignment of the five *G. sinense* and three of *Ganoderma* sp. enzymes that don´t cluster with other fungal TSs shows the non-canonical Asp-rich metal-binding motifs DxxxD characteristic of UbiA-type Class I enzymes, but not the second NxxxDxxxD motif of these TSs (Figure 2) (Li, 2016; Christianson, 2017; Rudolf and Chang, 2020). Instead, a second motif of these enzymes have the canonical NSE triad sequence of Class I sesquiterpene-diterpene synthases (Dickschat, 2016, 2019; Christianson, 2017). The canonical Class I TSs has two Asp-rich motifs to bind the trinuclear Mg^{2+} cluster for diphosphate abstraction from substrate; the first is a DDxxD motif, and the second is an NSE triad (Schmidt-Dannert, 2015; Rudolf and Chang, 2020). The eight proteins within these group show exactly the same (D(D/E)xx(D/E) motif, being DEYTD; whereas the NSE motif shows the consensus sequence ND(M/V/I)A(G)SYNRE (Figure 2). These motifs have been recently described in a new diterpene cyclase family of the superfamily UbiA in the basidiomycete *Hericium erinaceum* (Yang et al., 2017). However, the *Ganoderma* spp. enzymes do not share the central G and the final D amino acids in the NSD motif of *H. erinaceum* UbiA-type enzyme. Also, in *Ganoderma* spp. the DxxxD motif is oriented nearest of the N-terminal than the NSE one, whereas in the protein of *H. erinaceum* the orientation of such motifs is inverted. Furthermore, NSE motif of *Ganoderma* spp. enzymes has practically the canonical sequence of the bacterial Class I diterpene synthases (Dickschat, 2016) which is also present in experimentally characterized fungal sesquiterpene synthases (López-Gallego et al., 2010a),

sharing the initial ND, the middle S, and the final E amino acids that are landmarks of such proteins. Additionally, the N-terminal/C-terminal orientation of DxxxD and NSE motifs of *Ganoderma* spp. enzymes are the same that those observed for canonical DDxxD/NSE motifs in bacteria (Dickschat, 2016), and all the enzymes have the R and the RY of bacterial monoterpene and sesquiterpene synthases (Figure 2), also involved for binding of the substrate and the Mg^{+2} (Dickschat, 2016). Such unique structural characteristics that combine non-canonical UbiA-type and canonical Class I sesquiterpene synthases are coherent with the phylogenetic results that separate it from the rest of analyzed enzymes.

When two arbitrary selected enzymes of these group (JGI106195 from *Ganoderma* spp. and PIL37688 from *G. sinense*) are analyzed for homology, the best templated found is the 1,8-cineole synthase from *Streptomyces clavuligerus* and the generated models fits well with this monoterpene synthase (Figure 3) with Ramachandran plots showing that the residues in favored region are higher than 97% (Supplementary Table II). Both modeled *Ganoderma* spp. enzymes generate optimal docking parameters using farnesyl diphosphate (FPP) as substrate (Supplementary Table III).

The lack of phylogenetic association of these subgroup Class I TS enzymes with crystalized or functionally characterized protein homologs difficulties the prediction on their catalytic capabilities. The non-canonical DxxxD characteristic of UbiA-type Class I enzymes, suggest that these eight enzymes of *Ganoderma* sp. and *G. sinense* can have similar enzymatic capabilities of the diterpene cyclase of the superfamily UbiA recently described in *H. erinaceum*, which are involved in the synthesis of a new kind of diterpenes using geranyl-geranyl pyrophosphate (GGPP) as substrate (Yang et al., 2017). On the other hand, the canonical NSE triad sequence of Class I sesquiterpene-diterpene synthases suggests that these enzymes have the same catalytic activities of the UbiA-type TSs. This is a superfamily of intramembrane prenyltransferases found both in prokaryotes and eukaryotes involved in the synthesis of ubiquinones, menaquinones, plastoquinones, hemes, chlorophylls, vitamin E, and structural lipids (Li, 2016). In fungi, these enzymes catalyze the transfer of a farnesyl diphosphate (FPP) to the polyketide-derived aromatic core in the biosynthesis of different meroterpenoids. The genomic analysis showed that the homologs of UbiA-type prenyl transferases and cyclases are more abundant in Basidiomycota compared to Ascomycota,

being together the 27% of prenyl-chain modifying enzyme homologs (Schmidt-Dannert, 2015).

It has been stated that the UbiA-type enzymes can be the result of convergent evolution or be the common ancestor of all Class I TSs (Schmidt-Dannert, 2015), but the wide complexity and diversity of UbiA superfamily, with thousands of proteins distributed in eight subfamilies, hinders the phylogenetic inferences (Li, 2016). By other side, an 1,8-cineole synthase has been cloned and experimentally characterized in the ascomycete fungi *Hypoxylon* sp., which it is not phylogenetically related with that of *S. clavuligerus* (Shaw et al., 2015), but both enzymes have the canonical DDxx(D/E) and NSE motifs. However, the lack of sequence similarity between *S. clavuligerus* and *Hypoxylon* sp. 1,8-cineole synthases and the robust homology modeling of the *Ganoderma* spp. enzymes using the bacterial enzyme as template, suggests that the enzymes in this subclade can synthetize 1,8-cineole or structurally similar monoterpenes like D-limonene (Shaw et al., 2015). The enzymes within this *Ganoderma* spp. group show a mosaicism structure that combines characteristics of canonical and non-canonical Class I TSs. By other side, homology modelling indicates that the enzyme 3D conformation and motifs conservation are more relevant than sequence similitude to synthetize similar metabolites. Interestingly, the analysis of terpene synthases of *Hypoxylon* sp. showed 11 enzymes phylogenetically unrelated with other fungal, bacterial and plant TSs (Shaw et al., 2015), suggesting a particular evolutionary pathway for terpene synthesis in this species. Thus, these group of enzymes appears to be subjected to a particular evolutive pathway within *G. sinense* and *Ganoderma* sp. species, a hypothesis that might be explored in the future. Such enzymes need to be further studied in order to get insights on its function and evolutive origin.

The larger clade groups TSs from different taxonomic origin, including fungi, human, bacterial and algae representatives. This clade is subdivided in four main subclades within which 19 TSs from *Ganoderma* spp. are distributed (Figure 1). Three *G. lucidum* enzymes (AHN91949, ABF57214, ABF57214) cluster together with a crystallized TS of *H. sapiens* and experimentally studied enzymes from yeast and filamentous fungi. In a different subclade, three *G. boninense* enzymes (VWO96958, VWO94988, VWP01048) lies in the same terminal clade from experimentally characterized enzymes of several basidiomycete species. In other of these subclades, two *G. boninense* (VWO95157, VWP00961) and one *G.*

lucidum (ACB37020) enzymes groups with Archean prokaryotic crystalized or experimentally characterized TSs of *Streptomyces pristinaespiralis*, *Archaeoglobus fulgidus* and *Aeropyrum pernix*. In this same subclade but in a different terminal branch, one *G. boninense* (VWP00963) and one *Ganoderma* sp. (JGI151299) enzymes groups with experimentally characterized enzymes from basidiomycete species (Figure 1). Finally, in a different subclade which subdivided in several terminal branches, three *G. lucidum* (APO20484, ADD60470, ADD60469), two *G. boninense* (VWP00962, VWP01049), one of each *G. sinense* (PIL24816) and *Ganoderma* sp. (JGI118462) are grouped with enzymes of different basidiomycetes, but this subclade also includes TSs from filamentous and yeast ascomycetes, the taxonomically complicated fungal pathogen *Pneumocystis carinii*, human, and even one of the eubacteria *Alicyclobacillus acidocaldarius*. The rest of seven *Ganoderma* sp. and five *G. sinense* TSs show a wide distribution in three different clades composed exclusively of enzymes from basidiomycete, mainly Polyporales, some of which are experimentally characterized.

The first subgroup of *Ganoderma* in the larger clade TSs showed structural relationships with enzymes within the UbiA-type Class I terpene synthases (TSs), coherent with their phylogenetic relationships. These enzymes are conformed exclusively by three *G. lucidum* proteins and show both Asp-rich metal-binding motifs DxxxDD and NxxxDxxxD of UbiA-type cyclase that are reminiscent but not identical to the DDxxD and NSE/DTE motifs found in canonical Class I TSs (Li, 2016; Rudolf and Chang, 2020). The proteins within this group show exactly the same (D(D/E)xx(D/E) and similar NSE motifs, being DTIEDD and NI(I/A/L)RD(F/Y)(R/L/H)ED, respectively (Figure 4), but the three *G. lucidum* protein has the same sequence in both motifs and the NSE motif is the same between the basidiomycetes of the group. In the obtained phylogeny, *G. lucidum* enzymes cluster with crystalized TS of *Homo sapiens* and functionally characterized enzymes of several fungal ascomycete species and the basidiomycete *Inonotus obliquus*.

As previously stated, the UbiA-type has been associated to the synthesis of meroterpenoids. Interestingly, the previous studied fungal enzymes within this subgroup have been functionally characterized as classical squalene synthases (SQS). Early in the study of the ERG9 enzyme from the yeast *S. cerevisiae* it has been shown to contain a PEST motif characteristic of short half-life proteins, one to four membrane spanning domains, and a N-

terminus with signal sequence of integration into endoplasmic reticulum membrane (Jennings et al., 1991; Fegueur et al., 1991). Later, it has been shown that the monomeric enzyme catalyzes the two-step conversion of farnesyl diphosphate (FPP) to squalene using presqualene diphosphate as substrate in the presence of Mg^{2+} and NADPH (Zhang et al., 1993). The functional redundancy between the ERG9 enzyme, the fission yeast *Schizosaccharomyces pombe* P29704 protein, and the human SQS included in this study was posteriorly probed (Robinson et al., 1993). By other side, the corresponding coding genes for *Fusarium fujikuroi* (Zhao et al., 2010), *Mortierella alpina* (Huang et al., 2015), and *I. obliquus* (Zheng et al., 2013) have been cloned and heterologous expressed, showing its capability to synthetize squalene using farnesyl diphosphate (FPP).

The proteins ABF57214 and AHN91949 of *G. lucidum* within this clade were modelling using as template the human squalene synthase (PDB:1EFZ) that lies in the same clade (Figure 3, Supplementary Table II). Also, a docking analysis was performed using FPP as substrate (Supplementary Table III). Both modelling and docking parameters are robust, in concordance with the phylogenetic results. Results here obtained suggest that in fungi this subgroup of UbiA-type Class I enzymes can participate both in the synthesis of meroterpenoids and squalene, and this can be the function of the three enzymes of *G. lucidum* within this group. Furthermore, the involvement of these enzymes in the squalene synthesis implies that they are also involved in the synthesis of triterpenes, by the further conversion of such substrate in 2,3-oxidosqualene by a squalene epoxidase (SQE), the final intermediary in such pathway (Schmidt-Dannert, 2015). By other side, at least 21 different meroterpenoids has been reported in *G. lucidum* in last five years (Peng and Qiu, 2018) and new metabolites of this kind are the described continuously for this species (Luo and Cheng, 2019; Cai et al., 2021), thus the catalytic capabilities of the three UbiA-type enzymes of *G. lucidum* here described needs to be further studied to optimize the production of the metabolites with medical and biotechnological relevance. No one of the putative proteins of *G. lucidum* found in this group have been experimentally characterized, but the structural and phylogenetic analysis of the AbiU-type proteins within this group indicates that such enzymes possess similar catalytic and biochemical properties to these previously studied enzymes, something that must be useful in its further characterization.

The ABF57213 and ABF57214 proteins within this UbiA-type group are 100% identical, which strongly suggest that they come from a duplication event in the genome of *G. lucidum*, appearing to be the unique duplicate gene coding for TSs in *Ganoderma* spp. Unique or low copy number of TS genes are a common characteristic of fungi. When searching for di-terpene synthases (di-TSs) genes, 39 out of 48 genomes analyzed showed only copy, with few genomes showed two or three copies or paralogous (Fischer et al., 2015). Although in the agarical *Gymnopus luxurians* three copies of di-TSs were detected, it was not possible to determine if it arose for duplication event within the genome of the species. Whereas for the ascomycete *Claviceps purpurea* the clustering of the two copies found suggest a duplication event within the genome of such species. The other basidiomycete in which the copy number of a TS synthase gene has been documented was *Armillaria gallica*, in which only one copy of the gene encoding a protoilludene synthase was found (Engels et al., 2011). The mechanism denominated RIP (repeat induced point mutations) that prevents gene duplication events has been described in the ascomycete *Neurospora crassa* (Galagan and Selker, 2004). Such mechanism is responsible that only 0.1% of the protein-coding genes represent duplicates in *N. crassa*. This is a defense mechanism against the mobilization of mobile genetic elements, which are responsible of gene duplication events. Furthermore, RIP is only one of six mechanisms described in fungi to inhibits the TE elements and includes methylation induced premeiotically (MIP), meiotic silencing by unpaired DNA (MSUD), sex-induced silencing (SIS), somatic quelling (cosuppression), and several cotranscriptional RNA surveillance processes (exemplified by spliceosome-coupled and nuclear RNAi [SCANR]) (Gladyshev, 2017). Of all these mechanisms, the SIS and SCANR have been described in the basidiomycete species *Cryptococcus neoformans*, a human pathogen. All this can help to explain the low copy number of TS genes in *Ganoderma* spp., but the presence of TE silencing mechanisms remains to be demonstrated in mushroom basidiomycetes. By other side, it will be interesting to elucidate the mechanism by which one event of duplication that generates ABF57213 and ABF57214 copies were able to escape for duplication prevention mechanism. Interestingly, it has been recently showed that that the presence of the *erg-10a* and *erg-10b* genes coding for ERG-10a and ERG-10b proteins that have redundant enzymatic functions in *N. crassa*. Both are sterol C-5 desaturases that catalyze the same step during ergosterol biosynthesis, a key compound in the terpene

biosynthesis (Herzog et al., 2020). The evidence suggests that the *erg-10a* gene arose by horizontal gene transfer from a species in the basidiomycete order Tremellales to a species in the Pezizomycotina ascomycete order.

In a second subclade, three *G. boninense* enzymes (VWO96958, VWO94988, VWP01048) cluster with experimental characterized Class I terpene synthases of several basidiomycetes (Figure 1). These enzymes include the Cop6 of *Coprinopsis cinerea* that synthesize α-cuprenene (Lopez-Gallego et al., 2010b), Omp9 and Omp10 enzymes of *Omphalotus olearius* that synthesizes α/β-barbatenes and daucene/*trans*-(E)-Dauca-4(11),8-diene metabolites, respectively (Wawrzyn et al., 2012). The other basidiomycete enzymes in this subclade are the ShSTS1 enzyme from *Stereum hirsutum* that synthesize α/β-barbatenes (Nagamine et al., 2019) and the Fompi1 enzyme of *Fomitopsis pinicola* that synthesize α-cuprenene (Wawrzyn et al., 2012). The FgCLM1 enzyme of the ascomycete *Fusarium graminearum* that synthesize longiborneol (McCormick et al., 2010) is also included in this group. Interestingly, α/β-barbatenes are also produced by *Fusarium verticillioides* (Dickschat et al., 2011), this can explain why the FgCLM1 enzyme of the closely related species *F. graminearum* groups with the basidiomycete enzymes in this subclade but is not necessarily the same enzyme in both species. The multiple alignment of the enzymes in this subclade shows that there is not clear correspondence of the motifs in the well characterized enzymes with those of *G. boninense* (data not shown). The canonical DDxxD motif is evident in *S. hirsutum*, but not in the other enzymes, whereas de canonical NSE motif is evident in all enzymes but *G. boninense* ones. Despite this, the phylogenetic clustering and the structural relationships of the metabolites synthesized between this group strongly indicates that the three *G. boninense* enzymes in this group are canonical Class I terpene synthases related to the synthesis of barbetenes, cuprenene or closely structural related sesquiterpenes. One of the *G. lucidum* enzyme (ACB37020) cluster together with an experimentally characterized *Wolfoporia cocos* TS (Figure 1) which catalyzes the farnesyl diphosphate (FPP) synthesis from geranyl diphosphate (GPP) and isopentenyl diphosphate (IPP) (Wang et al., 2014). The highly identity and clustering of these two enzymes have been previously observed, but not in a robust multiple sequence alignment and phylogenetic analyses, and not assignation to a TS class (Wang et al., 2014). Both enzymes have two DDxxD motifs with the same sequence that are DDMMD and DDFLD (Figure 5). These Class I TSs are

homodimers with their active sites oriented in parallel fashion (Christianson, 2017). Thus, the phylogenetic analysis and structural relationship with the characterized enzyme of *W. cocos* strongly suggest that the ACB37020 enzyme of *G. lucidum* is a farnesyl diphosphate synthase (FPPS).

Two *G. boninense* enzymes (VWP00961 and VWP095157) cluster near prokaryotic TSs, including those crystalized enzymes of *Streptomyces pristinaespiralis* (PDB:4OKZ), *Archaeoglobus fulgidus* and *Aeropyrum pernix* (Figure 1). The gene coding (Genbank ID: WP005317515) for the *S. pristinaespiralis* terpene synthase within this group has been cloned and the enzyme has been experimentally characterized by its heterologous expression in *Escherichia coli* (Baer et al., 2014). This enzyme is a selina-4(15),7(11)-diene synthase (SdS) belonging to the Class I terpene cyclases which uses only farnesyl-PP (FPP) as substrate and produces also germacrene B in minor amounts than the main product. The motif ^{174}YTLMRLYDGAT^{184} determines the G1/2 helix breakage at the active site of such enzyme and Arg178 (central R) has been found to be the PPi sensor, which is highly conserved in bacteria (Baer et al., 2014). Interestingly, the multiple alignment between this enzyme and others in the same clade or nearest subclades showed that such motif is not shared between these enzymes, but the secondary structure predictions within the alignment showed that the number of helixes and the G1/2 breakage is shared by most of enzymes (Figure 6). Due to the length of the *G. boninense* enzyme (VWP00961), that is the double of the rest of enzymes within this subclade, this enzyme was omitted in the multiple alignment.

By other side, the enzymes of *A. fulgidus* (PDB:4TQ3) and *A. pernix* (PDB:4OD5) have been described has homologs and members of the superfamily of UbiA membrane-embedded isoprenoid synthases previously mentioned (Cheng and Li, 2014; Huang et al., 2014). Both enzymes catalyze the synthesis of all-trans polyprenyls by repeated addition of isopentenyl pyrophosphate (IPP). It is remarkable that the enzymes of all fungal species within this subclade but those of *G. boninense* (VWP00961 and VWP095157) share the functional NALYDRDVD and YEFD motifs of *A. fulgidus* (PDB:4TQ3) and *A. pernix* (PDB:4OD5) proteins (Figure 6). However, the VWP095157 *G. boninense* enzyme showed amino acid sequences that resemble both motifs, although the latter in a different location within the alignment (Figure 6). Thus, despite phylogenetic relatedness, the enzymes within this subclade showed limited amino acid identity but similar folding pattern and are

functionally diverse. Furthermore, both *G. boninense* enzymes within the clade have a twofold difference in amino acid length.

Interestingly, when seeking templates in SWISS-MODEL website for modelling VWO95157 *G. boninense* protein, the best parameters were obtained using the Get3-Get4-Get5 complex from *S. cerevisiae* (PDB: 5bwk1d; Supplementary Table II), which are involved in the GET pathway to target proteins from ribosome to the endoplasmic reticulum membrane (Gristick et al., 2014). This strongly suggest that such protein belongs to the UbiA membrane-embedded isoprenoid synthases above mentioned that efficiently uses FPP as substrate (Supplementary Table III). However, results here obtained make difficult to assign a putative function to *G. boninense* enzymes and opens the question on the origin of the fungal enzymes within the subclade. It might be possible that adding more sequences of the UbiA superfamily can aid to obtain a refinement of the putative functional characteristics of the *G. boninense* enzymes of this subclade.

The next subclade includes enzymes of *G. boninense* and *Ganoderma* sp. with those of several basidiomycetes (Figure 1); however, the length of *G. boninense* VWP00963 enzyme generated an alignment that difficulties to observe the functional motifs (data not shown), thus it was eliminated of the multiple alignment. When the *Ganoderma* sp. enzyme JGI:151299 is aligned with other TSs within this subclade, the shared functional motifs are revealed (Figure 7). The founding motifs in this subclade are non-canonical and do not allow to assign a putative function to the enzymes within this subclade. Surprisingly, the best template found to modelling VWP00963 enzyme was the mitochondrial complex I (PDB:6yj4) from the ascomycetous yeast *Yarrowia lipolytica* (Grba and Hirst, 2020) (Supplementary Table II). This can be attribute to transmembrane domains maybe shared by both enzymes and suggest that such *G. boninense* protein are and UbiA-type TS attached to ER membrane. Docking results suggest that this TS use FPP as substrate (Supplementary Table III), but further analyses are necessary to determine the kind of metabolites synthesized by VWP00963 enzyme.

An interestingly subclade includes a combination of enzymes from *Homo sapiens*, the bacteria *Alicyclobacillus acidocaldarius*, the ascomycetes *Cephalosporium caerulens, Daldinia eschscholzii Schizosaccharomyces pombe*, the human pathogens *Candida albicans* and *Pneumocystis carinii*, and several basidiomycetes, including one *Ganoderma* sp.

(JGI:118462) and *G. sinense* (PIL24816), and three *G. lucidum* (APO20484, ADD60470, ADD60469) enzymes (Figure 1). When aligned, the enzymes within this clade do not show to share functional motifs (data not shown); however, when only the basidiomycete enzymes within this subclade are aligned, they shown to share a modified DCTAEA (DCTGEG in fungi here analyzed) and four QW motifs of eukaryotic oxidosqualene cyclases (OSC), a Class II triterpene cyclases (Racolta et al., 2012) (Figure 8). Furthermore, the squalene-hopene-cyclase from *A. acidocaldarius* (PDB:1sqc) that are in the same clade is the best templated for modelling the APO20484 from *G. lucidum*, JGI:118462 from *Ganoderma* sp. and PIL24816 *G. sinense* enzymes (Figure 3, Supplementary Table II), and the squalene as substrate generates robust docking parameters (Supplementary Table III). Thus, both phylogenetic and modelling results strongly indicates that the four *Ganoderma* spp. enzymes within this clade are Class II squalene cyclases.

A big clade in the phylogenetic tree obtained is composed only by basidiomycete TSs, which is divided in three main subclades (Figure 1). Thus, phylogenetic evidence suggests that the enzymes within these subclades arose early in the Basidiomycota group. Two *Ganoderma* sp. (JGI143866, JGI164758) and one *G. sinense* (PIL26225) cluster together in the first Basidiomycota subclade. The multiple alignment shows the canonical DDxxD and NSD motifs, as well as the R and RY residues relevant for binding of the substrate and the Mg^{2+} (Figure 9) described for bacterial Class I sesquiterpene synthases (Dickschat, 2016). Such motifs, particularly the NSD one, are closely similar to those in the *Streptomyces clavuligerus* (ATCC27074) sesquiterpene synthase that synthetizes (−)-isohirsut-1-ene. Despite the lack of overall amino acid sequence similarity, the location of the motifs and structural conformation of *Ganoderma* spp. enzymes with that of *S. clavuligerus* are similar (data not shown). This suggests that such subclade of basidiomycete enzymes, particularly those of *Ganoderma* spp., have the similar catalytic activities to the *S. clavuligerus* sesquiterpene synthase. When modelling the enzymes JGI164758 from *Ganoderma* sp. and PIL26225 from *G. sinense* within this clade the best template was the geosmin synthase (PDB: 5dz2) from *S. coelicolor* (Supplementary Table II), and the docking analysis show that these enzymes can process FPP (Supplementary Table III). Thus, structural analysis indicates that enzymes of *Ganoderma* spp. and other of the other basidiomycetes in this clade are sesquiterpene synthases.

The second subclade within the Basidiomycota clade includes one *G. sinense* (PIL:35773) and one *Ganoderma* sp. (JGI:57679) enzymes (Figure 1). Interestingly, this subclade combines a non-canonical DxxxD motif of bacterial UbiA-type cyclase with the canonical NSE motif of Class I terpene synthases (Christianson, 2017; Rudolf and Chang, 2020) (Figure 10). In most enzymes of the basidiomycete species within this subclade, including those of *Ganoderma* spp., this second motif has a remarkable identity with the corresponding one of bacteria 2-methylisoborneol (2-MIB) synthases, particularly with those of the one of the variants of actinomycete *S. coelicolor* with the NDLYSYTKE motif (Dickschat, 2016). Experimental and bioinformatics evidence showed that 2-MIB synthases are present in several cyanobacteria species (Izaguirre and Taylor, 2004; Wang et al., 2011). Functional motifs of 2-MIB synthases are similar between actinomycete and cyanobacteria, but whereas the NSE one is clearly defined, the second motif in such taxa seems like a combination or intermediate between canonical DDxxD and non-canonical DxxxD motifs. That being because, several of these motifs have the first DD amino acids and three central amino acids instead of two, and not all have the final D (Wang et al., 2011; Dickschat, 2016). By other side, the best template found to model the two enzymes within this clade was the spiroviolene synthase (PDB:6tiv) from *Streptomyces violens* (Supplementary Table II), a bacterial class I diterpene cyclase (Schriever et al., 2021) that can use GGPP as substrate (Supplementary Table III).

Previous evidence and results here obtained do not precisely clarify the origin of enzymes within this basidiomycetes subclade. It might be suggested that the enzymes in this basidiomycete subclade are 2-MIB synthases that has been acquired by horizontal gene transfer (HGT) from an actinomycete or cyanobacteria to ancestral basidiomycete with posterior divergence that originates the DxxxD motif within this last taxon. The origin of TS in fungi by HGT from bacteria has been demonstrated (Jia et al., 2019), but alternative hypothesis like convergent evolution, duplication and gene recombination documented in plant TS cannot be excluded (Cseke et al., 1998; Pichersky and Lewinsohn, 2011). Several previous works describe the production of 2-MIB by ascomycete fungi (Börjesson et al., 1993; La Guerche et al., 2006; Moularat et al., 2008; Sadoughi et al., 2015), However, to the best of our knowledge, 2-MIB production by basidiomycetes have not been reported, nor the enzymes associated to their synthesis in fungal taxa. Both actinomycetes and cyanobacteria

used the same mechanism to synthetize 2-MIB, which consist in the methylation of GDP by methyl transferase (GDPMT) followed by cyclization of methyl-GDP by MIB synthase (MIB) (Giglio et al., 2011; Wang et al., 2011). Thus, it is possible that ascomycetes and basidiomycetes use similar synthesis mechanisms, something that needs to be studied in future works.

In order to refine the probable functional diversity of the third Basidiomycota, this was divided in three minor subclades (Figure 1). The first minor subclade includes one *G. sinense* (PIL23378) and one *Ganoderma* sp. (JGI:57109) enzymes (Figure 1) and the multiple alignment with other enzymes of the cluster shows an interesting motifs sequence (Figure 11). As in the previous subclade, the first motif of some enzymes seems to be a combination of the non-canonical DxxxD associated to the previously described UbiA-type terpene cyclases (Rudolf and Chang, 2020) with the canonical DDxxD motif of canonical terpene synthases (Dickschat, 2016). However, regarding the *Ganoderma* spp. enzymes, this motif is closer to the non-canonical one. On the other hand, the NDS motif shows high sequence conservation within the members of the clade and the *G. sinense* enzyme lacks the initial N residue (Figure 11). Also, these enzymes show as well as the R and RY residues relevant for binding of the substrate and the Mg^{2+}. Interestingly, NDS motif has high identity with the corresponding one at the C-terminal domain of the geosmin synthase of *Nostoc punctiforme*, but the TS of this cyanobacterium is nearly two-fold longer than those of basidiomycetes within this subclade (Agger et al., 2008; Giglio et al., 2008). In concordance with these structural signatures, the best template to model the enzymes within this subclade is the geosmin synthase (PDB:5dz2) from *S. coelicolor* (Figure 3, Supplementary Table II) using FPP as substrate (Supplementary Table III). Geosmin production has been documented in basidiomycetes, including *Armillaria mellea*, *Cortinarius herculeus*, *Cortinarius hinnuleus*, *Cystoderma amianthinum*, *Cystoderma carcharias*, and *Pholiota squarrosa* (Breheret et al., 1999; Müller et al., 2013; Arnold et al., 2016), but as far as we know, it has been not reported for *Ganoderma* spp. Based in this evidence on the NSD motif resemblance, the R, RY key amino acids, and the DxxxD/DDxxD like motif, as well as the modelling and docking results, it can be suggested that the *Ganoderma* spp. and other basidiomycete enzymes within this subclade might be a geosmin synthase like enzyme, but further experimental studies are needed to proof this.

The second minor subclade includes one *Ganoderma* sp. (JGI:119170) and one *G. sinense* (PIL24516) enzymes (Figure 1). When some selected enzymes within this group were aligned, a very interesting motif pattern emerges (Figure 12). All these enzymes showed a highly conserved non-canonical DxxxD (DNISD) motif characteristic of UbiA-type cyclases (Rudolf and Chang, 2020). A second NSE (NDIFSYN) motif characteristic of Type I terpene synthases (Dickschat, 2016) and conserved in most enzymes is also present in this group. Finally, a QW like motif that expands by 21 amino acids is present in the C-terminal extreme of the aligned enzymes, characteristic of Class II bacterial triterpene cyclase, including squalene-hopene cyclases (SHC) and eukaryotic oxidosqualene cyclases (OSC), in which such motif normally expands 16 amino acids (Racolta et al., 2012). Furthermore, these enzymes also share the highly conserved R amino acid and RY dyad (Figure 12) important for binding of the substrate and the Mg^{2+} of bacterial TS (Dickschat, 2016). To the best of our knowledge, this combination of motifs has not precedent in any fungi and seeking for an explanation probably requires demonstrating several evolutionary mechanisms acting in combination as the HGT from bacteria (Jia et al., 2019), duplication and gene recombination (Cseke et al., 1998; Pichersky and Lewinsohn, 2011), previously mentioned. The best template to model the enzymes within this subclade is the geosmin synthase (PDB:5dz2) from *S. coelicolor* (Figure 3, Supplementary Table II) using FPP and germacrenediol as substrates (Supplementary Table III). The combination of motifs in these *Ganoderma* spp. TSs difficult to stablish putative function and terpenes produced by enzymes in this group is difficult to elucidate due its motifs combination but modelling results can suggest the synthesis of geosmin-like metabolites.

The final minor subclade within the basidiomycete clade shows the canonical DDxxD and NSE motifs of bacterial Class I terpene cyclases (Figure 13). Multiple alignment was conducted with arbitrary selected enzymes within the subclade, showing that both motifs are highly conserved. Whereas the DDxxD motif differs only in one amino acid, most of the NSE motifs showed SYN sequence besides the conserved first two and final amino acids. Such motifs are present in mono, di and sesquiterpene synthases, thus making impossible to have a more precisely hypothesis on the kind of terpene produced by enzymes within this subclade. As in the case of the enzymes in the two previous subclades within this big Basidiomycota clade, the best template to modeling the two *Ganoderma* sp. (JGI:58158,

JGI:58881) and the *G. sinense* (PIL:35630) enzymes in this subclade are from *Streptomyces* spp. proteins (Figure 3, Supplementary Table II). However, whereas for the closely phylogenetically related (PIL:35630, JGI:58158) the best templated was the selinadiene synthase from *S. pristinaespiralis* (PDB:4okm) that uses FPP as substrate (Supplementary Table III), for the other *Ganoderma* sp. enzyme (JGI:58881) was the spiroviolene synthase (PDB:6tiv) from *S. violens* previously described, a bacterial class I diterpene cyclase (Supplementary Table II) that can use GGPP as substrate (Supplementary Table III).

Conclusion

The present work assigned putative functions to 39 TSs from four different *Ganoderma* spp., using a combination of bioinformatic tools that included a robust phylogenetic analysis, molecular modeling using crystallized proteins as templates, and docking analysis with the two substrates commonly recognized by different groups of TSs. The results here obtained showed a clade of TSs of *Ganoderma* spp. with no defined relationships to other TSs, another group with phylogenetic and structural relationships between prokaryotes and eukaryotes, and a large specific clade of basidiomycete TSs. This suggests at least three evolutionary trajectories in the different TSs of *Ganoderma* spp.: possible ancestral TSs or acquired through horizontal transfer, which are present in diverse taxonomic groups; TSs that were generated within Basidiomycota; and enzymes specific to the *Ganoderma* genus. Functional analysis shows Class I and Class II TSs in *Ganoderma* spp., with great versatility for the synthesis of both cyclic and non-cyclic terpenes. Experimental strategies can be designed for the cloning and heterologous expression of the genes of the enzymes analyzed here, for the synthesis of specific terpenes based on the functional hypotheses here proposed.

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Phylogenetic and structural analysis of *Ganoderma* spp. terpene synthases

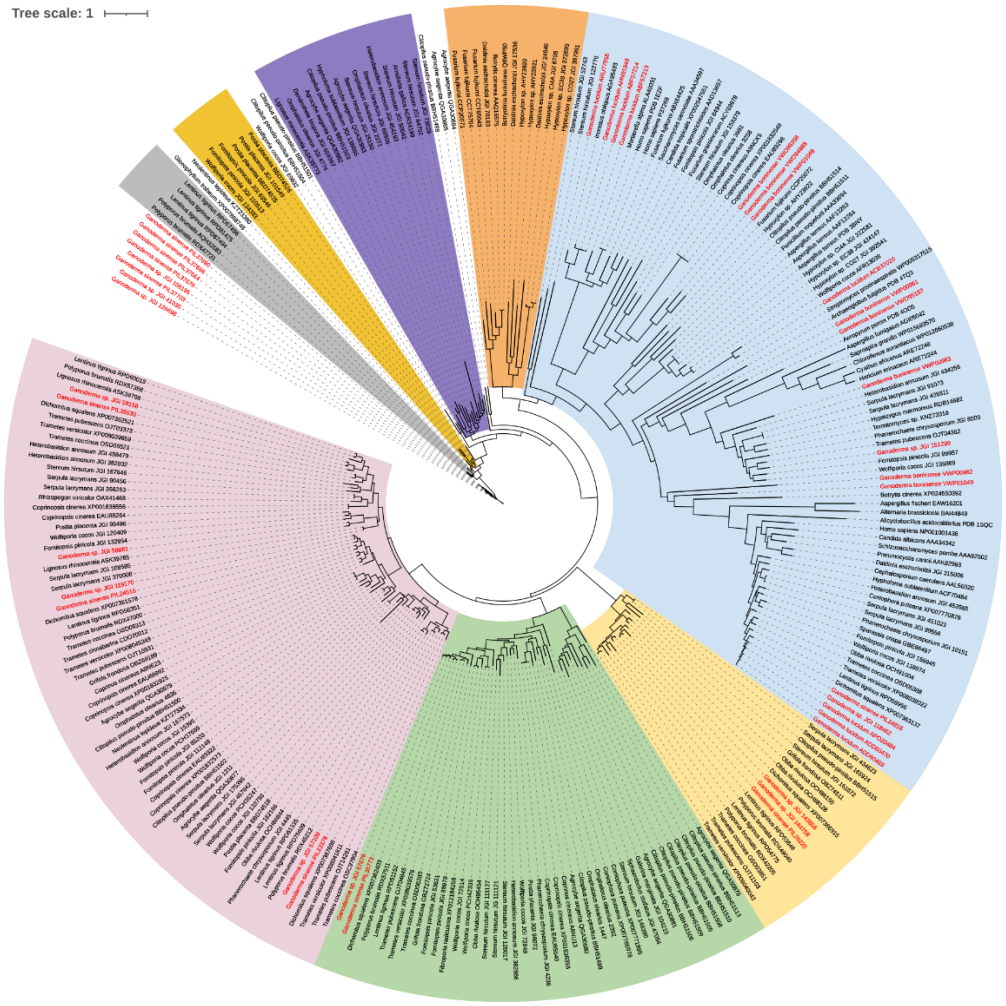

Figure 1. Phylogenetic analysis of the terpene synthases retrieved from databases. Sequences selected from the NCBI GenBank and JGI appear with their corresponding accession number. The tree was generated with Maximum Likelihood (ML) and Bayesian Inference (BI) but only the ML tree is shown because the BI tree is congruent with the same grouping pattern. In each node the bootstrap (1000 iterations) values above 50 and the Bayesian probability values above 0.5 are shown. Main phylogenetic clades are highlighted in different colors and *Ganoderma* spp. enzymes are depicted in red.

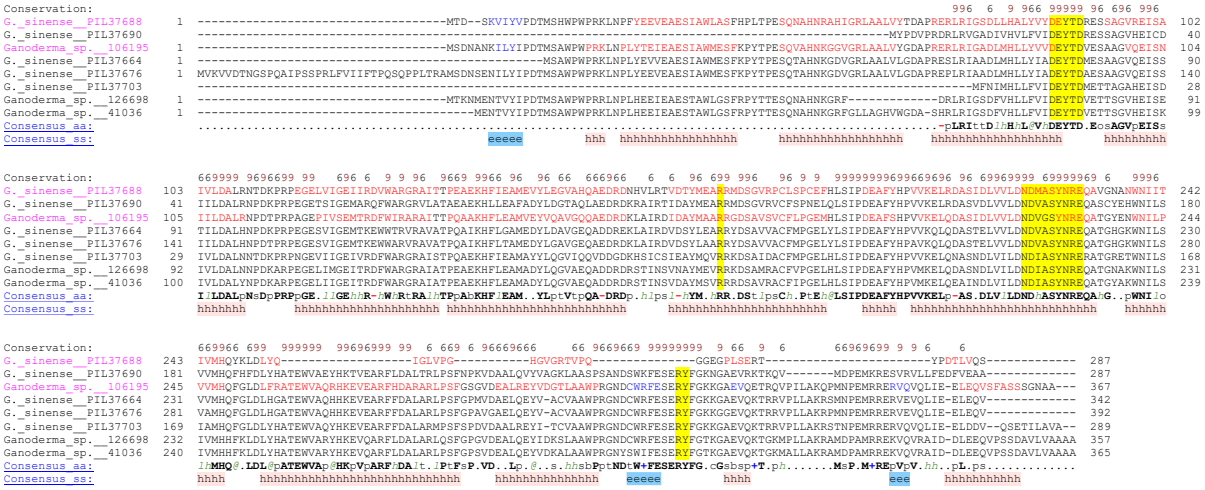

Figure 2. Multiple alignment of *Ganoderma* spp. terpene synthases not grouped in the phylogenetic tree. The Asp-rich metal-binding motifs DxxxD and NSE, and the R/RY aa residues involved in substrate and Mg^{+2} binding are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

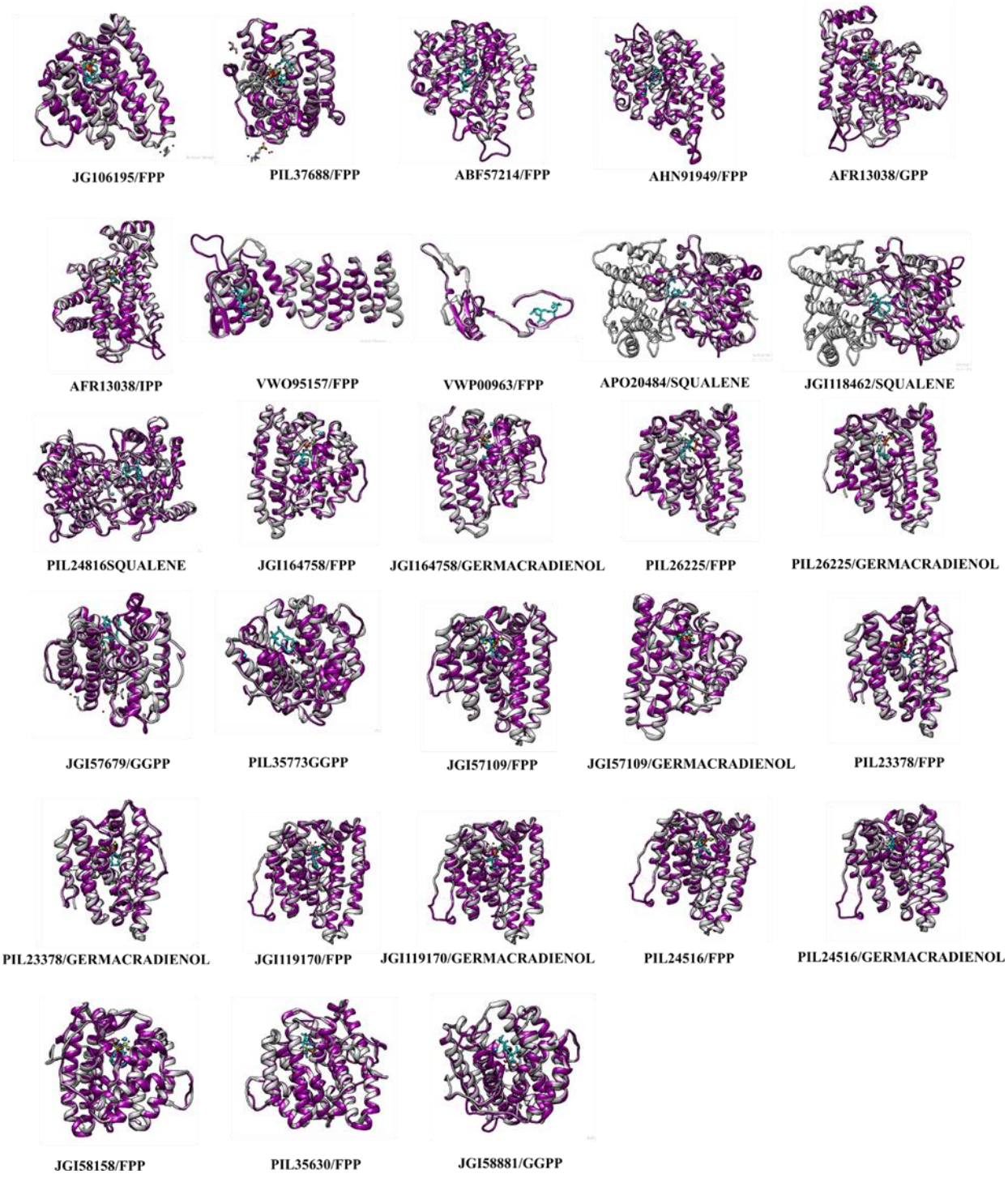

Figure 3. Homology modelling and docking analysis of *Ganoderma* spp. terpene synthases. Below each model the ID of the enzyme and the substrate for docking are showed. For modelling and docking parameters see Material and Methods and Supplementary Tables II and III. The magenta color corresponds to *Ganoderma* spp. enzymes and the grey one to templates for modelling.

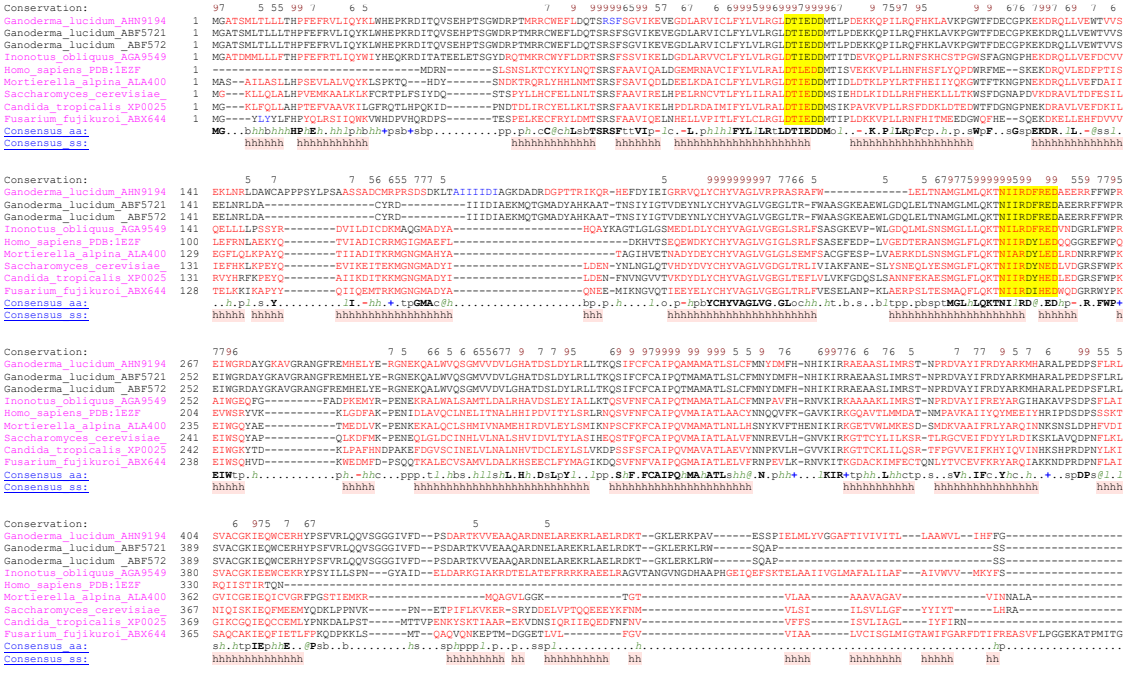

Figure 4. Multiple alignment of *Ganoderma* spp. and SQSs amino acid sequences cluster together in the phylogenetic tree. The Asp-rich metal-binding motifs DxxxDD and NxxxDxxxD of UbiA-type cyclase are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e. Besides each species name the ID of Genbank or JPG accession for each sequence are depicted. For facility, the final four amino acids of the *F. fujikuroi* TS are not depicted.

Figure 5. Alignment of *Ganoderma lucidum* and *Wolfoporia cocos* terpene synthases grouped in the phylogenetic tree. The Asp-rich metal-binding motifs DDxxD and NDE are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

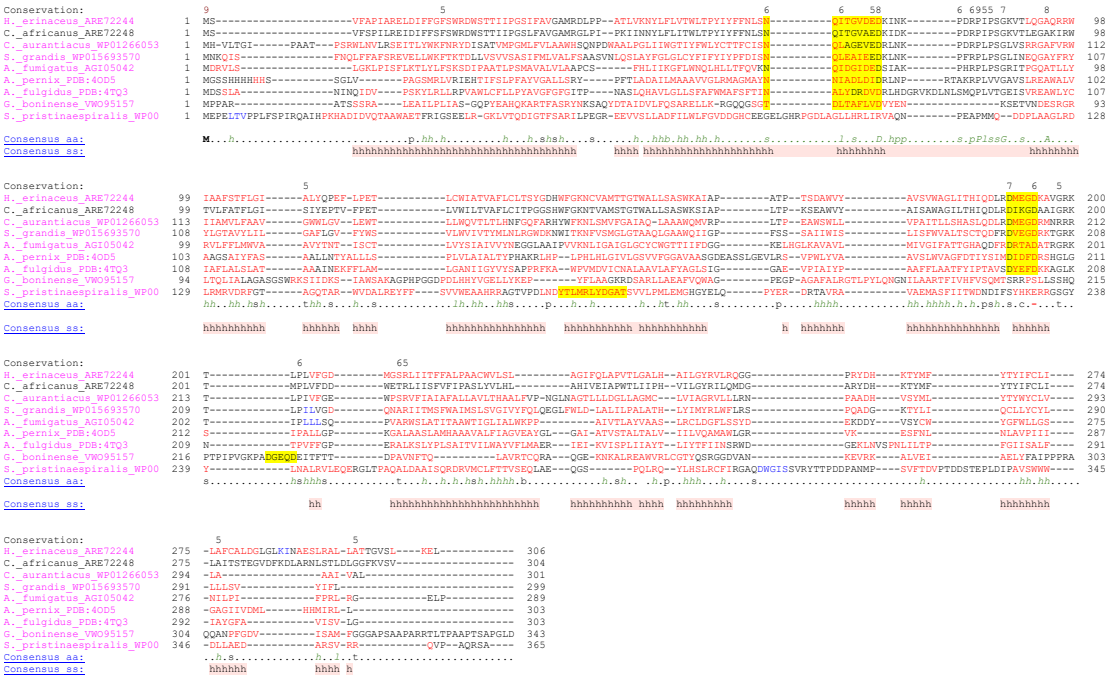

Figure 6. Alignment of *Ganoderma* spp. and terpene synthases grouped in the phylogenetic tree. The motif ^{174}YTLMRLYDGAT^{184} of the *Streptomyces pristinaespiralis* crystalized enzyme that determines the G1/2 helix breakage at the active site is highlighted in yellow. The NALYDRDVD and YEFD conserved aspartate-rich motifs characteristic of the UbiA family found in the *Archaeoglobus fulgidus* enzyme are also highlighted with the putative same motifs of the other sequences. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

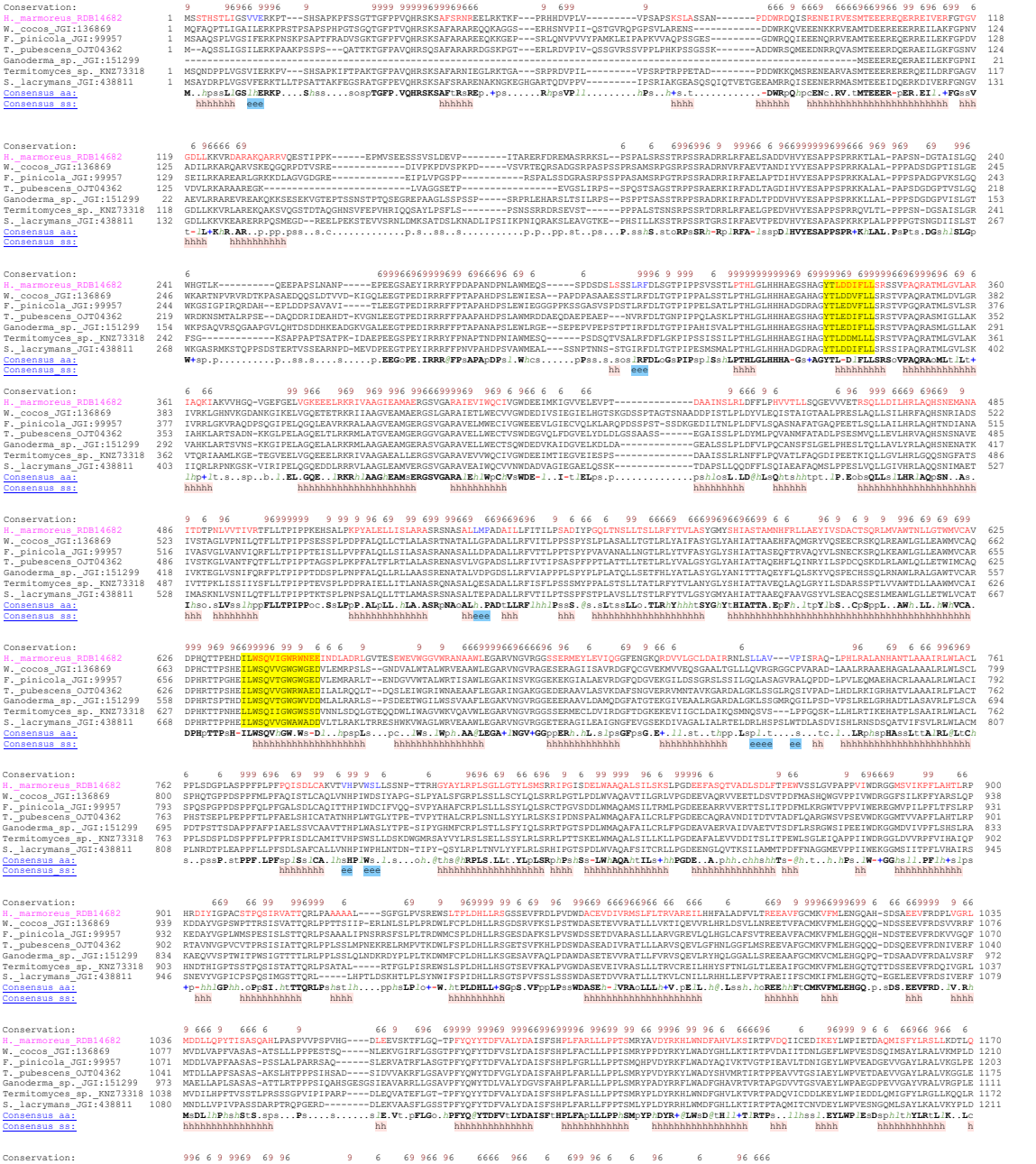

Figure 7. Alignment of *Ganoderma* sp. and other basidiomycete terpene synthases grouped in the phylogenetic tree. The expanded DDxxD and QW motifs are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

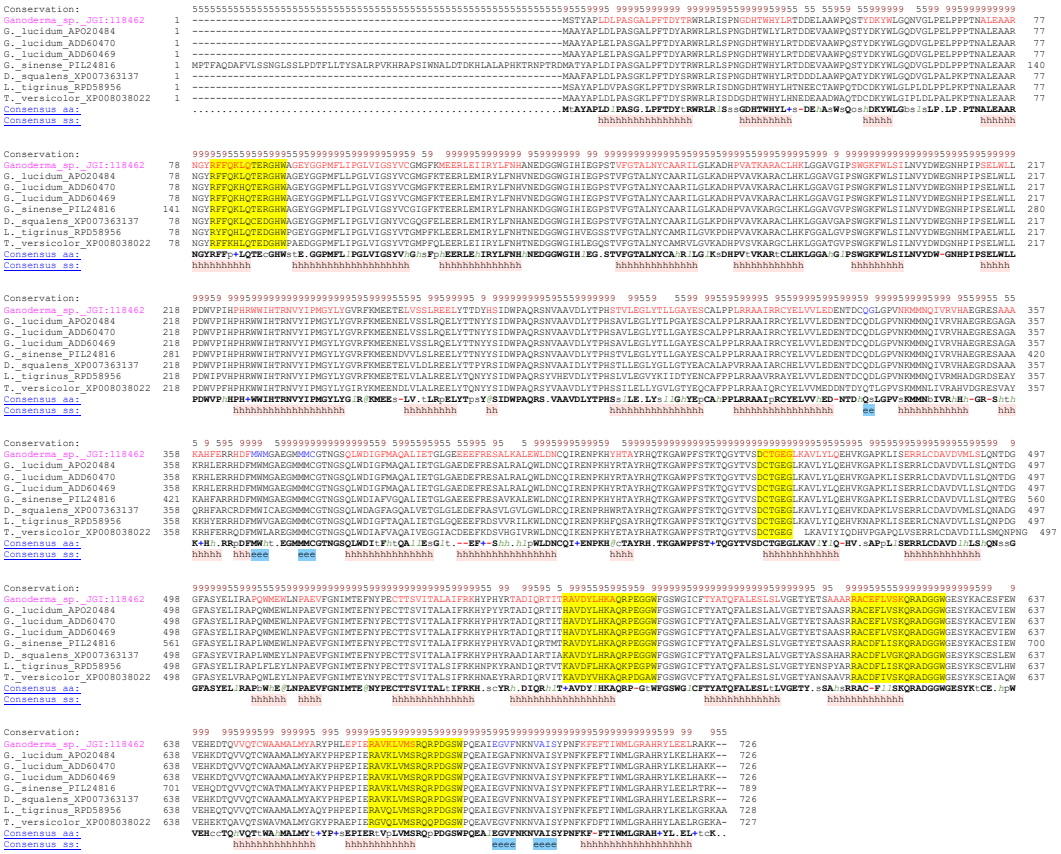

Figure 8. Alignment of *Ganoderma* sp. and other basidiomycete triterpene cyclases grouped in the phylogenetic tree. The modified DCTAEA and four QW motifs are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

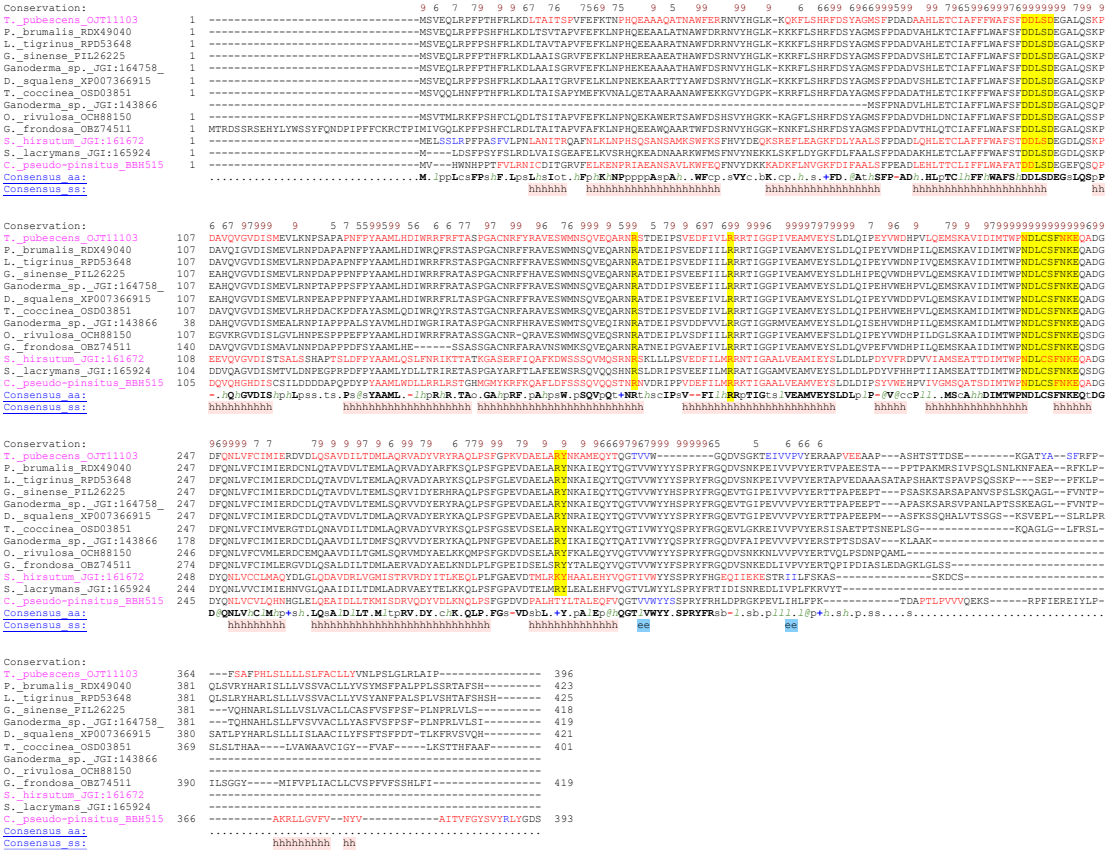

Figure 9. Alignment of *Ganoderma* sp. and other basidiomycete putative sesquiterpene synthases grouped in the phylogenetic tree. Canonical DDxxD and NSD motifs, as well as the R and RY residues relevant for binding of the substrate and the Mg^{2+} are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

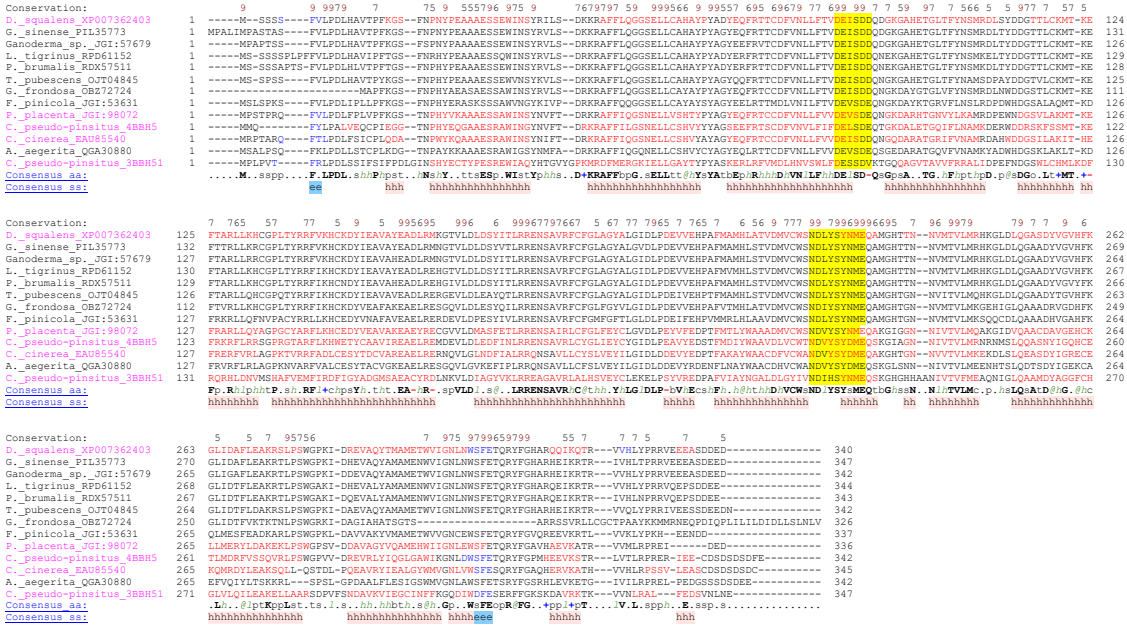

Figure 10. Alignment of *Ganoderma* sp. and other basidiomycete putative 2-methylisoborneol (MIB) related synthases grouped in the phylogenetic tree. Non-canonical DxxxD motif of bacterial UbiA-type cyclase and the canonical NSE motif of Class I terpene synthases are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

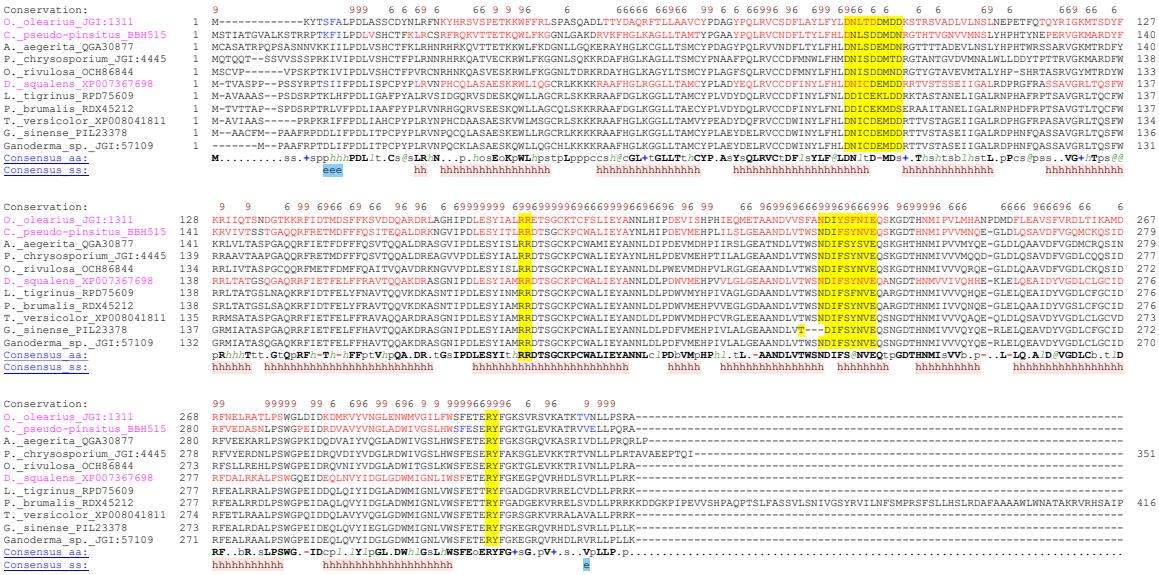

Figure 11. Alignment of *Ganoderma* sp. and other basidiomycete putative sesquiterpene synthases grouped in the phylogenetic tree. DDxxD and NSD motifs, as well as the R and RY residues relevant for binding of the substrate and the Mg^{2+} are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

Figure 12. Alignment of *Ganoderma* sp. and other basidiomycete terpene synthases grouped in the phylogenetic tree. Non-canonical DxxxD motif of bacterial UbiA-type cyclase, the canonical NSE motif of Class I terpene synthases, expanded QW motif, and the R and RY residues relevant for binding of the substrate and the Mg^{2+} are highlighted in yellow are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

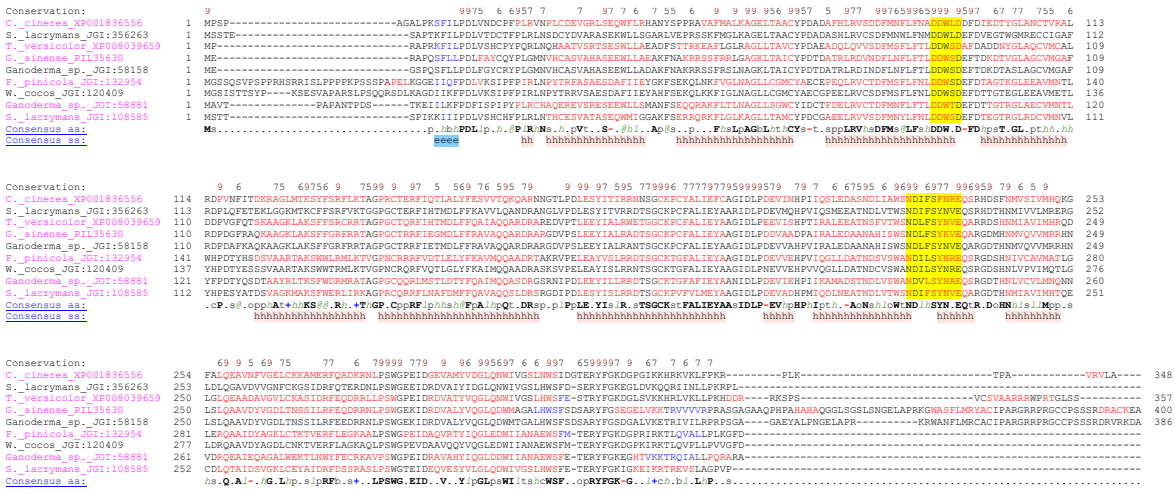

Figure 13. Alignment of *Ganoderma* sp. and other basidiomycete terpene cyclases grouped in the phylogenetic tree. The canonical DDxxD and NSE motifs of Class I terpene cyclases are highlighted in yellow are highlighted in yellow. The alignment was performed in PROMALS3D web server (http://prodata.swmed.edu/promals3d/promals3d.php). The first line in each block shows conservation indices for positions with a conservation index above 4. The last two lines show consensus amino acid sequence (Consensus_aa) and consensus predicted secondary structures (Consensus_ss). The first and last residue numbers of each sequence in each alignment block are shown before and after the sequences, respectively. Consensus predicted secondary structure letters: alpha-helix: h; beta-strand: e.

Supplementary Table I. Proteins used for phylogenetic analysis.

Taxa	JGI/Genbank ID	Species
Basidiomycota	QGA30877	*Agrocybe aegerita*
	QGA30878	*Agrocybe aegerita*
	QGA30879	*Agrocybe aegerita*
	QGA30880	*Agrocybe aegerita*
	QGA30881	*Agrocybe aegerita*
	QGA30882	*Agrocybe aegerita*
	QGA30883	*Agrocybe aegerita*
	QGA30884	*Agrocybe aegerita*
	QGA30885	*Agrocybe aegerita*
	AGR34199	*Armillaria gallica*
	BBH51498	*Clitopilus pseudo-pinsitus*
	BBH51499	*Clitopilus pseudo-pinsitus*
	BBH51500	*Clitopilus pseudo-pinsitus*
	BBH51501	*Clitopilus pseudo-pinsitus*
	BBH51502	*Clitopilus pseudo-pinsitus*
	BBH51503	*Clitopilus pseudo-pinsitus*
	BBH51504	*Clitopilus pseudo-pinsitus*
	BBH51505	*Clitopilus pseudo-pinsitus*
	BBH51506	*Clitopilus pseudo-pinsitus*
	BBH51508	*Clitopilus pseudo-pinsitus*
	BBH51509	*Clitopilus pseudo-pinsitus*
	BBH51510	*Clitopilus pseudo-pinsitus*
	BBH51511	*Clitopilus pseudo-pinsitus*
	BBH51513	*Clitopilus pseudo-pinsitus*
	BBH51514	*Clitopilus pseudo-pinsitus*
	BBH51515	*Clitopilus pseudo-pinsitus*
	XP007770878	*Coniophora puteana*
	XP007771895	*Coniophora puteana*
	XP007765978	*Coniophora puteana*
	EAU89322	*Coprinopsis cinerea*
	EAU85540	*Coprinopsis cinerea*
	EAU89298	*Coprinopsis cinerea*
	XP001832573	*Coprinopsis cinerea*
	XP001836556	*Coprinopsis cinerea*
	XP001832925	*Coprinopsis cinerea*
	XP001836356	*Coprinopsis cinerea*
	XP001832549	*Coprinopsis cinerea*

EAU85264	*Coprinopsis cinerea*
EAU88892	*Coprinopsis cinerea*
A8NU13	*Coprinus cinereus*
A8NCK5	*Coprinus cinereus*
A8NE23	*Coprinus cinereus*
ARE72248	*Cyathus africanus*
JGI:659367	*Dendrothele bispora*
XP007362403	*Dichomitus squalens*
XP007362521	*Dichomitus squalens*
XP007361578	*Dichomitus squalens*
XP007366915	*Dichomitus squalens*
XP007363137	*Dichomitus squalens*
XP007367698	*Dichomitus squalens*
XP012184256	*Fibroporia radiculosa*
JGI:53631	*Fomitopsis pinicola*
JGI:88978	*Fomitopsis pinicola*
JGI:132954	*Fomitopsis pinicola*
JGI:110513	*Fomitopsis pinicola*
JGI:85203	*Fomitopsis pinicola*
JGI:112148	*Fomitopsis pinicola*
JGI:162166	*Fomitopsis pinicola*
JGI:99957	*Fomitopsis pinicola*
JGI:159945	*Fomitopsis pinicola*
JGI:84944	*Fomitopsis pinicola*
JGI:63546	*Fomitopsis pinicola*
JGI:104215	*Galerina marginata*
VWP00962	*Ganoderma boninense*
VWP01049	*Ganoderma boninense*
VWP01048	*Ganoderma boninense*
VWP00963	*Ganoderma boninense*
VWP00961	*Ganoderma boninense*
VWO96958	*Ganoderma boninense*
VWO95157	*Ganoderma boninense*
VWO94988	*Ganoderma boninense*
APO20484	*Ganoderma lucidum*
ADD60470	*Ganoderma lucidum*
AHN91949	*Ganoderma lucidum*
ACB37020	*Ganoderma lucidum*
ABF57213	*Ganoderma lucidum*
ABF57214	*Ganoderma lucidum*
ADD60469	*Ganoderma lucidum*
ARU77555	*Ganoderma lucidum*

PIL35773	*Ganoderma sinense*
PIL35630	*Ganoderma sinense*
PIL37703	*Ganoderma sinense*
PIL37688	*Ganoderma sinense*
PIL24516	*Ganoderma sinense*
PIL37676	*Ganoderma sinense*
PIL37664	*Ganoderma sinense*
PIL26225	*Ganoderma sinense*
PIL24816	*Ganoderma sinense*
PIL37690	*Ganoderma sinense*
PIL23378	*Ganoderma sinense*
JGI:57679	*Ganoderma* sp.
JGI:58158	*Ganoderma* sp.
JGI:58881	*Ganoderma* sp.
JGI:106195	*Ganoderma* sp.
JGI:119170	*Ganoderma* sp.
JGI:126698	*Ganoderma* sp.
JGI:143866	*Ganoderma* sp.
JGI:151299	*Ganoderma* sp.
JGI:164758	*Ganoderma* sp.
JGI:118462	*Ganoderma* sp.
JGI:41036	*Ganoderma* sp.
JGI:57109	*Ganoderma* sp.
XP007868748	*Gloeophyllum trabeum*
OBZ72724	*Grifola frondosa*
OBZ69189	*Grifola frondosa*
OBZ74511	*Grifola frondosa*
ARE72244	*Hericium erinaceus*
JGI:382866	*Heterobasidion annosum*
JGI:458479	*Heterobasidion annosum*
JGI:382802	*Heterobasidion annosum*
JGI:167573	*Heterobasidion annosum*
JGI:434256	*Heterobasidion annosum*
JGI:453585	*Heterobasidion annosum*
JGI:454193	*Heterobasidion annosum*
ACF70484	*Hypholoma sublateritium*
JGI:138665	*Hypholoma sublateritium*
RDB14682	*Hypsizygus marmoreus*
AGA95493	*Inonotus obliquus*
RPD61152	*Lentinus tigrinus*
RPD60019	*Lentinus tigrinus*
RPD67494	*Lentinus tigrinus*

RPD67496	*Lentinus tigrinus*
RPD81425	*Lentinus tigrinus*
RPD56351	*Lentinus tigrinus*
RPD54775	*Lentinus tigrinus*
RPD53648	*Lentinus tigrinus*
RPD58956	*Lentinus tigrinus*
RPD75609	*Lentinus tigrinus*
RPD61335	*Lentinus tigrinus*
ASK39765	*Lignosus rhinocerotis*
ASK39773	*Lignosus rhinocerotis*
ASK39768	*Lignosus rhinocerotis*
KZT23360	*Neolentinus lepideus*
KZT27334	*Neolentinus lepideus*
OCH86844	*Obba rivulosa*
OCH95454	*Obba rivulosa*
OCH88128	*Obba rivulosa*
OCH88150	*Obba rivulosa*
OCH91004	*Obba rivulosa*
JGI:4774	*Omphalotus olearius*
JGI:2271	*Omphalotus olearius*
JGI:1311	*Omphalotus olearius*
4636	*Omphalotus olearius*
1447	*Omphalotus olearius*
2392	*Omphalotus olearius*
3258	*Omphalotus olearius*
3981	*Omphalotus olearius*
JGI:4238	*Phanerochaete chrysosporium*
JGI:4445	*Phanerochaete chrysosporium*
JGI:8500	*Phanerochaete chrysosporium*
JGI:10151	*Phanerochaete chrysosporium*
RDX45212	*Polyporus brumalis*
RDX57511	*Polyporus brumalis*
RDX57358	*Polyporus brumalis*
RDX47000	*Polyporus brumalis*
RDX47731	*Polyporus brumalis*
RDX49040	*Polyporus brumalis*
RDX42205	*Polyporus brumalis*
AQH32583	*Polyporus brumalis*
BBD74518	*Postia placenta*
JGI:99496	*Postia placenta*
JGI:101549	*Postia placenta*
JGI:98072	*Postia placenta*

BBD74526	*Postia placenta*
BBD74525	*Postia placenta*
OAX41468	*Rhizopogon vinicolor*
JGI:90456	*Serpula lacrymans*
JGI:356263	*Serpula lacrymans*
JGI:108585	*Serpula lacrymans*
JGI:370008	*Serpula lacrymans*
JGI:175395	*Serpula lacrymans*
JGI:457642	*Serpula lacrymans*
JGI:165924	*Serpula lacrymans*
JGI:434623	*Serpula lacrymans*
JGI:438811	*Serpula lacrymans*
JGI:91073	*Serpula lacrymans*
JGI:99556	*Serpula lacrymans*
JGI:451021	*Serpula lacrymans*
GBE86497	*Sparassis crispa*
JGI:47084	*Sphaerobolus stellatus*
JGI:159379	*Stereum hirsutum*
JGI:161672	*Stereum hirsutum*
JGI:167646	*Stereum hirsutum*
JGI:146390	*Stereum hirsutum*
JGI:128017	*Stereum hirsutum*
JGI:50042	*Stereum hirsutum*
JGI:122776	*Stereum hirsutum*
JGI:52743	*Stereum hirsutum*
JG:111121	*Stereum hirsutum*
JGI:111127	*Stereum hirsutum*
JGI:64702	*Stereum hirsutum*
JGI:73029	*Stereum hirsutum*
JGI:69906	*Stereum hirsutum*
JGI:25180	*Stereum hirsutum*
KNZ73318	*Termitomyces sp.*
CDO70012	*Trametes cinnabarina*
OSC97804	*Trametes coccinea*
OSD06380	*Trametes coccinea*
OSD06521	*Trametes coccinea*
OSD08313	*Trametes coccinea*
OSD03851	*Trametes coccinea*
OSD05388	*Trametes coccinea*
OJT04845	*Trametes pubescens*
OJT03373	*Trametes pubescens*
OJT10931	*Trametes pubescens*

	OJT04362	*Trametes pubescens*
	OJT11103	*Trametes pubescens*
	OJT14281	*Trametes pubescens*
	XP008039579	*Trametes versicolor*
	XP008039659	*Trametes versicolor*
	XP008045349	*Trametes versicolor*
	XP008040042	*Trametes versicolor*
	XP008038022	*Trametes versicolor*
	XP008041811	*Trametes versicolor*
	PCH35247	*Wolfiporia cocos*
	JGI:72514	*Wolfiporia cocos*
	JGI:72849	*Wolfiporia cocos*
	PCH42336	*Wolfiporia cocos*
	JGI:120409	*Wolfiporia cocos*
	JGI:89832	*Wolfiporia cocos*
	JGI:15395	*Wolfiporia cocos*
	PCH37689	*Wolfiporia cocos*
	JGI:134393	*Wolfiporia cocos*
	JGI:133798	*Wolfiporia cocos*
	JGI:136869	*Wolfiporia cocos*
	JGI:138974	*Wolfiporia cocos*
	AFR13038	*Wolfiporia cocos*
Ascomycota	BAI44849	*Alternaria brassicicola*
	EAW16201	*Aspergillus fischeri*
	AGI05042	*Aspergillus fumigatus*
	PDB:3BNY	*Aspergillus terreus*
	AAF13264	*Aspergillus terreus*
	Q6WP50	*Botryotinia fuckeliana*
	AAQ16575	*Botrytis cinerea*
	XP024550392	*Botrytis cinerea*
	AAA34342	*Candida albicans*
	XP002547551	*Candida tropicalis*
	AAL56020	*Cephalosporium caerulens*
	JGI:17536	*Daldinia eschscholzii*
	JGI:315006	*Daldinia eschscholzii*
	JGI:24646	*Daldinia eschscholzii*
	JGI:70183	*Daldinia eschscholzii*
	CCP20071	*Fusarium fujikuroi*
	ABX64425	*Fusarium fujikuroi*
	CCT65043	*Fusarium fujikuroi*
	CCT75704	*Fusarium fujikuroi*
	CCP20072	*Fusarium fujikuroi*

	ACY69978	*Fusarium graminearum*
	AAD13657	*Fusarium sporotrichioides*
	AHY23922	*Hypoxylon* sp.
	AHY23920	*Hypoxylon* sp.
	AHY23921	*Hypoxylon* sp.
	CI4A JGI:6706	*Hypoxylon* sp.
	CI4A JGI:322581	*Hypoxylon* sp.
	CO27 JGI:397991	*Hypoxylon* sp.
	CO27 JGI:392541	*Hypoxylon* sp.
	EC38 JGI:372695	*Hypoxylon* sp.
	EC38 JGI:424147	*Hypoxylon* sp.
	AAA33694	*Penicillium roqueforti*
	AAK82993	*Pneumocystis carinii*
	AAA34597	*Saccharomyces cerevisiae*
	AAA92502	*Schizosaccharomyces pombe*
	AAF13263	*Spergillus terreus*
Other eukarya	NP001001438	*Homo sapiens*
	P37268	*Homo sapiens*
	PDB:1EZF	*Homo sapiens*
	ALA40031	*Mortierella alpina*
	WP015693570	*Saprospira grandis*
Prokayotes	PDB:4OD5	*Aeropyrum pernix*
	PDB:1SQC	*Alicyclobacillus acidocaldarius*
	PDB:4TQ3	*Archaeoglobus fulgidus*
	WP012660538	*Chloroflexus aurantiacus*
	WP005317515	*Streptomyces pristinaespiralis*

Supplementary Table II. *Ganoderma* spp. proteins selected for homology modelling and docking analysis.

***Ganoderma* PROTEINS ID**	**PDB TEMPLATE ID**	**PDB DESCRIPTION**	**SUBSTRATES PUBCHEM ID[1]**	**RESIDUES IN FAVOURED REGIONS OF THE RAMACHANDRAN PLOT**
JGI 106195	5nx6 1A	1,8-cineole synthase from *Streptomyces clavuligerus* in complex with 2-fluoroneryl diphosphate	FPP (445713)	97.7
PIL37688	5nx6 1A	1,8-cineole synthase from *Streptomyces clavuligerus* in complex with 2-fluoroneryl diphosphate	FPP (445713)	98.0
ABF57214	1ezf 1A	Human squalene synthase	FPP (445713)	98.3
AHN91949	1ezf 1A	Human squalene synthase	FPP (445713)	98.1
AFR13038	4ga3 1A	Human Farnesyl Diphosphate Synthase in Complex with BPH-1260	GPP (445995)/IPP (1195)	98.3
VWO95157	5bwk 1d	Get3-Get4-Get5 intermediate complex from *Saccharomyces cerevisiae*	FPP (445713) doi: 10.1128/AEM.02017-09	95.1
VWP00963	6yj4 1q	*Yarrowia lipolytica* complex I	FPP (445713) doi: 10.1128/AEM.02017-09	100

APO20484	1sqc 1a	Squalene-hopene-cyclase from *Alicyclobacillus acidocaldarius*	Squalene (638072) doi: 10.1126/science.277.5333.1811	96.5
JGI118462	1sqc 1a	Squalene-hopene-cyclase from *Alicyclobacillus acidocaldarius*	Squalene (638072) doi: 10.1126/science.277.5333.1811	96.2
PIL24816	1sqc 1a	Squalene-hopene-cyclase from *Alicyclobacillus acidocaldarius*	Squalene (638072) doi: 10.1126/science.277.5333.1811	96.4
JGI164758	5dz2 1a	Geosmin synthase from *Streptomyces coelicolor* N-terminal domain complexed with three Mg^{2+} ions and alendronic acid	FPP (445713)/Germacrenediol (16667385) doi: 10.1021/acs.biochem.5b01143	99.4
PIL26225	5dz2 1a	Geosmin synthase from *Streptomyces coelicolor* N-terminal domain complexed with three Mg^{2+} ions and alendronic acid	FPP (445713)/Germacrenediol (16667385) doi: 10.1021/acs.biochem.5b01143	99.0
JGI57679	6tiv 1a	SVS_A2 protein (205-DREMH-209 /205-AQDLE-209 mutant) from ancestral sequence reconstruction *Streptomyces* sp. CWA1	GGPP (447277) doi: 10.1021/jacs.0c10214	96.3
PIL35773	6tiv 1a	SVS_A2 protein (205-DREMH-209 /205-AQDLE-209 mutant) from ancestral sequence reconstruction *Streptomyces* sp. CWA1	GGPP (447277) doi: 10.1021/jacs.0c10214	96.9
JGI57109	5dz2 1a	Geosmin synthase from *Streptomyces coelicolor* N-terminal domain complexed with three Mg^{2+} ions and alendronic acid	FPP (445713)/Germacrenediol (166673	100

			85) doi: 10.1021/acs.biochem.5b01143	
PIL23378	5dz2 1a	Geosmin synthase from *Streptomyces coelicolor* N-terminal domain complexed with three Mg^{2+} ions and alendronic acid	FPP (445713)/Germacrenediol (16667385) doi: 10.1021/acs.biochem.5b01143	99.7
JGI119170	5dz2 1a	Geosmin synthase from *Streptomyces coelicolor* N-terminal domain complexed with three Mg^{2+} ions and alendronic acid	FPP (445713)/Germacrenediol (16667385) doi: 10.1021/acs.biochem.5b01143	99.4
PIL24516	5dz2 1a	Geosmin synthase from *Streptomyces coelicolor* N-terminal domain complexed with three Mg^{2+} ions and alendronic acid	FPP (445713)/Germacrenediol (16667385) doi: 10.1021/acs.biochem.5b01143	98.8
JGI58158	4okm 1a	Selinadiene Synthase apo and in complex with diphosphate *Streptomyces pristinaespiralis* ATCC 25486	FPP (445713) doi: 10.1002/anie.201403648	98.4
PIL35630	4okm 1a	Selinadiene Synthase apo and in complex with diphosphate *Streptomyces pristinaespiralis* ATCC 25486	FPP (445713) doi: 10.1002/anie.201403648	98.0
JGI58881	6tiv 1a	SVS_A2 protein (205-DREMH-209 /205-AQDLE-209 mutant) from ancestral sequence reconstruction *Streptomyces* sp. CWA1	GGPP (447277) doi: 10.1021/jacs.0c10214	97.8

[1] Abbreviatures: FPP, farnesyl diphosphate; GGPP, geranyl-geranyl pyrophosphate.

Supplementary Table III. Docking parameters obtained for *Ganoderma* spp. terpene synthases.

PROTEIN ID	SUBSTRATE[1]	GLOBAL ENERGY (kcal/mol)	ATTRACTIVE VdW (kcal/mol)	REPULSIVE VdW (kcal/mol)	ACE (kcal/mol)	HYDR
JGI 106195	FPP	-18.91	-17.83	4.92	2.09	
PIL37688	FPP	-27.64	-13.20	4.09	-8.20	
ABF57214	FPP	-28.96	-17.11	3.53	-4.61	
AHN91949	FPP	-19.73	-21.41	12.15	1.19	
AFR13038	GPP	-20.27	-14.69	0.99	0.46	
AFR13038	IPP	-14.95	-11.50	6.14	-2.13	
VWO95157	FPP	-36.92	-18.42	4.97	-8.68	
VWP00963	FPP	-14.78	-10.49	4.65	-4.34	
APO20484	Squalene	-38.39	-20.54	8.88	-9.96	
JGI118462	Squalene	-40.98	-15.67	1.59	-13.27	
PIL24816	Squalene	-41.25	-19.85	3.02	-9.81	
JGI164758	FPP	-27.60	-18.36	1.74	-1.21	
JGI164758	Germacrenediol	-27.11	-13.20	2.08	-5.71	
PIL26225	FPP	-32.26	-18.06	5.27	-6.05	
PIL26225	Germacrenediol	-31.26	-13.20	2.31	-8.38	
JGI57679	GGPP	-30.05	-18.35	4.90	-4.83	

PIL35773	GGPP	-16.92	-18.97	6.63	3.04
JGI57109	FPP	-27.53	-17.34	2.95	-2.55
JGI57109	Germacrenediol	-27.69	-12.52	2.50	-6.90
PIL23378	FPP	-30.63	-15.99	6.36	-7.67
PIL23378	Germacrenediol	-29.13	-13.00	2.67	-7.42
JGI119170	FPP	-31.46	-16.67	5.44	-6.93
JGI119170	Germacrenediol	-31.39	-12.04	0.68	-8.99
PIL24516	FPP	-31.81	-16.75	0.94	-5.13
PIL24516	Germacrenediol	-33.57	-12.89	3.08	-10.64
JGI58158	FPP	-27.53	-17.60	1.28	-1.71
PIL35630	FPP	-24.90	-17.82	0.73	0.55
JGI58881	GGPP	-28.55	-23.42	5.27	1.33

[1] Abbreviatures: FPP, farnesyl diphosphate; GGPP, geranyl-geranyl pyrophosphate.

VI. DISCUSIÓN GENERAL

Los agroecosistemas constituyen el tipo de bioma más grande de la Tierra (Ellis y Ramankutty, 2008), ocupando un tercio de la superficie terrestre libre de hielo del planeta (Ramankutty et al., 2008). La agricultura es un medio de sustento relevante para el 40 % de la población mundial, contribuyendo con aproximadamente el 30 % del producto interno bruto en los países de bajos ingresos. Además de alimentos, las actividades agrícolas también proporcionan, fibra, biocombustibles y otros productos para la población humana. Por lo anterior, existe una presión cada vez mayor sobre la agricultura para satisfacer las necesidades de la población humana actual y futura.

Los plaguicidas constituyen insumo agrícola importante, que han sido relevantes para el incremento de la producción de la producción agrícola desde la segunda mitad del siglo pasado (Soares y Porto, 2009). No obstante, diversos factores entre los que se pueden incluir el cambio climático y las mismas prácticas de producción y control de plagas han contribuido al incremento de enfermedades ocasionadas por agentes fitopatógenos (Christensen et al., 2011), ocasionando un uso inadecuado de plaguicidas químicos sintéticos.

Los hongos y oomicetes fitopatógenos ocasionan pérdidas considerables pre y postcosecha de cultivos de relevancia agronómica (Drenth y Guest, 2016); Fisher et al., 2020; Fones et al., 2020). Entre los patógenos de mayor relevancia agrícola se encuentran *Magnaporthe oryzae*, *Botrytis cinerea*, *Fusarium oxysporum* y *Colletotrichum* spp. (Dean et al., 2012). Por su parte, entre los diez oomicetes de mayor relevancia agrícola como patógenos de diversos cultivos se encuentran seis especies del género *Phytophthora*, incluyendo *P. infestans* y *P. parasitica* (Kamoun et al., 2015). En varias de estas especies de hongos y oomicetes se ha reportado la resistencia a fungicidas y plaguicidas empleados comúnmente para su control (Leroux et al., 2002; Matson et al., 2015; Dowling et al., 2020). Por lo anterior, en este trabajo se incluyeron algunas de dichas especies de microorganismos fitopatógenos como cepas de prueba para la inhibición del crecimiento por parte de *Irpex lacteus* y *Ganoderma* sp.

Entre las estrategias consideradas para el combate de microrganismos fitopatógenos, se ha explorado el uso de metabolitos derivados de fuentes naturales, como plantas, bacterias, algas y hongos, para el reemplazo de los fungicidas químicos convencionales (Abdel-Hafez et al., 2015; Chen et al., 2019; Raihan et al., 2021; Wang et al., 2021). En particular, los extractos y metabolitos secundarios de diversas especies de hongos han mostrado actividad inhibitoria contra hongos y oomicetes fitopatógenos (Rodrigo et al., 2022). Sin embargo, en el caso

particular de los basidiomicetes dichos estudios se han realizado principalmente a partir de extractos/metabolitos del cuerpo fructífero o del micelio (Stajić et al., 2017), siendo menos frecuente al análisis de metabolitos extracelulares obtenidos de cultivos líquidos (Shen et al., 2017).

Los metabolitos antifúngicos de hongos incluyen principalmente a policétidos, seguido de terpenos y péptidos no ribosomales (Rodrigo et al., 2022). Para le caso particular de los basidiomicetes se incluyen a dichos metabolitos también polisacáridos y proteínas (Stajić et al., 2017). En concordancia con reportes previos de hongos en general y de basidiomicetes en particular, este estudio se encontraron terpenos y policétidos de manera mayoritaria en los metabolitos extracelulares de las cepas de *Irpex lacteus* y *Ganoderma curtisii* analizadas en el presente trabajo. Aunque los terpenos prevalecieron cobre los policétidos, quizá debido al hecho previamente comentado de que los estudios mayoritarios son de metabolitos intracelulares sobre los extracelulares. Así, es posible que los terpenos se secreten en mayor proporción que los policétidos, al menos en especies del género *Ganoderma*, algo que debe ser evaluado con mayor detalle a futuro.

Entre las estrategias que se han empleado para incrementar o diversificar la producción de metabolitos extracelulares se encuentran las modificaciones del medio y las condiciones de cultivo, la modulación de la expresión de reguladores transcripcionales asociados a genes del metabolismo secundario, la expresión heteróloga de genes clonados, modificaciones en el control epigenético y el co-cultivo de dos especies fúngicas, entre otras (Baral et al., 2018; Harvey et al., 2018: Poças-Fonseca et al., 2020; Yu et al., 2021). El co-cultivo se ha empleado con éxito en la producción y caracterización de metabolitos secundarios en *Ganoderma applanatum* (Xu et al., 2018; Yao et al., 2018) e *I. lacteus* (Zhou et al., 2018; Sadahiro et al., 2020; Shi et al., 2020). En el caso de *Ganoderma* spp. también se ha empleado la expresión heteróloga (Wang et al., 2018; Chu et al., 2019). Así, a futuro será interesante evaluar estas estrategias en las cepas de *I. lacteus* y *G. curtisii* aquí estudiadas, con la finalidad de caracterizar con mayor precisión su diversidad metabólica y encontrar metabolitos antifúngicos particulares para fitopatógenos de difícil control.

El análisis genómico de los genomas disponibles en bases de datos públicas posibilitó la evaluación de las relaciones filogenéticas y estructurales de las terpeno sintasas de *Ganoderma* spp. Esto no fue posible en *I. lacteus* debido a que la versión del genoma disponible para esta

especie durante la realización del presente trabajo todavía era una versión preliminar. La combinación del análisis genómico *in silico* y experimental optimiza el descubrimiento de nuevos metabolitos con actividad farmacológica (Ziemert et al., 2016), por lo que es necesario obtener la secuencia del genoma de *G. curtisii* para tener un mejor conocimiento de su capacidad para la síntesis de metabolitos secundarios. Esto permitirá ubicar los clusters de genes biosintéticos responsables de la síntesis de terpenos, policétidos y péptidos no ribosomales en dicha especie, como se ha evaluado en otros basidiomicetos (Lackner et al., 2012; Gressler et al., 2021). Al mismo tiempo, el conocimiento genómico de las cepas aquí analizadas permitirá la aplicación de herramientas genético- moleculares para la producción eficiente y específica de metabolitos antes mencionadas, como la sobreexpresión, la expresión heteróloga y el silenciamiento (Xiao y Zhong, 2016).

VII. CONCLUSIÓN

El uso de fungicidas químicos sintéticos ha contribuido al aumento de la producción agrícola, pero también ha ocasionado problemas ambientales y de salud. El análisis de nuevas especies y/o nuevos aislados geográficos de basidiomicetes comestibles y medicinales constituye una fuente de nuevos metabolitos con actividad farmacológica, en particular de sustancias con actividad antifúngica hacia hongos fitopatógenos. El análisis de cepas locales de especies de basidiomicetes silvestres posibilita el desarrollo de biotecnología local, aplicada a la solución de problemas agrícolas ocasionados por variantes específicas de hongos fitopatógenos. Las cepas de *G. curtisii* e *I. lacteus* aquí estudiadas producen una gran variedad de metabolitos antifúngicos con potencial para su uso en el control de plagas a nivel local y global. La diversidad de metabolitos aquí estudiadas es basal, ya que no se utilizó ninguna condición de inducción, por lo que todavía falta mucho para conocer el potencial de producción de metabolitos secundarios de las cepas de estudio. Las cepas de estudio inhibieron el crecimiento de hongos y oomicetes fitopatógenos, presentando una composición distinta en el metaboloma extracelular, por lo que se acepta la hipótesis del presente trabajo.

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XI. APÉNDICE

ARTÍCULO

La batalla entre humanos y bacterias patógenas

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Las bacterias patógenas han acompañado desde siempre al ser humano

Las bacterias patógenas han afectado la sa- lud humana desde los inicios del proceso de civilización, o incluso desde antes. En **huesos de indígenas** peruanos, cuya antigüedad se calcula en mil años, se encontró ácido desoxirribonucléico (ADN) de *Mycobacterium pinnipedii,* una bacteria cercana a *Mycobacterium tuberculosis*, causante de la tuberculosis humana. Aunque una especie cercana a estas dos, ya existía hace cinco mil años.

Algunas enfermedades infecciosas bacterianas, han provocado altos niveles de mortandad en periodos muy cortos de tiempo. La **Pandemia Justiniana**, ocurrida en el Norte de África y Europa entre los años 541 y 767 y ocasionada por la bacteria *Yersinia pestis*, es un ejemplo de ello. Esta misma

bacteria causó la llamada **Muerte Negra** en la Europa medieval, la cual provocó el fallecimiento de una tercera parte de la población europea de entonces. Aunque había duda sobre el origen de la mortandad tan elevada de ese periodo, el ADN de la bacteria, recuperado de esqueletos humanos antiguos, indica que este patógeno fue el culpable.

Un ejemplo histórico más reciente es la mortandad masiva de población indígena en México durante el periodo 1545–1550, por una enfermedad que los indígenas llamaban *cocoliztli*. Investigadores de Alemania, Estados Unidos de América (EUA) y Suiza, en colaboración con científicos del Instituto Nacional de Antropología e Historia de México, han relacionado al *cocoliztli* con una variante de la bacteria *Salmonella enterica***,** denominada **serotipo Paratyphi C.** Los investigadores llegaron a este resultado debido a que esqueletos de un cementerio indígena en Oaxaca, tenían el ADN de esta bacteria, por lo que proponen que se trató de la denominada fiebre entérica (tiofidea/paratifoidea), que es la enfermedad provocada por el patógeno identificado.

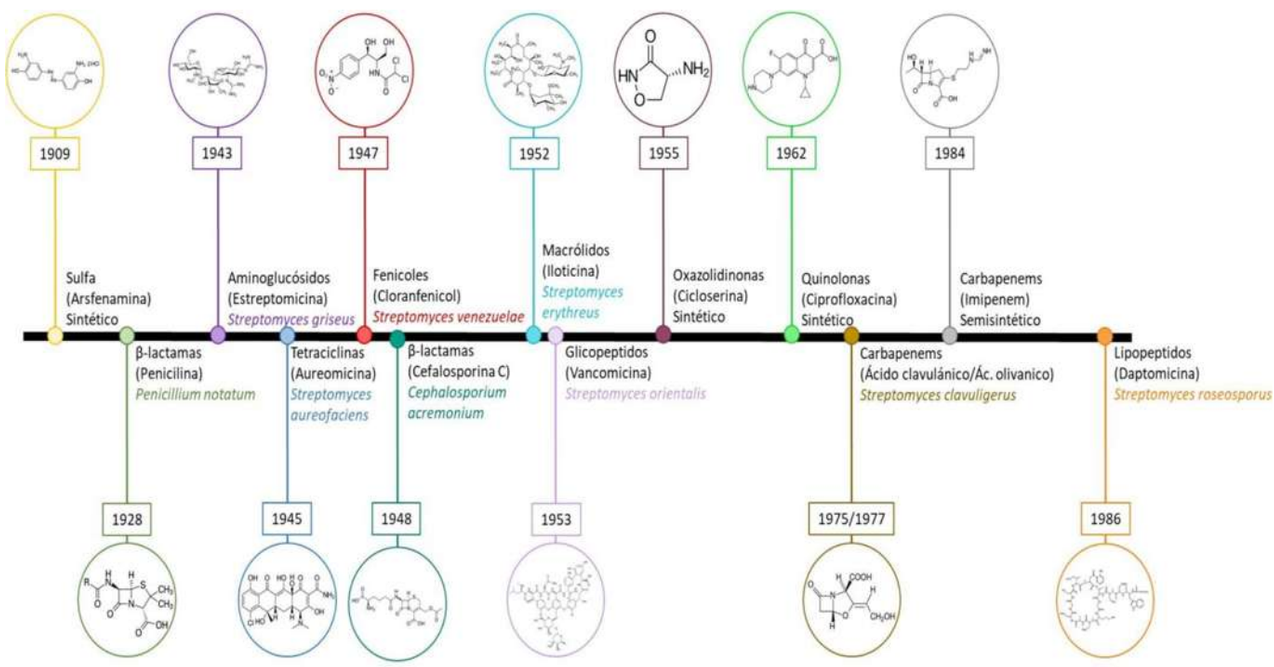
Estos casos sirven para ejemplificar, cómo el ser humano ha padecido enfermedades ocasionadas por bacterias que han representado **un serio problema de salud** desde que inició su expansión por la Tierra. Pero, ¿Cómo se pudo empezar a combatir eficazmente éstas y otras enfermedades causadas por bacterias? A partir del descubrimiento e introducción de **los antibióticos**.

Los antibióticos, una herramienta para combatirlas bacterias patógenas

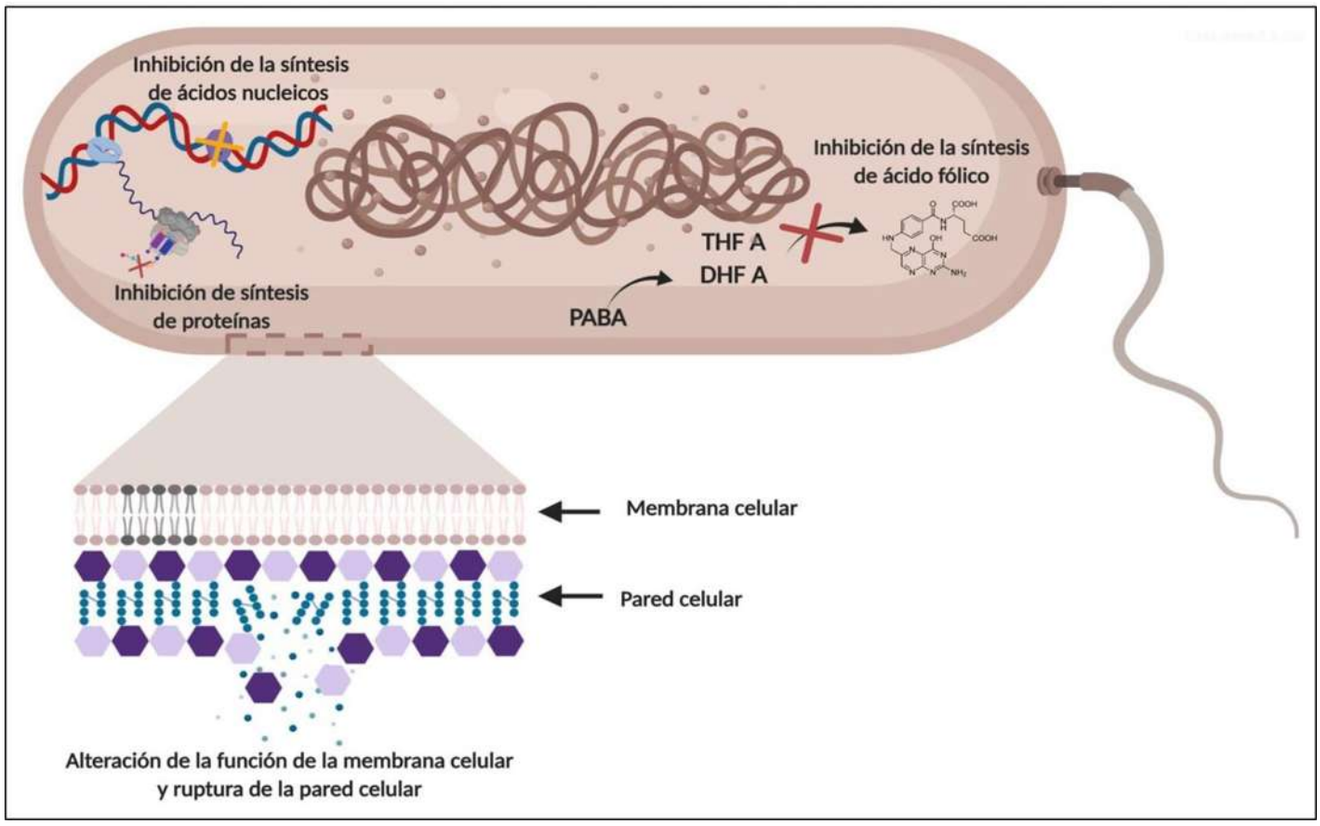
Los antibióticos son compuestos orgánicos que inhiben el crecimiento bacteriano o matan a las bacterias, y están dirigidos a moléculas o estructuras específicas dentro de las células bacterianas. Comúnmente se acepta que los antibióticos son de **origen natural**, al ser producidos por especies de distintos grupos biológicos. Algunos otros científicos también reconocen como antibióticos, a moléculas totalmente **sintetizadas** por el hombre en laboratorios farmacéuticos, aunque no todos están de acuerdo en esto. Un tercer grupo serían los antibióticos **"híbridos"**, en los que una parte de la molécula es natural y la otra sintética.

Si se aceptan las moléculas sintéticas, puede considerarse que el uso de antibióticos inició con la **arsfenamina** en 1910 y la posterior introducción del **prontosil** en 1932. Pero si por el contrario, solo se consideran las moléculas de origen natural, entonces debemos tomar en cuenta el año 1928, cuando Alexander Fleming descubrió la **penicilina** —antibiótico producido por hongos del género *Penicillium*—, la cual tuvo su primer uso terapéutico en 1941. En este sentido, al hablar de antibióticos de origen natural, estas dos fechas pueden considerarse como las relevantes para el inicio del uso de antibióticos.

Los antibióticos pueden dividirse en grupos de acuerdo con sus características químicas. Entre los primeros antibióticos utilizados, la arsfenamina y el prontosil pertenecen a las **sulfonamidas**, mientras que la penicilina al grupo de las **β-lactamas.** Con el tiempo se descubrieron nuevos antibióticos, como los **aminoglucósidos**, y los de más reciente introducción como los **betalactámicos,** macrólidos y quinolonas. Otra forma de clasificar los antibió-



Fechas de descubrimientos de antibióticos relevantes, organismos de los que fueron aislados y de aquellas que son moléculas sintéticas



Mecanismos de acción de algunos grupos de antibióticos

ticos es por la manera en que atacan las distintas funciones vitales de las bacterias.

En este sentido, están aquellos que rompen la integridad de la célula al impedir que se forme la **pared celular,** o desestabilizando la estructura de la membrana plasmática, estructuras que delimitan a las células bacterianas. Otros afectan la función de un complejo de moléculas denominado **ribosoma,** inhibiendo la síntesis de proteínas. También están aquellos que impiden la replicación o **copiado del ADN,** algo que la bacteria requiere hacer cada vez que va a dividirse; o bien los que bloquean el proceso para generar los mensajes que surgen de las secuencias de ADN necesarios para la síntesis de proteínas, denominado **transcripción.**

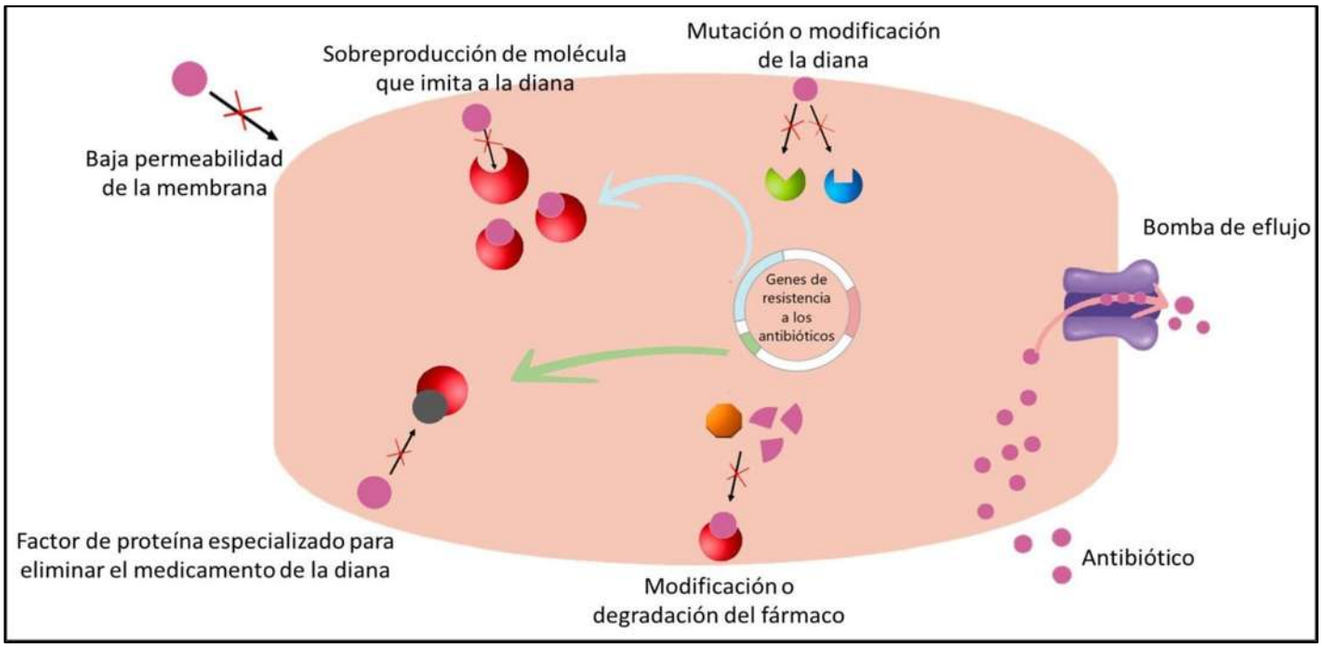
Las bacterias pueden adquirir resistencia a los antibióticos

¿Por qué a pesar de que los primeros antibióticos aparecieron desde inicios del siglo pasado, se continúa con el descubrimiento e incorporación de nuevos grupos?

Esto se debe a que **las bacterias patógenas han adquirido resistencia** a cada nuevo tipo de antibiótico que el hombre ha utilizado para combatirlas, de manera que, cada vez es más difícil curarnos de enfermedades infecciosas bacterianas.

Actualmente se conocen muy bien las principales estrategias que las bacterias emplean para desarrollar resistencia a los antibióticos. Un primer mecanismo de defensa es **impedir que el antibiótico entre a la célula**, modificando las características de la membrana que la rodea. Si el antibiótico entra, las bacterias tienen proteínas en esta membrana que funcionan como "bombas", de manera similar a las que sacan agua de un pozo, para expulsar al antibiótico del interior celular antes de que pueda causarle daño. Si todavía quedan moléculas de antibiótico dentro de la célula bacteriana, otro tipo de proteínas puede degradar al antibiótico, destruyendo los enlaces químicos que conforman la molécula, para hacerlo inofensivo. Las bacterias también pueden modificar la estructura química de la molécula, blanco sobre la que actúa el antibiótico, para que ya no la pueda identificar, evitando así el daño. Estas estrategias dan una idea de cómo en su interacción con la medicina humana, **las bacterias han desarrollado mecanismos exitosos de defensa**.

Una bacteria puede contar con más de uno de estos mecanismos de defensa, presentando resistencia a tres o más antibióticos de los distintos grupos químicos, una característica denominada **multirresistencia**. Una bacteria multirresistente es muy difícil de combatir para un médico y pue-de causar la muerte del paciente. Como si esto no fuera lo suficientemente preocupante, las bacterias que poseen estos mecanismos de resistencia los heredan a las **células "hijas"** cuando se dividen, y además, también transfieren a otras bacterias de distinta especie, moléculas de ADN que contienen las **instrucciones para la resistencia**. Esto ocurre en ríos, lagos, suelo y en los alimentos, y también en nuestro cuerpo, una vez que la bacteria patóge- na se encuentra con las bacterias que lo habitan de



manera natural, comúnmente sin causarnos daño. Estas interacciones permiten que aquellas bacterias que no eran resistentes, puedan adquirir resistencia a un antibiótico o tornarse multirresistentes.

Fuentes de nuevos antibióticos

La gran diversidad biológica del planeta constituye una **"farmacia natural"** en la cual es posible encontrar nuevas sustancias orgánicas para atacar con éxito a las bacterias patógenas multirresistentes.

Las plantas han sido consideradas desde hace tiempo una **fuente importante de nuevos antibióticos**, debido a la documentación de su eficacia en el uso tradicional de distintas culturas en todo el mundo. Las hojas, las raíces, los tallos y el fruto de una planta pueden contener una gran variedad de compuestos orgánicos, algunos de los cuales son efectivos contra bacterias patógenas.

Desde el descubrimiento de la penicilina, **los hongos han sido continuamente evaluados** para encontrar nuevos antibióticos. Una ventaja de este grupo de organismos, es que algunos de esos compuestos son "arrojados" al medio de cultivo en los que crecen comúnmente en el laboratorio, lo que facilita su recuperación.

Otro grupo microbiano ampliamente estudiado son las **actinobacterias**, en las cuales se ha identificado una gran variedad de antibióticos, incluidos algunos de uso actual. Más recientemen- te, los investigadores han puesto su atención en distintas **especies marinas,** como algas, esponjas y corales. Debido a que la investigación en estos grupos biológicos todavía es incipiente, se cree que los primeros antibióticos descubiertos en estos organismos, son apenas el inicio de la descripción de sustancias con diferentes mecanismos de acción, que pueden ser eficientes para combatir distintas enfermedades causadas por bacterias.

Sin duda alguna, la investigación de los compuestos producidos por distintos grupos de organismos, permitirá encontrar antibióticos eficaces contra bacterias que representan grandes riesgos actuales y futuros para la salud humana. Pero, así como las bacterias han respondido generando mecanismos para resistir a los antibióticos que se usan en la actualidad, seguramente irán modificando su ADN y sus células para resistir a los nuevos antibióticos. Esta será **una larga batalla** entre el ingenio humano para combatir enfermedades bacterianas, y la capacidad evolutiva de las bacterias para crear mecanismos que les permitan combatir cada nuevo antibiótico. No creemos que ni a nosotros, los autores de este trabajo, ni al amable lector actual, nos toque ver el final de la historia y conocer al ganador.

sabermás

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