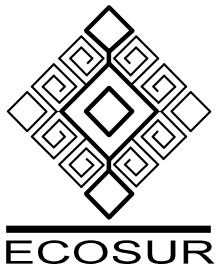

El Colegio de la Frontera Sur

Interacción de *Streptomyces galilaeus* CFFSUR-B12 con *Mycosphaerella fijiensis* Morelet, microorganismos no blanco y plantas de banano (*Musa* sp. AAA)

TESIS

presentada como requisito parcial para optar al grado de Doctor en Ciencias en Ecología y Desarrollo Sustentable

por

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RESUMEN

Se evaluaron *in vitro* las interacciones antagónicas entre las quitinasas extracelulares parcialmente puras, sobrenadante de cultivo y una suspensión de esporas ($1x10^9$ u.f.c. ml^{-1}) del actinomiceto *Streptomyces galilaeus* CFFSUR-B12 con el hongo ascomiceto *Mycosphaerella fijiensis* Morelet, agente causal de la Sigatoka Negra del Banano (SNB). En campo se evaluó la efectividad de las aplicaciones de la cepa CFFSUR-B12 (esporas y sobrenadante) para reducir la severidad de la SNB en plantas asperjadas con los agentes de biocontrol, asimismo se evaluó el efecto de las aplicaciones de estos agentes sobre la diversidad de microorganismos "no blanco" asociados al banano. También se determinó si la bacteria promueve el crecimiento y se establece como endófito en las plantas de banano.

Las quitinasas de CFFSUR-B12 inhibieron parcialmente la elongación del tubo germinativo y el crecimiento micelial de *M. fijiensis*, mientras que el sobrenadante de cultivo y la suspensión de esporas inhibieron por arriba del 90% el crecimiento del hongo. Las quitinasas no inhibieron la germinación de las ascosporas del hongo, mientras que el sobrenadante y las esporas la inhibieron en un 96 y 56%, respectivamente. En campo las plantas asperjadas con las esporas de CFFSUR-B12 presentaron significativamente menor severidad y cantidad de SNB, en comparación con las plantas tratadas con el fungicida mancozeb y agua (testigo). Después de ser asperjados en campo, los agentes de biocontrol no afectaron la diversidad de las comunidades microbianas asociadas a la rizósfera y filósfera de las plantas de banano. Por último, el actinomiceto no promovió el crecimiento vegetal ni tampoco se estableció como endófito (radicular o foliar) en las plantas de banano.

PALABRAS CLAVE: *Streptomyces galilaeus* / CFFSUR-B12 / *Mycosphaerella fijiensis* / Sigatoka Negra del Banano / quitinasas

I. INTRODUCCION

La agricultura convencional enfrenta actualmente varios retos y limitantes que deberá superar para ofrecer alimentos de calidad a una población en constante crecimiento, que según cifras del Banco Mundial, alcanzará los 9 mil millones de personas hacia el 2050. Para poder producir suficientes alimentos, los grandes desafíos por afrontar son el cambio climático, la disponibilidad de agua y la alta dependencia hacia los insumos (fertilizantes, agroquímicos y combustibles) derivados del petróleo. El panorama se ensombrece debido a que la agricultura convencional o moderna ha dejado una marcada huella ecológica que ha ido creciendo con el paso del tiempo: los suelos agrícolas están fuertemente degradados y agotados, se ha perdido diversidad (biológica y genética), y las plagas y enfermedades se han vuelto más difíciles de controlar. Esto ha complicado la situación financiera de los productores, quienes han empezado a resentir la “Ley de los rendimientos decrecientes” que implica una inversión mayor con cada vez menores niveles de producción (Altieri 1994, 1999; Altieri y Nicholls 2000, 2013).

Bajo esta perspectiva, el desarrollo de tecnologías de producción más limpias, suaves, sustentables y en armonía con el ambiente, es hoy en día una prioridad para poder garantizar la preservación y funcionalidad de los recursos naturales para las futuras generaciones (Ramírez et al. 2004). De esta manera, el control biológico se considera como una herramienta alternativa y sustentable para el manejo de las plagas que colonizan a los cultivos agrícolas (Bale et al. 2008; Feditchkina 2014), la cual utiliza a los enemigos naturales (microorganismos o sus metabolitos, parasitoides y a los depredadores) como agentes antagónicos de las plagas insectiles y de los fitopatógenos (Cook y Baker 1983).

En México y en la región del Soconusco, Chiapas, el control biológico de fitopatógenos por medio de microorganismos antagonistas es un campo que ha sido poco explorado, aún y cuando en la literatura existe un cúmulo de evidencia exitosa relacionada con el uso de estos agentes (Swiontek et al. 2014). La falta de adopción de estas tecnologías posiblemente esté relacionada con el desconocimiento del uso de estos agentes biológicos, así como una alta dependencia hacia el uso de los agroquímicos (van Lenteren 2012).

La cepa CFFSUR-B12, identificada como *Streptomyces galilaeus*, es un actinomiceto que pertenece a la colección microbiológica de ECOSUR-Tapachula. Las condiciones en las que esta cepa fue aislada de su hábitat natural, permitieron hipotetizar que el microorganismo podría ser un potencial agente de biocontrol de la Sigatoka Negra del Banano (SNB), debido a que: 1) al ser aislado como micoparásito del ascomiceto *Colletotrichum gloeosporioides* (antracnosis del cacao) podría ser un excelente productor de enzimas micolíticas (p. ej. quitinasas) y de metabolitos extracelulares antifúngicos, 2) al ser aislado sobre mazorcas de cacao, podría adaptarse a vivir en las condiciones adversas y cambiantes del ambiente aéreo de las plantas, similares a las condiciones de la filósfera (Andrews 1992; Blakeman y Fokkema 1982) en que se desarrolla la SNB, 3) está adaptado a vivir en las condiciones climáticas tropicales propias de la región, y 4) la bacteria ha demostrado *in vitro* tener una alta capacidad antagónica contra *C. gloeosporioides* (González 2007).

De esta manera y con el fin de contribuir en el conocimiento del control biológico de fitopatógenos en cultivos tropicales, en el presente trabajo se evaluaron las interacciones antagónicas entre la cepa CFFSUR-B12 con el hongo ascomiceto *Mycosphaerella fijiensis* Morelet, agente causal de la SNB. También se evaluó el efecto que tienen las aplicaciones de la suspensión de esporas y el sobrenadante de cultivo de CFFSUR-B12 sobre los microorganismos “no blanco” residentes de la rizósfera y filósfera del banano. Además, se estudió la interacción entre este actinomiceto y las plantas de banano para conocer si es capaz de promover el crecimiento vegetal y si tiene la habilidad de establecerse como endófito en las hojas y raíces del banano.

Este estudio es una caracterización de la cepa bacteriana CFFSUR-B12 en cuanto a su actividad quitinolítica y antifúngica, que pretende mostrar los beneficios de este microorganismo como agente de biocontrol para proponer a futuro una estrategia alternativa y amigable con el ambiente para el manejo de la SNB en nuestra región.

II. MARCO TEORICO

2.1 La Sigatoka Negra del Banano (SNB)

La Sigatoka Negra del Banano (SNB) es el principal problema fitosanitario que tienen que afrontar los productores de banano (*Musa* spp.), principalmente en áreas donde se cultivan clones triploides altamente susceptibles a la enfermedad, como es el caso del cultivar “Gran Enano (AAA)” producido en la región del Soconusco y en otros estados de México. La enfermedad está presente prácticamente en todas las zonas bananeras del mundo y es causada por el hongo *Mycosphaerella fijiensis* Morelet (Ascomycota: Dothideomycetes: Capnodiales: Mycosphaerellaceae), patógeno altamente virulento y agresivo considerado como hemibiotrófico, ya que es capaz de continuar su desarrollo después de que el tejido infectado ha muerto (Churchill 2011). El hongo presenta una fase sexual (*M. fijiensis* Morelet) y una asexual (*Paracercospora fijiensis* (Morelet) Deighton), teleomorfo y anamorfo, respectivamente (Figura 1). La fase sexual se caracteriza por la producción de ascosporas en pseudotecios que se forman en lesiones maduras, mientras que en la fase asexual se producen conidios que emergen de conidióforos formados en lesiones jóvenes (Stover 1980; Marín et al. 2003). Las ascosporas son la forma de inóculo que se dispersa entre plantas a través de la diseminación eólica (incluso a grandes distancias), mientras que los conidios infectan tejidos sanos cercanos a las lesiones dentro de la misma planta o de plantas vecinas, bien sea por escurrimiento o por salpique del agua de lluvia entre hojas (Agrios 2005). La mayor incidencia de la enfermedad está estrechamente relacionada con la temporada de lluvias, con humedades relativas superiores al 80 % y con temperaturas promedio entre 26 y 28 °C (Pérez et al. 2000; Manzo et al. 2005; Júnior et al. 2008). El tiempo de incubación de la enfermedad, es decir, el tiempo entre la infección y la aparición de los primeros síntomas puede variar de 13 hasta 35 días, dependiendo de las condiciones climáticas de cada región y la presión de inóculo (Marín et al. 2003). El daño provocado al banano consiste en una necrosis paulatina del tejido foliar infectado que debilita a la planta, reduce su capacidad fotosintética y su rendimiento, además de ocasionar una maduración prematura durante el transporte de la fruta proveniente de plantas severamente infectadas (Agrios 2005; Churchill 2011).

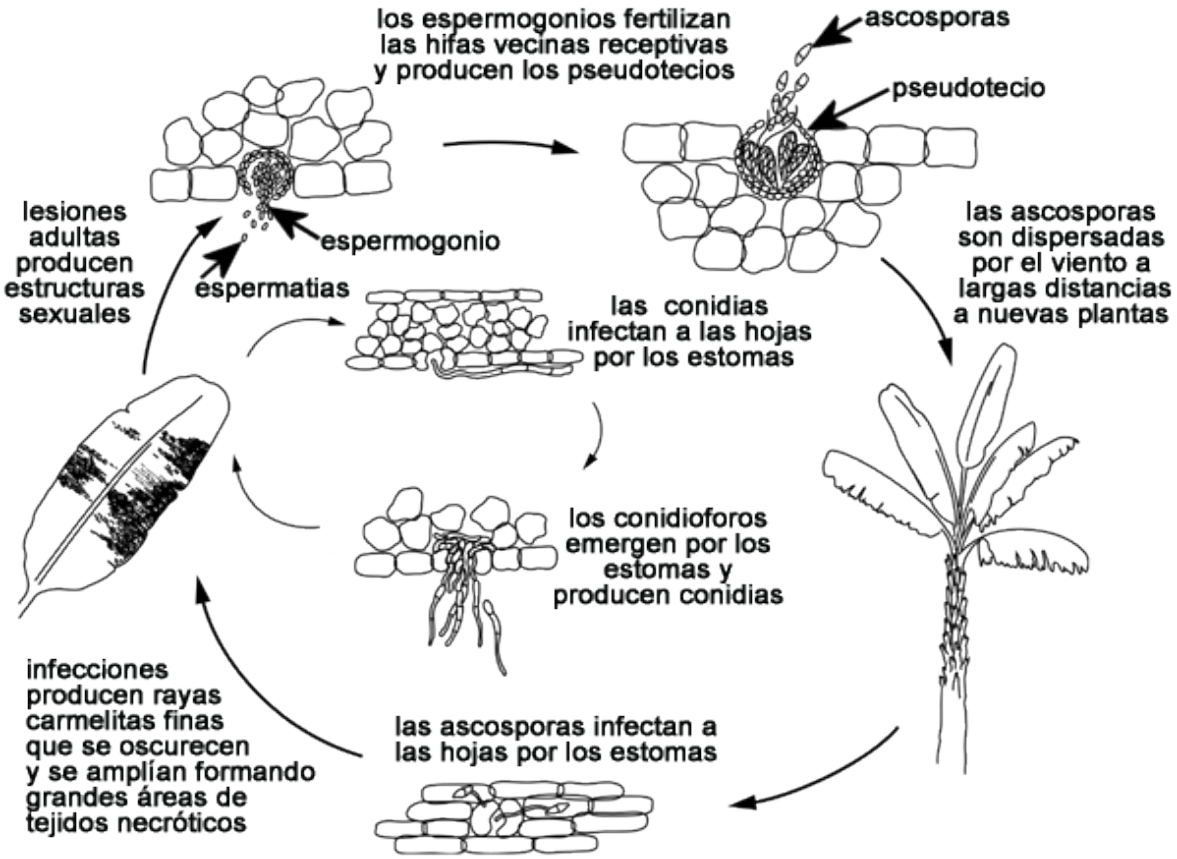

Figura 1. Ciclo biológico de la Sigatoka Negra del Banano (disponible en http://4.bp.blogspot.com/-2NCpumBM0JU/VDrbirOzuzI/AAAAAAAAACA/t4mUf-kMHXQ/s1600/1.gif)

Se distinguen varios estados de desarrollo de la SNB que se han agrupado en una escala que se ilustra en la Figura 2 (escala de Fouré):

Estado 1: Pequeñas lesiones o puntos blanco-amarillento a marrón de 1 mm de longitud denominadas pizcas, apenas visibles en el envés de las hojas.

Estado 2: Rayas o estrías cloróticas de 3-4 mm de longitud por 1 mm de ancho, de tonalidad amarillo verdoso y luego café.

Estado 3: Las rayas o estrías se alargan y amplían sin bordes definidos y de color café que pueden alcanzar hasta 2 cm de largo.

Estado 4: Manchas ovaladas de color café en el envés y negro en el haz.

Estado 5: Manchas negras rodeadas de un anillo negro y a veces un halo amarillento y centro seco ligeramente hundido.

Estado 6: Manchas con centro hundido de color blanco-grisáceo, con hundimiento de tejido, de coloración marrón clara, rodeada de tejido clorótico.

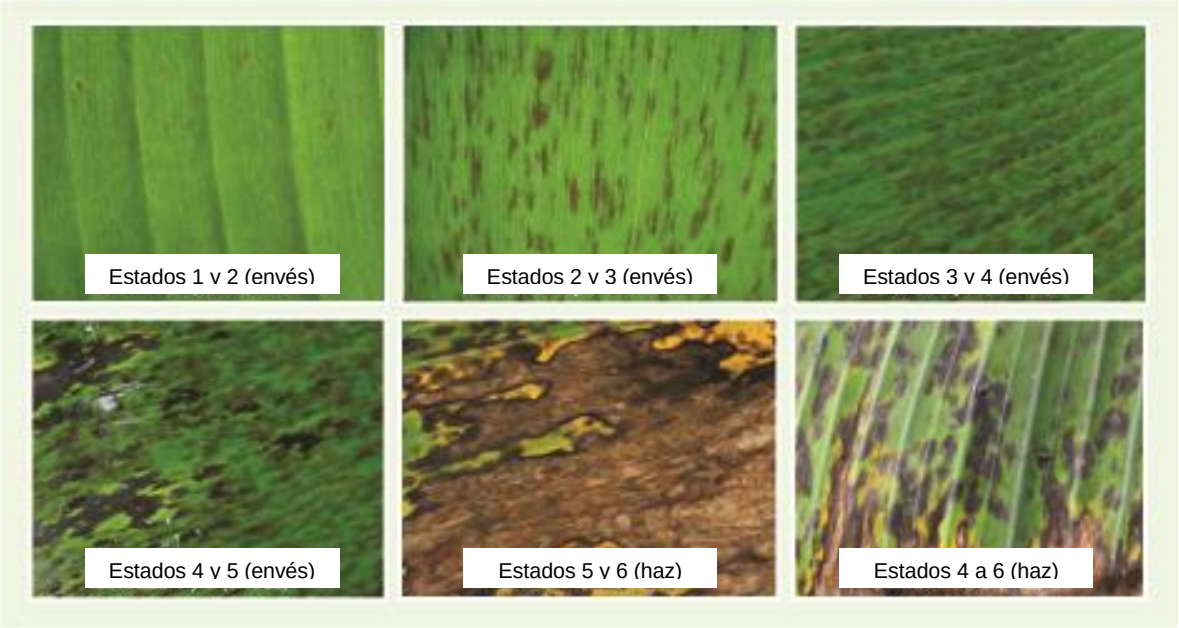

Figura 2. Estados de desarrollo de la Sigatoka Negra del Banano (Escala de Fouré) (Tomada de Alvarez et al. 2013).

Esta escala es ampliamente utilizada para evaluar el tiempo desarrollo de la SNB en las plantas infectadas (Carlier et al. 2002; Orozco-Santos 1998).

2.2 Manejo convencional de la SNB

El manejo de la SNB en las plantaciones bananeras es principalmente químico, con una frecuencia de una a dos aplicaciones semanales (Henriques et al. 1997) que junto con prácticas culturales como el deshoje y el deshije es utilizado para reducir la densidad de inóculo en la plantación (Orozco-Santos et al. 2008).

Se estima que el control de la enfermedad representa del 30 al 45 % de los costos totales de producción del banano (Marín et al. 2003; Manzo et al. 2005; Orozco-Santos et al. 2001), aunque en nuestra región algunos productores mencionan que esta cifra alcanza fácilmente el 50 % en temporadas de alta presión de inóculo. En nuestro país se

estima un gasto anual de 43 millones de dólares para el control de la SNB, que representan 430 toneladas de ingrediente activo en su mayoría sistémicos, unos 13 millones de litros del aceite agrícola “citrolina” (Orozco-Santos et al. 2001) y alrededor de 7 mil toneladas de fungicidas protectantes aplicados en las zonas bananeras (Orozco-Santos et al. 2008).

El principal fungicida aplicado es el mancozeb, un ditiocarbamato capaz de inducir tumoraciones, causar daño celular y en el ADN de mamíferos con exposición crónica a la molécula, lo cual se asocia con algunas enfermedades degenerativas incluyendo al cáncer (Bayoumi et al. 2002; Calviello et al. 2006; Mehrotra et al. 1987). Esta molécula y sus subproductos (Mn y etilentiourea) causan serios problemas de contaminación en suelos y cuerpos de agua cercanos a las plantaciones en donde se aplica por vía aérea este fungicida (Geissen et al. 2010). Además, el uso indiscriminado de agroquímicos puede traer como consecuencia una insensibilidad del patógeno hacia ciertas moléculas fungicidas, tal y como ya se ha reportado para el benomilo (Romero y Sutton 1998) y el propiconazol (Romero y Sutton 1997), lo que eleva notablemente los costos de producción.

2.3 Control biológico de la SNB

El control microbiológico representa una alternativa viable al control químico en el manejo de la SNB (Alvarez et al. 2013; Riveros et al. 2003), sin embargo la investigación sobre el control biológico de la enfermedad es limitada y se encuentra a nivel experimental.

Algunos trabajos han evaluado el antagonismo de algunas cepas de hongos (endófitos de banano y residentes de suelo) como *Trichoderma* spp. y *Fusarium oxysporum* (cepa no patogénica) contra *M. fijiensis* (Arzate-Vega et al. 2006; Osorio 2006) o como inductores de resistencia vegetal a la SNB (Barrios 2006). Asimismo, se han evaluado metabolitos extracelulares bacterianos: los filtrados de cultivo de varias cepas de *Bacillus* spp., demostraron *in vitro* una inhibición del desarrollo de *M. fijiensis* por arriba del 80 % (Ceballos et al. 2012). En condiciones de invernadero la aplicación de filtrados bacterianos retardó el desarrollo de la enfermedad y redujo el número de lesiones necróticas en plantas inoculadas con el patógeno (Cruz-Martín et al. 2010). Por

su parte, Riveros et al. (2003) encontraron que los filtrados bacterianos a diferentes concentraciones tuvieron un efecto inhibitorio de *M. fijiensis* variable, entre el 50 y el 100 %. Sánchez et al. (2002) aplicaron el fungicida natural F20 (CQF) contra la SNB en campo obteniendo resultados similares al control químico en el control de la enfermedad. Este producto es una mezcla de los antibióticos estreptotricina B y F, producidos por las cepas de *Streptomyces lavendofoliae* 383 y *St. rochei* F20, respectivamente. En la región del Soconusco, Chiapas, Holguin y colaboradores obtuvieron resultados comparables al control químico con mancozeb al aplicar una mezcla de microorganismos entre los que se encontraba *Serratia marcescens* y algunas cepas de *Streptomyces* (datos no publicados). González et al. (1996a, b) aislaron del filoplano de banano, bacterias quitinolíticas que mostraron porcentajes de inhibición *in vitro* entre el 60 y 75 %, mientras que en invernadero y en campo se logró controlar la enfermedad en un 84 y 40-60 %, respectivamente. Gutiérrez-Román et al. (2015) demostraron *in vitro* la alta capacidad antagónica de la cepa CFFSUR-B2 de *Serratia marcescens* y sus quitinasas en mezcla con el pigmento prodigiosina, para inhibir el desarrollo del tubo germinativo y el crecimiento micelial de *M. fijiensis*.

Algunos otros autores han evaluado la aplicación de sustratos específicos y selectivos sobre las hojas del banano para promover un incremento natural de microorganismos con propiedades líticas sobre el fitopatógeno. Los sustratos con base en quitina coloidal, harina de cebada, con urea y una solución mineral base, redujeron alrededor del 43-46 % las aplicaciones de fungicidas sintéticos para el control de la SNB (Patiño et al. 2007). La utilización de quitina coloidal tiene fundamento en que este biopolímero es un sustrato utilizado por muchas bacterias, e incluso por hongos, como fuente de carbono y energía que obtienen mediante digestión enzimática, lo induce una mayor producción de quitinasas (Williams y Robinson 1981).

2.4 El género *Streptomyces* como agente de biocontrol

El control biológico mediante la aplicación de microorganismos quitinolíticos ha surgido como una alternativa para sustituir, reducir o complementar el control químico de la SNB y minimizar los efectos desfavorables sobre el ambiente (Swiontek et al. 2014). Los actinomicetos resaltan dentro del biocontrol de fitopatógenos por su alta eficiencia

para inhibir el crecimiento de cepas de hongos altamente virulentas y agresivas, debido a su capacidad de producir enzimas micolíticas (Kämpfer 2012) y metabolitos antifúngicos (Qin et al. 2011). La gran habilidad antagónica del género *Streptomyces* ha sido ampliamente demostrada contra fitopatógenos como *Sclerotium rolsfii*, *Colletotrichum* spp. (Prapagdee et al. 2008; Shimizu et al. 2009), *Alternaria, Stemphylium*, *Fusarium* (Joo 2005; Quecine et al. 2008), *Mycosphaerella pinodes* (Mohamed y Benali 2010), entre otros múltiples ejemplos referidos en Shimizu (2011). El éxito de los microorganismos antagonistas como agentes de control biológico se ha relacionado principalmente con su capacidad de producir quitinasas extracelulares (Herrera-Estrella y Chet 1999) que degradan la quitina estructural de la pared celular de los hongos (Latgé et al. 2007, 2010; Bhattacharya et al. 2007; Swiontek et al. 2014).

Las quitinasas (EC 3.2.1.14) son enzimas que hidrolizan la quitina rompiendo los enlaces β-1,4 entre los monómeros de *N*-acetylglucosamina (NAG) que la componen (Muzzarelli 1999). Las quitinasas se clasifican en endo y exoquitinasas: las endoquitinasas rompen los enlaces aleatoriamente en sitios internos de la quitina generando oligómeros de NAG, mientras que las exoquitinasas actúan en el extremo no reductor de la cadena monomérica, donde las quitobiosidasas desprenden dímeros de NAG que son a su vez degradados a monómeros mediante la acción de las N-acetil glucosaminidasas o quitobiasas (Swiontek et al. 2014).

El género *Streptomyces* es conocido por la producción de una gran variedad de antibióticos (Procópio et al. 2012; Chater 2006; Watve et al. 2001) y alrededor de 7 mil compuestos secundarios extracelulares que pueden inhibir enzimas o alterar importantes procesos celulares vitales (Chater et al. 2010). Además, es un excelente productor de una amplia gama de enzimas extracelulares como celulasas, lipasas, lacasas, xilanasas, proteasas, glucanasas y quitinasas, para obtener carbono y nitrógeno de una variedad de biopolímeros disponibles en la naturaleza, como la quitina (Kämpfer 2012). La mayoría de las quitinasas reportadas para *Streptomyces* tienen masas moleculares que van desde los 20 kDa (Kim et al. 2003) a los 81 kDa (Haggag y Abdallh 2012), aunque recientemente se han reportado dos quitinasas de 114.8 kDa (Nagpure et al. 2014) y de 206.8 kDa (Fróes et al. 2012). *Streptomyces* producen mayormente quitinasas que pertenecen a la familia 18, aunque también producen una quitinasa de la familia 19 que solamente es

sintetizada por las plantas (Ohno et al. 1996; Watanabe et al. 1999). La maquinaria quitinolítica de *Streptomyces* varía en tamaño, número, modo de acción y de acuerdo con la cepa. En los genomas de *Streptomyces coelicolor* A3(2) (Bentley et al. 2002; Kawase et al. 2006) y de *Streptomyces albus* J1074 (Zaburannyi et al. 2014) se identificaron hasta 13 y siete posibles genes codificantes de quitinasas, respectivamente. Estas referencias dan cuenta de la variabilidad de quitinasas presentes en el género *Streptomyces*, por lo que es necesario hacer una caracterización específica de cada cepa bajo estudio.

De acuerdo con Baker y Cook (1974), el género tiene además de las anteriores características, varias ventajas biológicas para ser un buen agente de control biológico: produce inóculo en grandes cantidades, tiene la capacidad de esporular, de resistir, escapar o tolerar a otros antagonistas, puede invadir y ocupar sustratos orgánicos, y además es altamente adaptable a las mismas condiciones en las que se desarrolla el patógeno. Adicionalmente, algunas cepas de *Streptomyces* pueden establecer interacciones benéficas con las plantas mediante el establecimiento como residentes de rizósfera o filósfera, establecerse como endófitos y generar resistencia y/o promover el crecimiento vegetal (Franco et al. 2007; Lehr et al. 2008).

III. OBJETIVOS

3.1 Objetivo general

Estudiar la interacción de *Streptomyces galilaeus* CFFSUR-B12 con *Mycosphaerella fijiensis* Morelet *in vitro*, evaluar en campo la efectividad de las aplicaciones del actinomiceto sobre la severidad de la Sigatoka Negra del Banano (SNB), el efecto sobre microorganismos no blanco asociados al banano y estudiar las interacciones de la bacteria con las plantas de banano.

3.2 Objetivos específicos

1. Evaluar *in vitro* el efecto antagónico de las quitinasas, metabolitos extracelulares, sobrenadante de cultivo y suspensión de esporas de *St. galilaeus* CFFSUR-B12 sobre la germinación de ascosporas, elongación del tubo germinativo y crecimiento micelial de *M. fijiensis*.

2. Evaluar en campo la efectividad de la aplicación de la suspensión de esporas y sobrenadante de cultivo de *St. galilaeus* CFFSUR-B12 sobre la severidad de la Sigatoka Negra del Banano y el efecto sobre la microbiota asociada a la filósfera y rizósfera del banano.

3. Conocer si *St. galilaeus* CFFSUR-B12 es capaz de promover el crecimiento vegetal y establecerse como endófito en las plantas de banano.

IV. HIPOTESIS Y PREGUNTAS DE INVESTIGACION

Por ser un micoparásito, se hipotetizó que *St. galilaeus* CFFSUR-B12 produce quitinasas y metabolitos antifúngicos que podrían inhibir el desarrollo de *M. fijiensis*, y que podría ser un potencial agente de biocontrol de la Sigatoka Negra del Banano (SNB) sin afectar a la microbiota no blanco asociada a las plantas. Las preguntas que el presente estudio pretendió contestar son básicamente las siguientes: 1) ¿Existe una interacción antagónica entre las quitinasas, metabolitos extracelulares, esporas y sobrenadante de cultivo de *St. galilaeus* CFFSUR-B12 con *M. fijiensis*?, 2) ¿Es efectivo el actinomiceto CFFSUR-B12 como agente de biocontrol de la SNB en campo sin afectar la diversidad de otros microorganismos asociados al banano?, y 3) ¿Existe una interacción benéfica entre el actinomiceto y las plantas de banano que resulte en el establecimiento como endófito y en la promoción del crecimiento vegetal del banano?.

Las hipótesis de estudio del presente trabajo son las siguientes:

Ho_1: Las quitinasas, metabolitos, esporas y sobrenadante de cultivo de *St. galilaeus* CFFSUR-B12 inhiben *in vitro* la germinación de ascosporas, la elongación del tubo germinativo y el crecimiento micelial de *M. fijiensis*.

Ho_2: La suspensión de esporas y el sobrenadante de cultivo de la cepa CFFSUR-B12 son efectivos para reducir los niveles de severidad de la SNB bajo condiciones de campo, sin afectar la diversidad de microrganismos asociados a la rizósfera y filoplano del banano.

Ho_3: *St. galilaeus* CFFSUR-B12 se establece como endófito foliar y/o radicular y estimula el crecimiento vegetal en las plantas de banano.

V. MATERIALES Y METODOS

En los siguientes manuscritos que fueron enviados para su publicación, se describen los materiales y métodos utilizados durante los experimentos.

Con estos trabajos se cumplieron los objetivos específicos 1 y 2, los cuales contestan las preguntas referentes a 1) la interacción antagónica *in vitro* entre *St. galilaeus* CFFSUR-B12 y *M. fijiensis* y 2) la efectividad de los agentes de biocontrol para reducir la severidad de la SNB y el efecto sobre la diversidad de microorganismos no blanco.

1. **Moreno-Castillo B., M.F. Dunn, G.K. Guillén-Navarro, F. Holguín-Meléndez, M. Hernández-Ortiz, S. Encarnación-Guevara, G. Huerta-Palacios. 2015.** Antifungal performance of extracellular chitinases and culture supernatants of *Streptomyces galilaeus* CFFSUR-B12 against *Mycosphaerella fijiensis* Morelet. World Journal of Microbiology and Biotechnology 32:44. doi: 10.1007/s11274-015-1993-0

2. **Moreno-Castillo B., F. Holguín-Meléndez, M.F. Dunn, G.K. Guillén-Navarro, G. Huerta-Palacios. 2016**. *Streptomyces galilaeus* CFFSUR-B12 is highly effective as a biocontrol agent of the Black Sigatoka Disease but is benign towards banana-associated microbial communities. BioControl (enviado)

Una descripción general del flujo de las metodologías utilizadas en este trabajo se ilustra en el siguiente esquema (Figura 3).

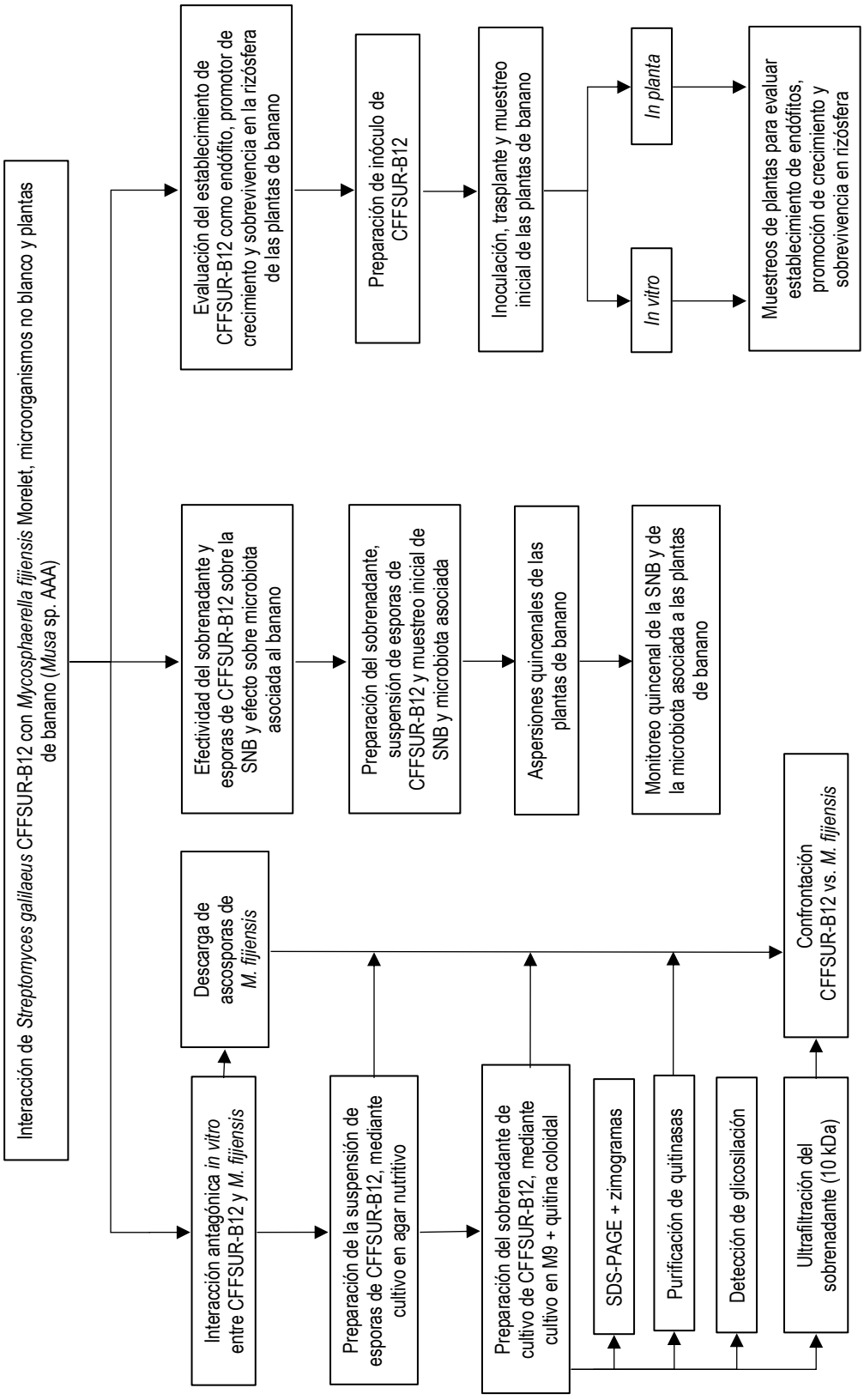

Figura 3. Flujo metodológico realizado durante el presente trabajo de investigación.

A continuación se describen de manera detallada, los métodos empleados para el ensayo de interacción entre *St. galilaeus* CFFSUR-B12 (en lo sucesivo citado como CFFSUR-B12) y las plantas de banano en condiciones de laboratorio y casas de sombra (objetivo específico 3). Con estos ensayos se determinó si el actinomiceto es capaz de promover el crecimiento y se establece como endófito en el banano (pregunta 3).

5.1 Promoción de crecimiento del banano en condiciones de casa de sombra

Preparación del inóculo

Este y los siguientes ensayos se realizaron durante el año 2012 en las instalaciones de ECOSUR-Unidad Tapachula. La cepa CFFSUR-B12 fue primeramente activada en medio Agar Nutritivo por 3-5 d a 26 ± 2 °C, hasta que las colonias esporularon (aspecto algodonoso y blancas). Posteriormente se cosecharon las esporas y se suspendieron en agua estéril. Se tomaron alícuotas de 100 µl y se inocularon en frascos conteniendo 50 ml de Caldo Nutritivo, los cuales fueron incubados a 150 rev. min^{-1}, 27 °C y en oscuridad durante 5 d hasta observar una saturación del medio con pequeñas agrupaciones blanquecinas y esféricas de micelio del actinomiceto. Posteriormente, el medio fue filtrado en tela tipo “cheeseclothe” y se recuperó el micelio que posteriormente fue suspendido en agua estéril. Se estimó una concentración de $1x10^{12}$ u.f.c. ml^{-1} de suspensión mediante el método de diluciones seriadas. Esta suspensión sirvió para inocular las raíces y hojas de las vitroplantas previo al transplante en macetas.

Preparación e inoculación de las vitroplantas de banano

Las plantas de banano utilizadas en este experimento fueron propagadas *in vitro* por micropropagación de ápices meristemáticos utilizando el medio MS (Murashige y Skoog 1962), considerando las fases de multiplicación (fitohormonas: 0.16 mg l^{-1} de Ac. Indol-3-Acético y 2.25 mg l^{-1} de 6-Bencilaminopurina), de regeneración (0.16 mg l^{-1} de Ac. Indol-3-Acético y 0.45 mg l^{-1} de 6-Bencilaminopurina) y de enraizamiento (sin fitohormonas) (Carlier et al. 2002; Martínez et al. 2009). Las vitroplantas con raíces bien desarrolladas y con una altura de 5-7 cm se consideraron aptas para pasarlas a una etapa de adaptación ambiental (aclimatación o endurecimiento) en macetas conteniendo 1 kg de un medio de soporte previamente esterilizado (arena de río y turba canadiense peat-moss en relación 1:1). Previo al trasplante, las vitroplantas fueron inoculadas con

CFFSUR-B12 en la raíz mediante inmersión por 30 min en una suspensión de esporas y micelio ($1x10^{12}$ u.f.c. ml^{-1}) en charolas de plástico; asimismo, 25 ml de esta misma suspensión fueron aplicados al pie de cada planta una vez transplantadas. La inoculación foliar de las plantas se hizo mediante la aspersión de la suspensión de esporas de CFFSUR-B12, utilizando un atomizador y asperjando las plantas a punto de goteo. Es importante, mencionar que las plantas inoculadas vía foliar ya contaban con un mes de aclimatación en las macetas. El testigo consistió de agua destilada estéril y un total de 18 plantas por tratamiento fueron inoculadas vía radicular y 10 vía foliar.

Las variables que se evaluaron fueron grosor de pseudotallo (solo con inoculación foliar), número de hojas funcionales por planta (tejido verde >50 % de la hoja), número de hojas nuevas y altura de planta. Con estas dos últimas se calculó la tasa de emisión foliar (hojas nuevas d^{-1}) y la tasa de crecimiento en altura (cm d^{-1}) que fueron analizadas mediante Análisis de Varianza (5 % de significancia). Las demás variables (datos crudos) fueron analizadas mediante un análisis de covarianza (5 % de significancia) en el paquete estadístico SAS (Covariance Structure Toepliz, Coef. AIC, The SAS Institute).

Se realizó un muestreo inicial y muestreos subsecuentes cada 7-10 d para monitorear el crecimiento de las plantas, durante 31 y 69 d para plantas inoculadas vía foliar y radicular, respectivamente. Asimismo, en cada uno de los muestreos se consideraron tres plantas de cada tratamiento para evaluar el establecimiento como endófito (en raíz u hoja según el sitio de inoculación) o como residente de rizósfera (solo para inoculadas en raíz) que se explica más adelante.

5.2 Promoción de crecimiento en biomasa del banano *in vitro*

Para conocer si CFFSUR-B12 ejerce alguna función sobre el crecimiento de las plantas, se llevó a cabo otro experimento en donde las vitroplantas se inocularon antes de ser pasadas a la etapa de enraizamiento en medio MS (sin hormonas). Para esto, diez vitroplantas axénicas de banano fueron inoculadas al pie (0.5 ml) o a las hojas por medio de un pincel, con una suspensión de esporas de CFFSUR-B12 (concentración de $1x10^{9}$ u.f.c. ml^{-1}), mientras que diez plantas testigo fueron solamente tratadas con agua estéril. Antes de pasar las plantas a enraizamiento y ser inoculadas, se tomaron los pesos (g) iniciales de las plantas bajo condiciones asépticas. Al cabo de 40 d las plantas se

volvieron a pesar y se calculó la tasa de incremento en biomasa (g d^{-1}), la cual se sometió a Análisis de Varianza (α=0.05).

5.3 Ensayo colorimétrico para la determinación de AIA

Este ensayo se hizo para saber si CFFSUR-B12 sintetiza ácido indol-acético (AIA) a partir del triptófano. El AIA es una fitohormona clave en la comunicación bacteria-planta y que favorece el establecimiento como endófito o como residente foliar, así como en la estimulación del crecimiento vegetal (Lindow y Brandl 2003; Hardoim et al. 2008). Este tipo de determinación colorimétrica es una prueba estándar cuando se busca seleccionar aquellos microorganismos con potencial para ser utilizados como promotores del crecimiento vegetal (Sziderics et al. 2007; Nimnoi et al. 2010). Esta prueba se realizó mediante el siguiente protocolo: 0.1 ml de una suspensión de esporas de CFFSUR-B12 ($1x10^{12}$ u.f.c. ml^{-1}) se inocularon por cada 50 ml de medio líquido compuesto de glucosa (5 g l^{-1}), extracto de levadura (25 mg l^{-1}) y L-triptofano (204 mg l^{-1}) (Sigma). El cultivo se incubó a 25 °C, 180 rev min^{-1} y oscuridad total durante 72 h. Un control negativo (sin inóculo) fue simultáneamente incubado. A las 72 h, se tomaron 1.5 ml del cultivo y se centrifugaron a 8000 X *g*, a 4 °C por 10 min (Sziderics et al. 2007). Del sobrenadante recuperado se tomó 1 ml y se reaccionó con 2 ml de reactivo de Salkowski (0.3 g de $FeCl_3$ en 8.14 ml de H_2SO_4 concentrado y 1.86 ml de agua destilada) por 30 min en oscuridad y a temperatura ambiente (Glickmann y Dessaux 1995). Una coloración rosa-púrpura indicó la presencia de AIA en el sobrenadante de cultivo. Un control positivo con Acido 3-Indol Acético (Sigma) fue preparado (100 µg ml^{-1}).

5.4 Establecimiento como endófito o residentes de rizósfera

Para detectar la presencia de CFFSUR-B12 como endófito radicular, secciones de 1 cm fueron cortadas de los extremos apicales de las raíces, se esterilizaron externamente y se colocaron 10 secciones en agar nutritivo en cada una de tres cajas Petri. Similarmente, para detectar el establecimiento como endófito foliar en las hojas jóvenes que fueron inoculadas, se cortaron discos foliares de 0.5 cm de diámetro, se esterilizaron externamente y se colocaron 10 discos sobre agar nutritivo en cada una tres cajas Petri. En ambos casos se determinó el porcentaje de secciones y discos

colonizados por CFFSUR-B12 después de incubar las cajas a 26 °C. Además, se preparó una solución madre mediante la maceración de tejido foliar en agua (relación 1:10, tejido:agua) y se hicieron diluciones seriadas de donde se tomaron alícuotas de 20 µl que se inocularon en agar nutritivo para determinar u.f.c. g^{-1} de tejido foliar. El proceso de esterilización externa del tejido de raíz u hoja muestreado se hizo con etanol 96 % por 30 s, luego con hipoclorito de sodio al 3 % por 3 min, etanol 70 % por 2 min y por último se enjuagaron tres veces con agua estéril (De la Cruz 2010).

Para determinar la presencia en la rizósfera de las plantas inoculadas en raíz, se tomó una muestra del sustrato adherido a las raíces y se diluyó en agua estéril (relación 1:10, suelo:agua). A partir de esta solución madre se hicieron diluciones seriadas, de donde se tomaron alícuotas de 20 µl que se inocularon en agar nutritivo para determinar u.f.c. g^{-1} de suelo mediante el método del número más probable.

Los muestreos de endófitos y rizósfera se realizaron a 36, 56, 84, 123 y 140 días después de la inoculación foliar y radicular. Se incluyó dentro de estos ensayos a la cepa *Serratia marcescens* CFFSUR-B2 que ha demostrado potencial como agente de biocontrol de la SNB (Gutiérrez-Román et al. 2015), para establecer comparaciones entre ambas bacterias en cuanto a la promoción de crecimiento (excepto en biomasa) y en cuanto al establecimiento como endófitos en las plantas de banano.

VI. RESULTADOS

6.1 Interacción antagónica *St. galilaeus* CFFSUR-B12–*M. fijiensis*

Como resultados de la evaluación *in vitro* de la interacción antagónica de las quitinasas, metabolitos extracelulares, suspensión de esporas y sobrenadante de cultivo de *St. galilaeus* CFFSUR-B12 sobre la germinación de esporas, elongación de tubo germinativo y crecimiento micelial de *M. fijiensis*, se presenta el siguiente manuscrito publicado en la revista científica World Journal of Microbiology and Biotechnology.

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ORIGINAL PAPER

Antifungal performance of extracellular chitinases and culture supernatants of *Streptomyces galilaeus* CFFSUR-B12 against *Mycosphaerella fijiensis* Morelet

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Abstract The tropical and mycoparasite strain *Streptomyces galilaeus* CFFSUR-B12 was evaluated as an antagonist of *Mycosphaerella fijiensis* Morelet, causal agent of the Black Sigatoka Disease (BSD) of banana. On zymograms of CFFSUR-B12 culture supernatants, we detected four chitinases of approximately 32 kDa (Chi32), 20 kDa (Chi20), and two with masses well over 170 kDa (ChiU) that showed little migration during denaturing electrophoresis at different concentrations of polyacrylamide. The thymol-sulphuric acid assay showed that the ChiU were glycosylated chitinases. Moreover, matrix assisted laser desorption ionization time-of-flight MS analysis revealed that the ChiU are the same protein and identical to a family 18 chitinase from *Streptomyces* sp. S4 (gil498328075). Chi32 was similar to an extracellular protein from *Streptomyces albus* J1074 (gil478687481) and Chi20 was non-significantly similar to chitinases from five different strains of *Streptomyces* ($P > 0.05$). Subsequently, Chi32 and Chi20 were partially purified by anion exchange and hydrophobic interaction chromatography and tested against *M. fijiensis*. Chitinases failed to inhibit ascospore germination, but inhibited up to 35 and 62 % of germ tube elongation and mycelial growth, respectively. We found that crude culture supernatant and living cells of *S. galilaeus* CFFSUR-B12 were the most effective in inhibiting *M. fijiensis* and are potential biocontrol agents of BSD.

Keywords *Streptomyces galilaeus* · Chitinases · Culture supernatant · *Mycosphaerella fijiensis*

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Introduction

Tropical banana plantations are continuously threatened by the highly virulent ascomycete *Mycosphaerella fijiensis* Morelet (anamorph *Paracercospora fijiensis*), causal agent of Black Sigatoka Disease (BSD; Churchill 2011). BSD-resistant varieties of economically important banana cultivars are not available and high-input chemical-based management, along with cultural practices, are required to keep disease losses under reasonable economic thresholds (Marín et al. 2003; Orozco-Santos et al. 2008). Synthetic fungicides are aerially-sprayed weekly on banana plantations (Henriques et al. 1997), which has led to public concern about potential hazardous effects on human health (Bayoumi et al. 2002; Calviello et al. 2006; Mehrotra et al. 1987) and natural resources (Geissen et al. 2010). Biocontrol of BSD is a promising but poorly explored alternative (Ceballos et al. 2012; Marín et al. 2003) and the use of chitinolytic microorganisms has been proposed to replace, reduce or complement chemical disease management programs (Gohel et al. 2006; Nagpure et al. 2014a; Brzezinska et al. 2014).

Some strains of *Streptomyces* have desirable features as biocontrol agents (Cook and Baker 1983). In addition to producing antifungal metabolites (Qin et al. 2011) and antibiotics (Chater 2006; Procópio et al. 2012; Watve et al. 2001), they also synthesize a variety of extracellular

chitinases that play a key role in biocontrol of fungal pathogens (Herrera-Estrella and Chet 1999) by targeting the chitin present as the major structural component in their cell walls (Bhattacharya et al. 2007; Gohel et al. 2006; Latgé 2007). These chitinases are also produced to decompose chitin in nature for nutrient and energy acquisition (Kämpfer 2012; Williams and Robinson 1981). The biocontrol potential of chitinase-producing *Streptomyces* strains has been widely demonstrated (Joo 2005; Mohamed and Benali 2010; Nagpure and Gupta 2013; Prapagdee et al. 2008; Quecine et al. 2008; Shimizu 2011).

Chitinases (EC 3.2.1.14) break down chitin into its monomers *N*-acetylglucosamine (NAG) by hydrolyzing β-1,4 glycosidic linkages between amino sugar units (Muzzarelli 1999). Endochitinases randomly split the chain, producing NAG chitooligomers. The exochitinases are divided in chitobiosidases (EC 3.2.1.29) or chitobiases (EC 3.2.1.30), which release NAG dimers and monomers from the polymer, respectively (Duo-Chuan 2006; Brzezinska et al. 2014). Streptomycetes mainly produce family 18 chitinases, along with a family 19 chitinase normally synthesized only by plants (Ohno et al. 1996; Watanabe et al. 1999).

Extracellular bacterial chitinolytic machineries are normally mixtures of endo and exochitinases that vary in a strain-dependant manner. Nagpure et al. (2014b) detected five chitinases in zymograms of culture supernatants of *Streptomyces violaceusniger* MTCC 3959, although up to 13 and seven putative genes were identified in the *Streptomyces coelicolor* A3(2) (Bentley et al. 2002; Kawase et al. 2006) and *Streptomyces albus* J1074 genomes (Zaburannyi et al. 2014), respectively. In addition to producing chitinases, antagonistic activities of *Streptomyces* strains are derived from about 7000 small secondary metabolites (0.1–3 kDa) that may inhibit enzymes or alter cellular processes in other organisms (Chater et al. 2010).

The tropical strain CFFSUR-B12, identified recently as *Streptomyces galilaeus* (G. Huerta, unpublished data) was isolated as a parasite of the cocoa anthracnose fungus. Since CFFSUR-B12 is a mycoparasitic strain, its production of antifungal extracellular chitinases and metabolites was hypothesized and is supported by its highly antagonistic activity towards some phytopathogens, including that causing BSD. With the aim of evaluating and characterizing *S. galilaeus* CFFSUR-B12 as a potential biocontrol agent, we tested the in vitro antagonistic interaction of its extracellular chitinases, metabolites and culture supernatant on ascospore germination, germ tube elongation and mycelial growth of *M. fijiensis*. Additionally, matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was carried out to identify the *S. galilaeus* CFFSUR-B12 extracellular chitinases.

Materials and methods

Microorganisms and growth conditions

Streptomyces galilaeus CFFSUR-B12 was isolated from anthracnose (*Colletotrichum gloesporioides*) lesions present on cocoa cobs (*Theobroma cacao* L.). For chitinase induction, 1 ml of a spore suspension of CFFSUR-B12 (7.8×10^{16} CFU ml^{-1}) was inoculated per liter of minimal medium M9 (Quecine et al. 2008) containing 1 % colloidal chitin from crab shells as major carbon source [Sigma-Aldrich Co. LLC, prepared as described by Gómez Ramírez et al. (2004)], 1 g L^{-1} yeast extract, 0.1 mM $CaCl_2$ and 1 mM $MgSO_4$. To determine the time of maximum chitinase production, supernatants from pilot cultures were assayed daily for activity using 10 mM 4-nitrophenyl *N*-acetyl-glucosamine (Sigma-Aldrich) as substrate (Park et al. 2000). Cultures were grown in the dark at 29° C with shaking at 200 rpm.

Ammonium sulfate precipitation of chitinases from culture supernatants

Spores, mycelia and insoluble material were removed from a total of 4.3 L of 5-day M9+ colloidal chitin culture by centrifugation at 15,300×*g* for 20 min at 4 °C. The crude supernatant was vacuum-filtered through 0.45 µm pore size membranes (Millipore, USA) and concentrated approximately 20-fold by lyophilization. Before and after lyophilization, aliquots of the supernatants were stored at −20 °C for further antifungal assays (referred to below as crude culture supernatant and concentrated supernatant, respectively). Proteins from the concentrated supernatant were precipitated by adding portions of solid ammonium sulfate to a final concentration of 85 % of saturation at 4 °C with slow stirring overnight. Protein pellets recovered by centrifugation (15,300×*g*, 20 min, 4 °C) were resuspended in either 20 mM Tris–HCl (pH 7) or 50 mM Tris–HCl (pH 8) for SDS–polyacrylamide gel electrophoresis (PAGE) and chitinase purification, respectively. Pellets were exhaustively dialyzed against the corresponding buffer at 4 °C. The dialysates used for SDS-PAGE were centrifuged as described above and concentrated eight-fold by ultrafiltration (Spin-X® UF500, Corning, MWCO = 10 kDa). Protein concentrations were determined by the Bradford method, with bovine serum albumin as standard (Bradford 1976).

SDS-PAGE and in-gel detection of chitinases

SDS-PAGE was done following reported procedures (Kim et al. 2003; Trudel and Asselin 1989) with some modifications. Resolving gels with 8 or 15 % polyacrylamide, or

8 % layered over 15 % polyacrylamide (all containing 0.1 % SDS) were used to separate large and small chitinases, respectively. Chitinase samples were mixed with an equal volume of 2× SDS sample loading buffer lacking β-mercaptoethanol and were not heat-denatured (Liau and Lin 2008; Nagpure et al. 2014b). Electrophoresis was carried out at room temperature for 65 min at 200 V constant power. After electrophoresis, separating gels were incubated in distilled water for 15 min at room temperature: complete removal of SDS was not necessary due to the SDS-resistance of CFFSUR-B12 chitinases, as reported for other bacterial chitinases (Liu et al. 2009).

Chitinase activity in the gels was detected by placing them in contact with an 8 % polyacrylamide gel containing 0.01 % glycochitin as substrate, prepared by acetylation of glycol-chitosan (Trudel and Asselin 1989). Overlayed gels were incubated at 37 °C in a humid chamber for 1 or 2 h for the 8 and 15 % polyacrylamide separating gels, respectively. Resolving and overlay gels were separated in distilled water. Resolving gels were stained with Coomassie Brilliant Blue R-250 (Garfin 1990), while overlay gels were stained in Calcofluor white M2R (Sigma-Aldrich) and distained in distilled water. Lytic zones corresponding to glycochitin degradation in overlay gels were visualized and photographed under UV light (Trudel and Asselin 1989).

In-gel detection of glycosylated chitinases

In a separate electrophoretic assay, we carried out a thymol/sulphuric acid protocol (Gander 1984) to detect glycosylated chitinases after resolution of proteins by SDS-PAGE, along with in-gel detection of chitinases on zymograms as explained above. Zones in resolving gels with a pink/reddish coloration against a clear background indicated the presence of glycoproteins.

Identification of CFFSUR-B12 chitinases by MALDI-TOF MS

Protein bands with chitinase activity detected on zymograms were excised from the corresponding Coomassie-stained resolving gels and distained. Following reduction, alkylation and Trypsin digestion (Promega), they were analyzed on an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Flex Analysis 1.2 v SD1 Patch 2 software (Bruker Daltonics) was used to record the mass spectra. The search engine MASCOT software (http://www.matrixscience.com) was used to compare the mass spectra against those annotated in the NCBInr database (http://www.ncbi.nlm.nih.gov/), with the following search parameters: taxonomy Bacteria (Eubacteria), peptide mass tolerance of ±200 ppm, one missed cleavage allowed, fixed modification of Propionamide (C) and oxidation of methionine as variable modification (Pérez-Vázquez et al. 2014; Garibay-Cerdenares et al. 2014). Scores above 86 were accepted ($P < 0.05$).

Partial purification of chitinases

Chitinases from culture supernatants were loaded on an anion exchange column (BioScale Q2, BioRad) equilibrated in 50 mM Tris HCl (pH 8) and eluted with a linear gradient of 0.3–0.6 M NaCl at a flow rate of 60 ml h^{-1}. One-millilitre fractions were collected, monitored at 280 nm for protein content and assayed for chitinase activity. Chitinase peak fractions eluting in the salt gradient were dialyzed overnight at 4 °C against 100 mM sodium phosphate buffer (pH 6.8) containing 2.4 M ammonium sulfate and loaded onto an Econo-Pac Methyl hydrophobic interaction chromatography (HIC) column (BioRad) equilibrated in the same buffer and eluted with a 2.4–0 M ammonium sulfate gradient at a flow of 120 ml h^{-1}. Fractions of 2 ml were collected, assayed for chitinase activity and read at 280 nm protein content. Chitinase purification was conducted at 4 °C. Chitinase activity was detected by spotting 3 µl of fractions onto a 8 % polyacrylamide gel containing 0.01 % glycochitin, incubating at 37 °C for 2 h under moist conditions and then treating with Calcofluor white M2R (Purushotham and Podile 2012). Lytic spots visualized and photographed under UV light were quantitated by densitometry using ImageJ software 1.47v (http://imagej.nih.gov/ij). The most active fractions were analyzed by SDS-PAGE with silver staining (Dodeca™ Silver stain Kit, BIORAD) and zymography as described above, assayed for protein content (Bradford 1976) and enzymatic activity by the dinitrosalicylic acid (DNS) method using *N*-acetylglucosamine (NAG) as standard (Miller 1959). One unit (U) of activity was defined as the amount of enzyme that released one µmol of NAG from colloidal chitin per minute.

Ascospore discharge

Discharge of *M. fijiensis* ascospores was done as described previously (Jacome et al. 1991; Pérez et al. 2002). Briefly, infected banana leaves were placed in a moist chamber for 24 h. Lesions bearing multiple mature pseudothecia on adaxial leaf surfaces (stage 6 on Fouré's scale) were stapled abaxial surface down to 5 × 5 cm pieces of cardboard and washed for 15 min under running tap water. Excess water was blotted off and cardboards were individually attached to petri dish lids and placed over base plates containing 1.5 % agar. After 1 h of incubation at 27 °C, cardboards were removed and ascospore discharges were inspected under magnification and marked by punching the agar with a sterile dissecting needle.

Inhibition of ascospore germination, germ tube elongation and mycelial growth of *M. fijiensis*

Inhibition of ascospore germination and germ tube elongation was assessed by directly applying 20 µl of each treatment to ungerminated ascospores, while mycelial growth reduction was assayed by applying treatments 24 h after germination. All assay plates were incubated at room temperature for 24 h. Growth was stopped by adding lactophenol solution and ascospores were photographed 48 h later. For each assay, photographs were taken from six to eight microscopic fields at 10× magnification with a Nikon Eclipse 80i microscope (Nikon Corporation, Japan) equipped with a Moticam2500® camera (Motic China Group CO. LTD.). In the germination assay, three hundred ascospores per treatment were inspected and counted for germinated and non-germinated ascospores, defined as those with germ tubes longer and shorter than 4 µm, respectively (Jacome et al. 1991). For germ tube elongation and mycelial growth inhibition tests, the lengths of germ tubes and mycelia were measured from 300 ascospores per treatment. Measures were taken by image analysis using the Motic® Image Plus 2.0 software (Motic China Group CO. LTD.).

Treatment preparations

Treatments in both antifungal assays were: (a) CFFSUR-B12 partially purified chitinases applied individually (ChiU, Chi32 and Chi20) or in a mixture (1:1:1) (v/v), (b) crude culture supernatant (CCS) (see chitinase precipitation section), (c) flow through metabolites fraction (Mets <10 kDa) and (d) protein-retained fraction ($P > 10$ kDa) obtained by ultrafiltration of CCS (MWCO = 10 kDa); (e) protein fraction retained ($P > 14$ kDa) after dialysis (MWCO = 14 kDa) of the CFFSUR-B12 concentrate supernatant (see chitinase precipitation section), and (f) CFFSUR-B12 spore suspension (3×10^{12} CFU ml^{-1}). Enzymatic activity and protein concentration was measured in all treatments by the DNS and Bradford methods, respectively, except for the bacterial spore suspension. After ultrafiltration, the retained protein fraction ($P > 10$ kDa) was diluted with distilled water to its initial volume. ChiU was also diluted to equal the enzymatic activity of the $P > 14$ kDa treatment.

Statistical analysis

For the antifungal assays, the percent inhibition with respect to untreated ascospores (control) was calculated (Ceballos et al. 2012; Pérez et al. 2002). Inhibition of ascospore germination was analyzed by homogeneity of proportions (Chi square test, $P < 0.05$), while data from germ tube and mycelia growth inhibition were subjected to ANOVA and Tukey's test ($P < 0.05$) for means comparison, using the R© software (Ver. 2.14.0, The R Foundation for Statistical Computing).

Results

SDS-PAGE and zymogram analyses of chitinases

Following resolution of *S. galilaeus* CFFSUR-B12 culture supernatant by SDS-PAGE, zymogram analysis revealed four distinct chitinase activities (Fig. 1). These included two low molecular mass chitinases of approximately 20 (Chi20) and 32 kDa (Chi32) that are within the size range expected for bacterial chitinases (Fig. 1). In addition, two very intense lytic zones corresponding to proteins with apparent masses well over 170 kDa (ChiU) were detected. These consistently showed little migration during electrophoresis in gels with different polyacrylamide concentrations (Fig. 1). These high molecular mass chitinases were the most active and abundant in CFFSUR-B12 culture supernatants as revealed by Coomassie staining, zymograms, total protein content and enzymatic activity (Bradford and DNS methods, respectively; Table 1).

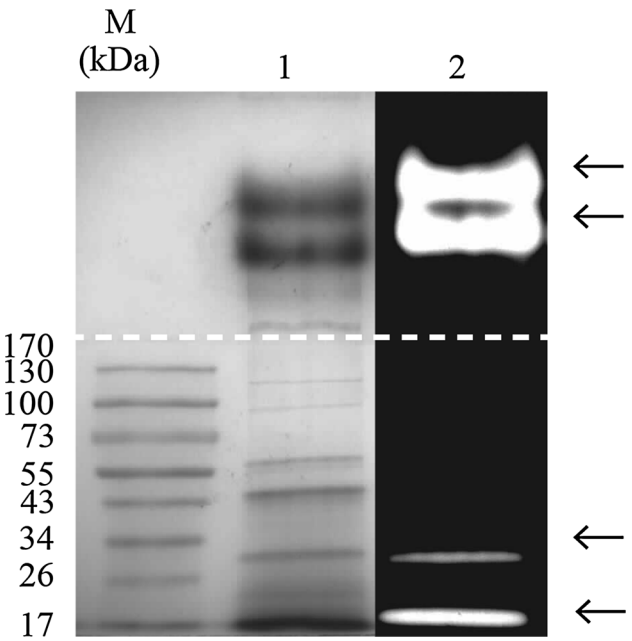

Fig. 1 Detection of chitinase activity from *S. galilaeus* CFFSUR-B12 culture supernatants on Coomassie-stained SDS-PAGE gels (*lane 1*) and activity-stained zymograms (*lane 2*) from SDS-PAGE with two concentrations of polyacrylamide 8 % (*top*) and 15 % (*bottom* demarcated by *dotted line*). The zymogram overlay shows chitinolytic activity as white zones (*arrows*). *Lane M* is the protein molecular mass marker with sizes indicated in kDa

Detection of glycosylated chitinases

We clearly detected a not-well-resolved pink-reddish zone that corresponded to the ChiU lytic zones on zymograms (Fig. 2). This demonstrates that the ChiU are glycosylated and helps explain their >170 kDa molecular masses.

Identification of CFFSUR-B12 chitinases by MALDI-TOF MS

The oligopeptides resulting from digestion of both ChiU bands were analyzed with MASCOT software and were similar to a glycosyl-hydrolase family 18 chitinase from *Streptomyces* sp. S4 (gil498328075, score: 95, expect: 0.0073 and 0.0065, for upper and lower ChiU bands, respectively) from the NCBInr database. Fragments represented 14 and 16 % of the total protein sequence of the annotated chitinase, which contains 628 amino acid residues and has a molecular mass of 66 kDa. These results show that the two bands of ChiU activity arise from the same protein.

Tryptic peptides from Chi32 showed similarity with a secreted protein from *Streptomyces albus* J1074 (gil478687481, score: 90, expect: 0.0087), a coverage of 19 % of the total protein that has 733 residues and a molecular mass of 78.7 kDa. Finally, Chi20 was non-significantly similar to chitinases from five different strains of *Streptomyces* (score = 72, $P > 0.05$).

Partial purification of chitinases

The >170 kDa molecular mass chitinases (ChiU) did not bind to the anion exchange column but were both obtained in the flow-through fraction, which did not contain detectable Chi32 and Chi20 activities (Fig. 3). The fraction containing both ChiU was used in the antifungal assays.

To separate Chi32 and Chi20 the enzymes were partially purified 7.1 and 28.7-fold, respectively, by anion exchange and HIC (Table 1; Fig. 3). Zymography of the purified preparations showed that each contained only the chitinase activity targeted in the purification. Silver staining showed that ChiU and Chi20 preparations contained a number of other (non-chitinolytic) proteins (Fig. 3), but the purity of the Chi32 preparation could not be clearly determined.

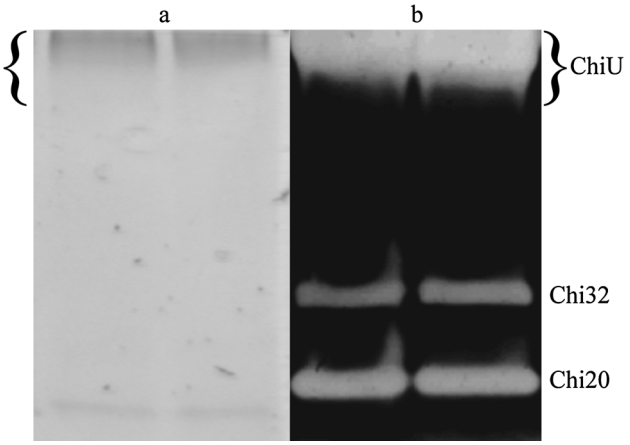

Fig. 2 Detection of glycosylated chitinases of *S. galilaeus* CFFSUR-B12 by the thymol/sulphuric acid method after resolution on SDS-PAGE. Brackets in **a** indicate the zone of high glycoprotein detection, which corresponds to ChiU unresolved lytic zones on zymograms (brackets in **b**). *Bottom bands* in **a** correspond to the dye front

Inhibition of ascospore germination, germ tube elongation and mycelia growth of *M. fijiensis*

CFFSUR-B12 partially purified chitinases had virtually no effect on *M. fijiensis* ascospore germination, either individually or in combination. In contrast, CCS inhibited ascospore germination by 96 %. When the CCS protein fraction ($P > 10$ kDa) was separated from CCS metabolites (Mets <10 kDa) and tested separately, ascospore inhibition was drastically reduced (1.4 and 5 % inhibition). Similarly, the protein fraction retained after dialysis of concentrated supernatant ($P > 14$ kDa) only inhibited ascospore germination by 8 %, despite of its higher chitinase content. Living cells of CFFSUR-B12 inhibited germination by 56 % (Figs. 4, 6).

Table 1 Purification of chitinases from *S. galilaeus* CFFSUR-B12

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Culture supernatant	45.6	13.872	0.304	1.0	100
Ammonium sulfate precipitation	11.0	4.938	0.449	1.48	35.6
Anion exchange					
ChiU	1.094	0.305	0.279	0.91	2.2
Hydrophobic interaction					
Chi32	0.026	0.056	2.154	7.08	0.4
Chi20	0.028	0.244	8.714	28.65	1.8

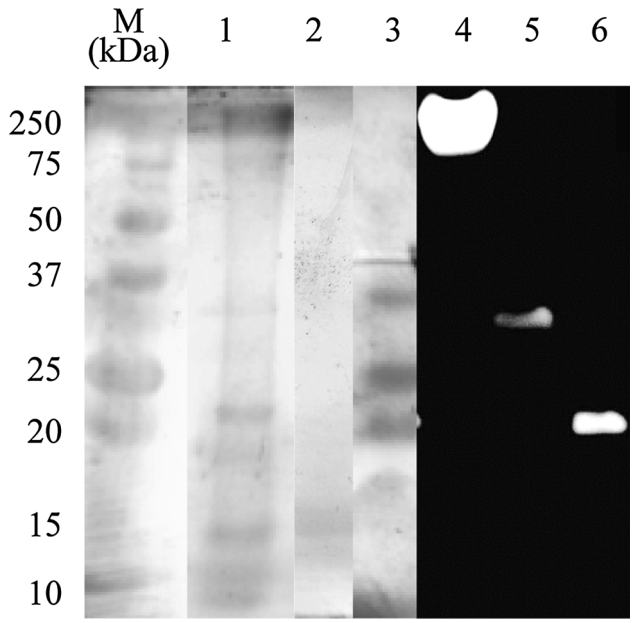

Fig. 3 Zymogram of partially purified chitinases from *S. galilaeus* CFFSUR-B12 supernatants. *Lanes 1*, *2* and *3* are silver stained gels of ChiU, Chi32 and Chi20 fractions, and *lanes 3*, *4* and *5* are the zymograms for ChiU, Chi32 and Chi20, respectively. The purity of Chi32 could not be clearly determined by silver staining. *Lane M* indicates protein molecular masses (kDa) in the protein standard

For *M. fijiensis* germ tube inhibition, the partially purified chitinases had a weak antagonistic effect. Chi20 applied individually was the most active (35 % inhibition), followed by ChiU (23 %) and Chi32 (22 %). When the partially purified chitinases were combined (ChiU-Chi32-Chi20), there was no increase in antifungal effect. In contrast, the CFFSUR-B12 spore suspension and CCS reduced germ tube growth 97 and 99 %, respectively. Meanwhile, CCS retained-protein ($P > 10$ kDa) and flow-through (Mets <10 kDa) fractions inhibited germ tube growth by 45 and 39 %, respectively. Despite its higher chitinase content, concentrated and dialyzed proteins ($P > 14$ kDa) had a substantial loss of inhibitory activity as compared to CCS, giving only 64 % inhibition of germ tube elongation (Figs. 5a, 6).

A notably greater inhibitory effect of most treatments was seen against actively growing *M. fijiensis* hyphae. Chi20 inhibited hyphal growth 63 %, while ChiU and Chi32 inhibited by 39 and 46 %, respectively. Inhibition was not increased when the chitinases were combined. Again, CFFSUR-B12 spore suspension and CCS were the most effective antifungal treatments (98 and 93 % of inhibition, respectively). The CCS protein fraction ($P > 10$ kDa) reduced mycelial growth 85 % despite of its low chitinase content, while flow-through fraction metabolites (Mets <10 kDa) inhibited by 53 %. The dialyzed protein fraction of the concentrate supernatant ($P > 14$ kDa) inhibited hyphal development 62 %, similar to Chi20 and the chitinase mixture (Fig. 5b).

Discussion

The SDS-PAGE and zymogram analyses of *S. galilaeus* CFFSUR-B12 culture supernatant revealed four clearly-resolved lytic bands corresponding to chitinases of 32 (Chi32) and 20 kDa (Chi20), and two bands of >170 kDa (ChiU). Chi32 and Chi20 are within the range reported (20–81 kDa) for *Streptomyces* chitinases (El-Sayed et al. 2000; Haggag and Abdallh 2012; Hoang et al. 2011; Joo 2005; Kim et al. 2003; Mukherjee and Sen 2006; Nagpure and Gupta 2013; Narayana and Vijayalakshmi 2009; Rabeeth et al. 2011; Singh et al. 1999; da Sobrinho et al. 2005; Brzezinska et al. 2013; Tsujibo et al. 1993). Chitinases with molecular mass >170 kDa, like ChiU, have not been reported in *Streptomyces*, with the highest mass currently reported being a 114.8 kDa chitinase found in *Streptomyces violaceusniger* MTCC 3959 culture supernatant (Nagpure et al. 2014b). In comparison to Chi32 and Chi20, ChiU was the more abundant and active against glycochitin, based on its total protein content and enzymatic activity, as well as its band intensity on Coomassie-stained gels and lytic zones on zymograms.

The high molecular mass of ChiU led us to suspect that these enzymes were post-translationally modified by glycosylation. Some authors have also suspected that unusually large chitinases (ca. 180 kDa) from *Serratia marcescens* NIMA detected on zymograms with glycochitin as substrate (Ruiz-Sánchez et al. 2005), were glycosylated. Glycosylation may increase enzyme activity (like ChiU on glycochitin) and is also involved in the export of proteins and/or chitinases out of the cell (Reguera and Leschine 2003; Shylaja and Seshadri 1989). It could account for some of the different chitinase isoforms occurring in chitinolytic microorganisms (Dahiya et al. 2006). That ChiU is glycosylated was confirmed by thymol/sulphuric acid assay, and a purification protocol for glycoproteins might be useful for resolving the ChiU proteins.

MALDI-TOF analysis showed that the two lytic bands detected on ChiU zymograms resulted from the same chitinase (*Streptomyces* sp. S4 family 18 glycosyl hydrolase, 66 kDa). The molecular mass of the annotated chitinase is significantly less than that of ChiU (>170 kDa), which might be explained by glycosylation of the latter protein. The resolution of ChiU into two bands on SDS-PAGE gels may have resulted from selective, partial deglycosylation during the procedure, or the presence of a protease in the CFFSUR-B12 culture supernatant that produces two active ChiU isoenzymes (Romaguera et al. 1992, Gal et al. 1998). These hypotheses remain to be clarified. Chi32 was similar to a 78.7 kDa secreted protein from *Streptomyces albus* J1074 that lacks chitinase

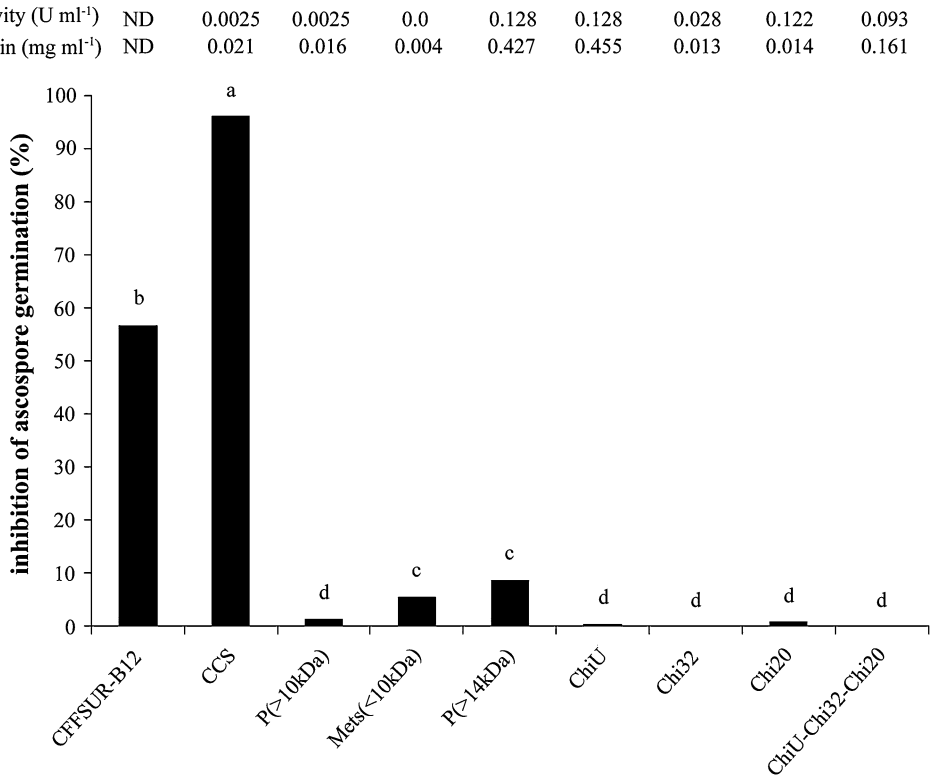

Fig. 4 Inhibitory effect of partially purified chitinases (ChiU, Chi32 and Chi20), crude culture supernatant (CCS), protein ($P > 10$ kDa) and metabolites (Mets <10 kDa) components of CCS, dialyzed protein component from concentrate supernatant ($P > 14$ kDa) and living cells (CFFSUR-B12) of *S. galilaeus* CFFSUR-B12 (3×10^{12} CFU ml^{-1}) on *M. fijiensis* ascospore germination. Enzymatic activities (U ml^{-1}) and protein concentration (mg ml^{-1}) of the treatment samples given at the top of the figure (ND = not determined). *Bars* indicate inhibition calculated relative to untreated ascospores (control). *Different letters on bars* indicate statistically significant differences ($P < 0.05$)

activity. The molecular mass differences between the two proteins suggests that Chi32 is a monomeric form of the *S. albus* protein. The chitinase activity of Chi32 was consistently demonstrated in our assays. We speculate that Chi20 is a chitinase unlike others so-far reported in the NCBInr database, as it shows some of similarity to several annotated *Streptomyces* chitinases. In summary, we show that strain CFFSUR-B12 produces three different chitinases that are detected as four lytic bands on zymograms.

We tested the in vitro antagonistic activity of living cells, partially purified extracellular chitinases, and several other fractions (protein or small metabolite) isolated from CFFSUR-B12 culture supernatant against *M. fijiensis* Morelet, causal agent of BSD. We showed the effectiveness of strain CFFSUR-B12 living cells and culture supernatant in inhibiting (generally above 93 %) *M. fijiensis* ascospore germination, germ tube elongation and mycelial growth. The effectiveness of culture supernatants from 12 strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* in inhibiting ascospore germination and mycelial growth of *M. fijiensis* has been shown (Ceballos et al. 2012), but this is the first report for any *Streptomyces* strain.

In contrast to Broadway et al. (1995), who found that individual purified chitinases from *Streptomyces albidoflavus* NRRL B-16746 were able to inhibit the germination of *Botrytis cinerea* and *Fusarium oxysporum* spores, we found that CFFSUR-B12 chitinases (individually or in combination) did not account for the inhibitory activity of a crude culture supernatant on ascospore germination. The enzymes themselves accounted for 35 and 62 % inhibition of germ tube elongation and mycelia growth, respectively. Moreover, antifungal activity was not increased when a tripartite chitinase mixture was applied to *M. fijiensis*. Someya et al. (2001) found a two-fold increase in inhibition of *B. cinerea* spore germination when a chitobiosidase was mixed with an endochitinase from *Serratia marcescens* B2, as compared to individual chitinases. Synergism between chitinases in decomposing chitin has also been demonstrated (Brurberg et al. 1996; Gutiérrez-Román et al. 2014; Suzuki et al. 2002). The generally lower inhibitory activity of the CFFSUR-B12 chitinases that we observed may be due to the removal of proteins like chitin-binding proteins (CBP or CHB) that may stabilize or increase their enzymatic activities (Duo-Chuan 2006; Gutiérrez-Román et al. 2014; Joo 2005; Purushotham et al. 2012; Schrempf 2001; Suzuki et al. 1998; Brzezinska et al. 2014; Watanabe et al. 1997). These accessory proteins may have been lost during dialysis of the CFFSUR-B12 concentrated supernatant, or during chitinase purification.

We also found that the effective antifungal performance of CFFSUR-B12 crude culture supernatant against *M. fijiensis* required the combined action of extracellular metabolites and the protein fraction (including chitinases).

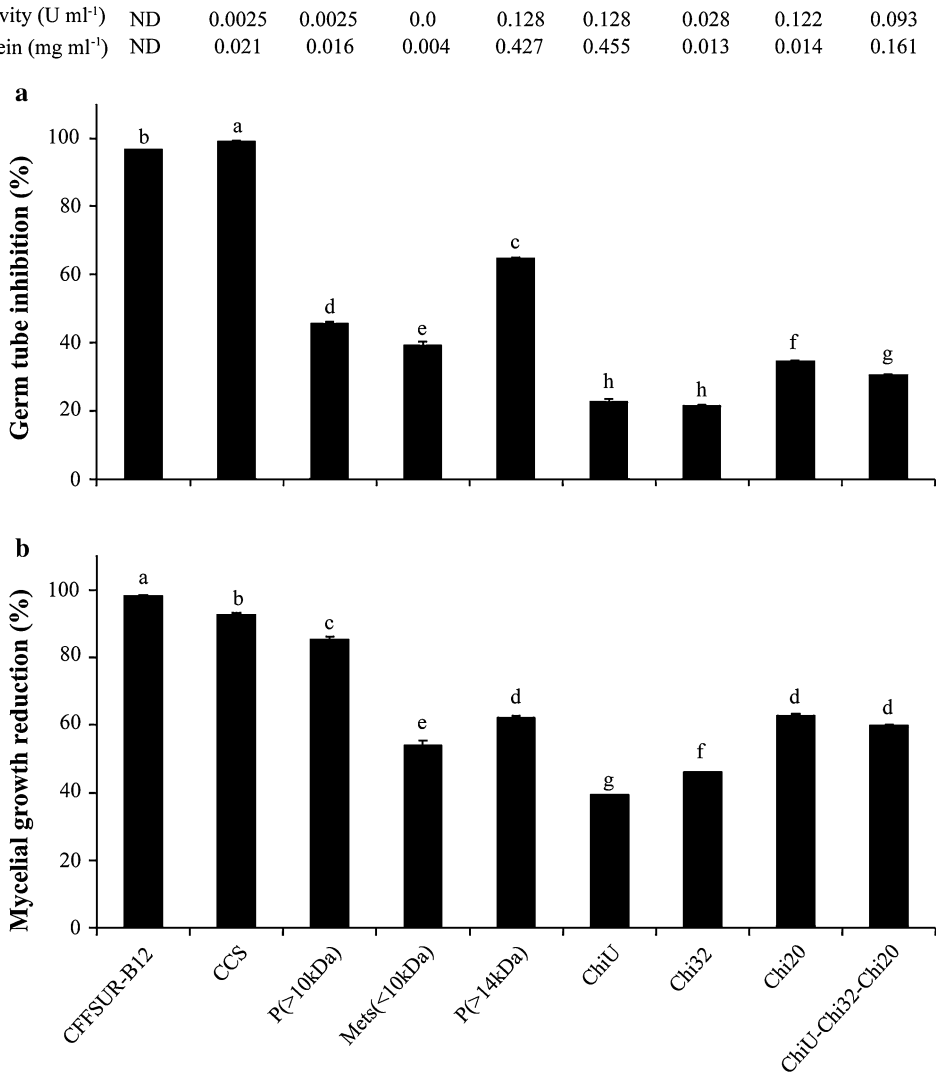

Fig. 5 Inhibition of *M. fijiensis* germ tube elongation (**a**) and mycelial growth (**b**), by partially purified chitinases (ChiU, Chi32 and Chi20), crude culture supernatant (CCS), protein ($P > 10$ kDa) and metabolites (Mets <10 kDa) components of CCS, dialyzed protein component from concentrate supernatant ($P > 14$ kDa) and living cells (CFFSUR-B12) from *S. galilaeus* CFFSUR-B12 (3×10^{12} CFU ml^{-1}). Enzymatic activities (U ml^{-1}) and protein concentration (mg ml^{-1}) are shown at the top panel **a** (*ND* not determined). *Black bars* indicate average percent inhibition relative to 300 untreated germ tube (**a**) and mycelia (**b**) controls. *Error bars* represent mean standard error and *different letters on bars* indicate statistically significant differences ($P < 0.05$)

When extracellular metabolites were separated from extracellular proteins and tested separately, both fractions had a greatly reduced inhibitory effect as compared to crude supernatant. Metabolites only explained about 50 % of total antifungal activity in the crude supernatant. Some authors have found that the simultaneous action of metabolites and cell-wall degrading enzymes is required for effective antifungal inhibition (Akutsu et al. 1993; El-Tarabily et al. 2000; Huang et al. 2013; Macagnan et al. 2008; Prapagdee et al. 2008). Apparently, chitinases may alter cell wall permeability or open entry points for metabolites in the hyphae, leading to a disruption of cellular processes (Quecine et al. 2008; Regev et al. 1996). A classic example is the synergistic effect of the red pigment prodigiosin and extracellular chitinases from *Serratia marcescens* against chitin-containing organisms (Someya et al. 2001; Tu et al. 2010).

CFFSUR-B12 spore suspension, along with crude supernatant, were the most effective treatments against *M. fijiensis*. Intimate contact between bacteria and fungus likely triggered production of extracellular metabolites and cell-wall degrading enzymes (Trejo-Estrada et al. 1998; Zhao et al. 2013). Mycolytic enzymes may include chitinases, glucanases, proteases and lipases (Nagpure et al. 2014b), while antibiotics (Procópio et al. 2012), chitin-synthetase inhibitors like nikkomycin and polyoxins (Schrempf 2001; Chater et al. 2010) and siderophores that sequester and limit pathogen access to iron (Macagnan et al. 2008) are included in the antifungal metabolites category.

From our results, we conclude that *S. galilaeus* CFFSUR-B12 spores and crude culture supernatant are potential biocontrol agents of *M. fijiensis*. However, more microcosm and field trials are required to elucidate the *in planta* antagonistic activity of the bacteria and extracellular compounds. Additionally, a suitable formulation of the biofungicide would be critical to extend its shelf-life and field survivorship without loss of antifungal properties

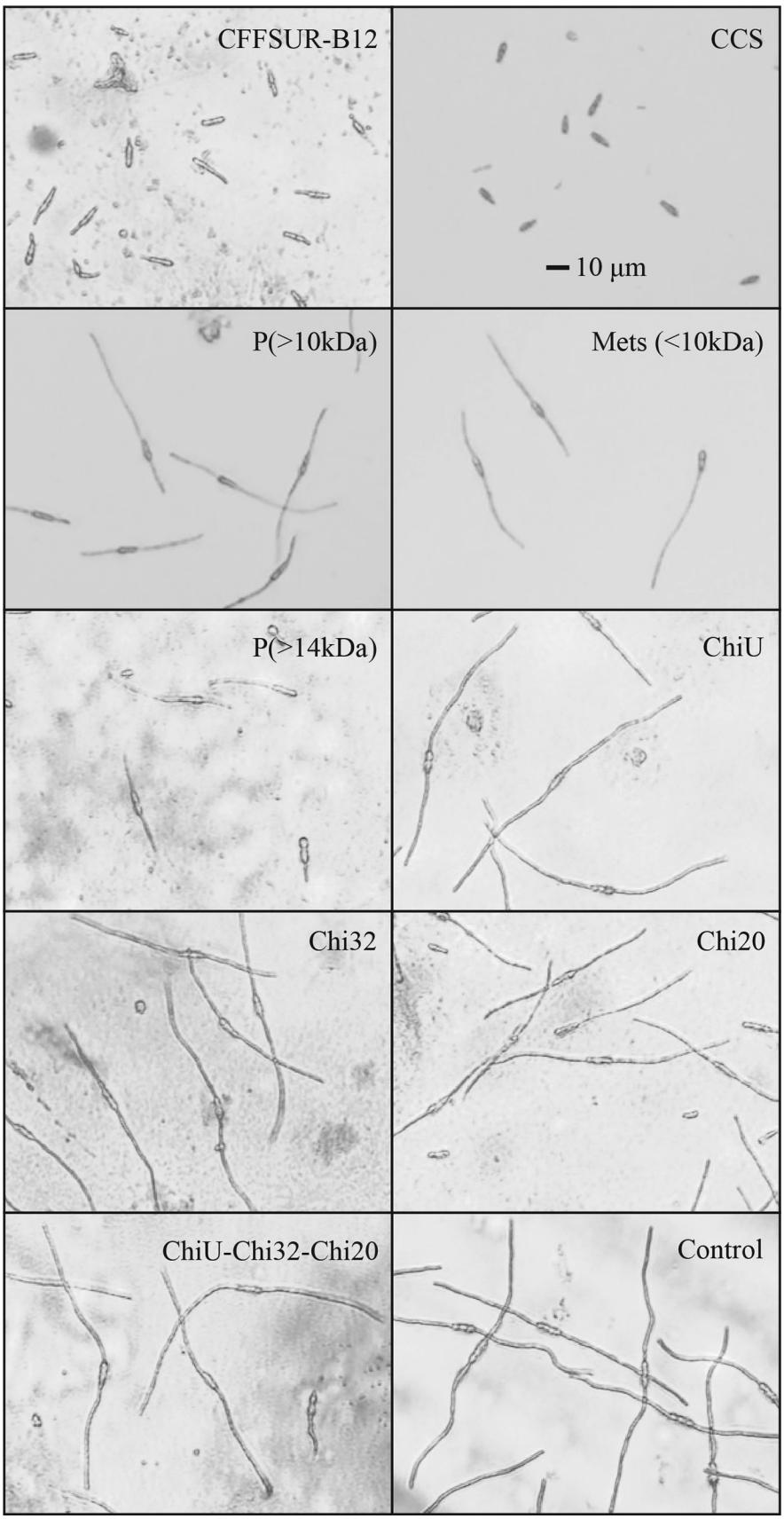

Fig. 6 Micrographs of 24 h old *M. fijiensis* ascospores discharged onto agar plates and treated with partially purified chitinases (ChiU, Chi32 and Chi20), crude culture supernatant (CCS), protein ($P > 10$ kDa) and metabolites (Mets <10 kDa) components of CCS, dialyzed protein component from concentrate supernatant ($P > 14$ kDa) and living cells (CFFSUR-B12) of *S. galilaeus* CFFSUR-B12 (3×10^{12} CFU ml^{-1}). Controls were untreated ascospores

(Gohel et al. 2006; Macagnan et al. 2006), especially considering that *M. fijiensis* is a foliar pathogen and the phyllosphere is a very difficult environment to inhabit (Andrews 1992; Blakeman and Fokkema 1982; Macagnan et al. 2008), as is the banana phylloplane (Ceballos et al. 2012).

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6.2 Efecto de *St. galilaeus* CFFSUR-B12 sobre la severidad de la SNB y microorganismos asociados al banano

Como resultado de la evaluación en campo de la efectividad de la aplicación del sobrenadante de cultivo y la suspensión de esporas de *St. galilaeus* CFFSUR-B12 sobre la severidad de la SNB y sobre la microbiota no blanco asociada a la filósfera y rizósfera del banano, se presenta el siguiente manuscrito que fue enviado a la revista BioControl.

BioControl

Streptomyces galilaeus CFFSUR-B12 is highly effective as a biocontrol agent of the Black Sigatoka Disease but is benign towards banana-associated microbial communities

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Abstract:	In this work, we tested Streptomyces galilaeus CFFSUR-B12 spore suspension and crude culture supernatant as biocontrol agents of the Black Sigatoka Disease (BSD) in the field. We sprayed a spore suspension (1x109 c.f.u. ml-1) and culture supernatant of strain CFFSUR-B12 on recently-flowered banana plants and found that spores reduced BSD severity over time, decreased the area under the BSD progress curve up to 29% and delayed disease progress up to 19 days in comparison to mancozeb and water-treated plants. Moreover, spore suspension had no deleterious effect on the diversity of banana root- and leaf-associated fungal, bacterial and actinomycete communities. Additionally, chitinases from culture supernatant were active and stable at ranges of temperature similar to those of the warm tropical conditions where banana is grown (29-37ºC). We concluded that spore suspension of strain CFFSUR-B12 is a potential biocontrol agent of BSD under field conditions and is not harmful to banana-associated microbiota.
Suggested Reviewers:	Jaime A. Gutierrez-Monsalve jagutierrez33@gmail.com He has recently evaluated the biocontrol agent Bacillus subtilis against the black sigatoka disease under field conditions.
	Sandra Mosquera smosquera@ucdavis.edu She has experience on field evaluation of bacterial biocontrol agents against the black

Streptomyces galilaeus CFFSUR-B12 is highly effective as a biocontrol agent of the Black Sigatoka Disease but is benign towards banana-associated microbial communities

Abstract

In this work, we tested *Streptomyces galilaeus* CFFSUR-B12 spore suspension and crude culture supernatant as biocontrol agents of the Black Sigatoka Disease (BSD) in the field. We sprayed a spore suspension ($1x10^9$ c.f.u. ml^{-1}) and culture supernatant of strain CFFSUR-B12 on recently-flowered banana plants and found that spores reduced BSD severity over time, decreased the area under the BSD progress curve up to 29% and delayed disease progress up to 19 days in comparison to mancozeb and water-treated plants. Moreover, spore suspension had no deleterious effect on the diversity of banana root- and leaf-associated fungal, bacterial and actinomycete communities. Additionally, chitinases from culture supernatant were active and stable at ranges of temperature similar to those of the warm tropical conditions where banana is grown (29-37ºC). We concluded that spore suspension of strain CFFSUR-B12 is a potential biocontrol agent of BSD under field conditions and is not harmful to banana-associated microbiota.

Key words: *Streptomyces galilaeus* / CFFSUR-B12 / Spore suspension / Black Sigatoka Disease / Chitinases

Introduction

The most challenging problem in commercial large-scale banana production is the Black Sigatoka Disease (BSD), caused by the ascomycete *Mycosphaerella fijiensis* Morelet (Churchill 2011). The management of BSD is mostly achieved by aerial spraying of synthetic fungicides every week or even twice a week in some regions, depending on favorable climatic conditions for disease development (Henriques et al. 1997; Orozco-Santos et al. 2008). BSD has an important economic impact on banana growers' budgets, since the disease may represent between 30 and 45% of banana total production costs (Marín et al. 2003; Orozco-Santos et al. 2001). Additionally, BSD chemical management has a negative impact on the environment because it pollutes natural resources (Geissen et al. 2010) and potentially has adverse effects on human health (Calviello et al. 2006; Mehrotra et al. 1987).

Biological control, especially using chitinolytic microorganisms or their metabolites (Swiontek et al. 2014), is an alternative to the current BSD chemical-based management that may reduce, substitute for or complement the conventional disease prevention programs. The BSD biocontrol literature is still limited, but there are some successful reports on isolating and *in vitro* or in field testing of the antagonistic effects of chitinolytic bacteria or their metabolites on *M. fijiensis* (González et al. 1996a, b; Ceballos et al. 2012; Gutiérrez-Román et al. 2015; Gutierrez-Monsalve et al. 2015).

Along with its other important benefits, BSD biological control also offers low risk of resistance development due to its “multisite” mode of action on phytopathogen metabolism (van Lenteren 2012), unlike the “monosite” mode of action of chemical fungicides (Romero and Sutton 1997, 1998; Vincelli 2002). Antagonistic microorganisms can produce 1) antibiotics, 2) chitin-synthase inhibitors like polyoxins and nikkomycins, 3) siderophores

that limit access to iron, and 4) mycolytic enzymes like chitinases, glucanases, proteases and lipases (Chater et al. 2010; Macagnan et al. 2008; Nagpure et al. 2014). Although biological control has been stated to be a safe approach since its implementation has low or no impact on non-target organisms (Suckling and Sforza 2014; Winding et al. 2004), it is necessary to clarify this assumption when the introduction of a new natural enemy in a new environment is planned (van Elsas and Migheli 1999; Follett and Duan 2000). Assessing non-target impacts on crop microbial communities is essential because these organisms provide multiple benefits to plants including protection (endophytes, plant resistance inducers and pathogen antagonists), nutrient intake (mycorrhizas, N fixers, rhizosphere residents, nutrient-cycles key players), growth (plant growth promoters, saprophytes) and soil contaminant removal or breakdown (van der Heijden et al. 2008).

We previously demonstrated *in vitro* high antifungal effects of a spore suspension and culture supernatant of *Streptomyces galilaeus* CFFSUR-B12 on *M. fijiensis* ascospore germination, germ tube elongation and mycelial growth (Moreno-Castillo et al. 2016). The present work was done with the aim of determining if CFFSUR-B12 spores and supernatant are effective as biocontrol agents of BSD under field conditions and how they affect non-target microorganisms. First, we characterized the chitinolytic activities and stability of the culture supernatant at different temperature and pH ranges. Next, we sprayed BSD naturally infected and recently-flowered banana plants with CFFSUR-B12 (spores and supernatant) in the field and evaluated non-target harmful effects on banana-associated microbial communities after exposure to the biocontrol agents.

Materials and Methods

Microorganism

Streptomyces galilaeus CFFSUR-B12 (henceforth referred as CFFSUR-B12) was isolated as mycoparasite on *Colletotrichum gloeosporioides*, causal agent of anthracnose in cocoa cobs (*Theobroma cacao* L.).

Treatment preparations

The spore suspension was prepared from CFFSUR-B12 grown on Nutrient Agar at 26°C for 72 h, or until abundant and homogenous white sporulation on the surface of colonies was observed. Spores were scrapped off, suspended in sterile-distilled water and adjusted to a final concentration of $1x10^9$ c.f.u. ml^{-1} before spraying onto the experimental plants.

To prepare the crude culture supernatant, one ml of a CFFSUR-B12 spore suspension ($1.3x10^{12}$ c.f.u. ml^{-1}) was inoculated per liter of minimal medium M9 (Quecine et al. 2008) supplemented with 1 g l^{-1} of yeast extract, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$ and 1% colloidal chitin from crab shells (Sigma-Aldrich Co. LLC) as major carbon and nitrogen source (Gómez-Ramírez et al. 2004). Cultures were incubated for five days in darkness at 29°C and 140 rev. min^{-1}. Afterwards, CFFSUR-B12 spores and mycelia were freeze-killed at -70°C for 24 h, removed by filtration through three layers of cheesecloth and then vacuum-filtered through nitrocellulose membranes (0.45 µm). Freshly prepared crude culture supernatant and spore suspension were employed for each spraying date in the field experiments.

Culture supernatant chitinase activity and chitinase stability at different pHs and temperatures

Since field conditions may affect culture supernatant enzymes activity and stability as a function of temperature and pH, we assessed chitinase activity at different temperatures and

121 pH values essentially as described by Park et al. (2000). Briefly, 0.5 ml reactions containing
122 300 µl of 0.1 M citrate-phosphate (pH 4 and 7) or Tris-HCl (pH 9) buffer, 100 µl of 10 mM
123 4 nitrophenyl *N*-acetyl-β-D- glucosaminide (PNG) (Sigma Chemical, St. Louis, MO, USA)
124 as substrate and 100 µl of crude culture supernatant as chitinase enzyme source were
125 incubated at 4, 10, 20, 29 and 37°C for 12 h. Reactions were stopped by adding 0.5 ml of
126 1M Na_2CO_3 and coloration was read at 405 nm. Assays at temperatures of 50°C and above
127 were not possible due to termodegradation of the substrate.
128 To assess stability of chitinases at pH values ranging from 4 to 9, 100 µl of crude culture
129 supernatants were incubated for 12 h at room temperature in 300 µl of buffers with
130 different pH values (4-7: citrate-phosphate; 8 and 9: Tris-HCl), and then assayed for
131 chitinolytic activity by adding 100 µl of the chromogenic substrate under standard
132 conditions (37°C and 12 h of incubation). Termostability was determined by pre incubation
133 of enzyme source alone from 4 to 70°C for 12 h and then assaying under standard
134 conditions. All assays were done in triplicate.
135 **Field assay**
136 The field trial was conducted in a 10 year-old commercial banana plantation (*Musa* sp.
137 AAA, clone "Francés") located in the "Finca El Carmen" (from Grupo Coliman S.A. de
138 C.V.) in the municipality of Mazatán, Chiapas, Mexico (14°54'44.7" N, 92°24'57.2" W, 18
139 m.a.s.l.). The experimental period was from January 22nd to April 7th, 2015 and no rains
140 were recorded during the course of the experiment. The experimental site was under
141 conventional crop management with the exception of fungicides sprayings.
142 Experimental plots were clusters of ca. 15 m diameter, and five recently-flowered plants
143 were chosen as experimental units. Treatments were terrestrially sprayed with a 15 l
144 motorized sprayer (Stihl®) and calibrated to 250 l ha^{-1}. A total of five sprayings were done

every 15 d and treatments evaluated in triplicate were: 1) CFFSUR-B12 spore suspension ($1x10^9$ c.f.u. ml^{-1}), 2) CFFSUR-B12 crude culture supernatant, 3) 0.1 % Dithane® 600 (mancozeb as active ingredient, 2.5 l ha^{-1}) (Dow AgroSciences), and 4) water (negative control). Disease severity was measured by the Stover's scale as modified by Gauhl, where grade 0= no BSD symptoms, grade 1= less than 10 spots per leaf, grade 2= up to 5% of the leaf area with necrosis, grade 3= necrotic tissue between 6% and 15%, grade 4= necrosis from 16% to 33%, grade 5= necrotic tissue from 34% to 50%, and grade 6= more than 50% of leaf area with necrotic tissue. The youngest leaves spotted (YLS) or in grade 1 (no more than ten BSD spots) and total number of leaves per plant (*N*) were determined. The BSD Infection Index (II) was also calculated according to the following formula (Carlier et al. 2002):

$$II\ (\%) = (\sum an/6N) \times 100 \quad (1)$$

Where *a* is the corresponding grade value in the scale (0 to 6), *n* corresponds to the number of leaves in each grade and *N* the total number of leaves per plant sampled.

To calculate the following BSD epidemiological parameters we considered two sampling intervals: the first was from 14 to 42 d and the second from 42 to 75 d after initial treatment sprayings:

The disease increase rate or apparent infection rate "r" of both intervals was calculated according to Van der Plank's logistic equation (1963):

$$r = [\ 1/t_2\text{-}t_1\ ]\ [\ \ln(X_2/1\text{-}X_2) - \ln(X_1/1\text{-}X_1)\ ] \quad (2)$$

Where t_1 and t_2 represent days after initial spraying (lower and upper limits on each interval, respectively), X_1 and X_2 are cumulative disease proportion at t_1 and t_2 while ln is the natural logarithm.

168 The area under disease progress curve (AUDPC) was computed by the trapezoidal
169 integration method using the following formula (Achicanoy 2000; Mohapatra et al. 2008):
170 $AUDPC = \sum [(II_1 + II_2)/2] [t_1 + t_2]$ (3)
171 Where II_1 and II_2 are the cumulative BSD infection indices at t_1 and t_2, respectively, being
172 t_1 and t_2 days after initial treatment spraying (lower and upper limits on each sampling
173 interval, respectively).
174 Finally, we calculated the factor Δt for each sampling interval with the following formula
175 (Whitney 1976):
176 $\Delta t = (1/r) \ln (x_0/x_{0S})$ (4)
177 Where Δt is the disease progress delay (in days) of one treatment (more effective)
178 compared to the other (less effective or control), r is the apparent infection rate at each
179 sampling interval previously calculated by Van der Plank's formula (1963), ln is the natural
180 logarithm, x_0 and x_{0S} are the disease proportions of the less and more effective treatments,
181 respectively.
182 **Effects on root and leaf cultivable associated bacteria**
183 To assess the effect of CFFSUR-B12 spore suspension and culture supernatant sprayings
184 on cultivable banana rhizosphere and phylloplane associated bacteria the same
185 experimental units from the field assay (previously described) were sampled from January
186 22nd to March 5th prior to each treatment spraying. Samples of ca. 50 g of mature leaves
187 (leaf 5 or 6) and ca. 50 g of rhizospheric soil were collected from each of the five plants on
188 each repetition. In the laboratory, these materials were homogenized and composite
189 representative subsamples of 5 g of leaf tissue and 2.5 g of rhizospheric soil were collected
190 per repetition. Each leaf or soil subsample was diluted in sterile extraction buffer [0.1 M
191 sodium phosphate buffer, pH 7 containing 0.1% (v/v) Tween 80] in a ratio 1:10 (grams of

192 sample: milliliters of buffer), then incubated for 1 h at 20°C and 150 rev. min^{-1} (Ceballos et
193 al. 2012). These solutions were serially diluted and 20 µl aliquots were spread in duplicate
194 onto nutrient agar plates (pH 7), then incubated for 24-48 h at 26°C. Plates with 30-300
195 colonies were selected and colonies of each colonial morphology group were counted.
196 Shannon-Wiener (H') were calculated and analyzed for each treatment sample (Moreno
197 2001).

198 **Effects on non-cultivable rhizosphere microbiota by PCR-DGGE**

199 *DNA extraction*. To assess non-target effects on non-cultivable rhizosphere fungi, bacteria
200 and actinomycete communities, DNA extractions from banana soil rhizosphere were done
201 in duplicate on January 22nd (initial sample before treatment spraying) and then after one
202 (February 5th) and three consecutive treatment sprayings (March 5th, 2015). DNA was
203 extracted with the DNA Powersoil™ extraction kit (MoBio, Carlsbad, CA, US) following
204 the manufacturer's instructions.

205 *PCR conditions*. Soil metagenomic DNA extractions were used as templates to amplify the
206 16S rDNA fragments of bacteria and actinomycetes, and a portion of the 18S rDNA for
207 fungi. Amplifications were done in a Mastercycler thermal cycler (Eppendorf) with the
208 primers (from Macrogen) and temperature conditions used by Guillén-Navarro et al. (2015)
209 and Das et al. (2007) with slight modifications in annealing temperature and primer
210 extension times (Table 1). Each 25 µl-reaction mixture consisted of 12.5 µl of 2X
211 MasterMix (Promega), 25 pmol of each forward and reverse primer, 5.5 µl of nuclease-free
212 water and ca. 2 ng of template DNA. Amplicons were enriched three times. For
213 actinomycetes, a nested PCR was done: first, 2 µl of amplicons from primers F243-R1378
214 were used as template for a second amplification using the primer pairs F984GC-R1378
215 (Heuer et al. 1997).

Denaturing Gradient Gel Electrophoresis (DGGE). Ten µl of enriched amplicons from each group (370 bp for fungi and 433 bp for bacteria and actinomycetes) were loaded on 10% polyacrylamide gels (w/v) in 1X TAE buffer and subjected to DGGE on a DCode™ mutation detection system (Biorad) under a 20-60% denaturing gradient of formamide and urea. Amplicons were electrophoresed at a constant voltage of 90 V at 60°C for 8 h in 1X TAE running buffer. Gels were stained with 1X SYBR Green o SYBR Gold (Invitrogen) diluted in running buffer and photographed under UV light. Images were inspected visually and ribotype profiles obtained from treatments on each sample date from two replicates were compared. Ribotypes were progressively numbered from gel top to bottom and bands with similar migration distance were considered as corresponding ribotypes between treatments and control. Presence or absence of bands were labeled as 1 or 0, respectively, and binary matrices were constructed from ribotypes on each microbial group (Fromin et al. 2002; Liu et al. 2007).

Statistical analysis

Data from chitinolytic activity and stability of CFFSUR-B12 culture supernatant were analyzed by one-way ANOVA (α=0.05). Time-course evolution of BSD infection indices (II), youngest leaves spotted (YLS), total leaves per plant (*N*) and Shannon-Wiener diversity indices were subjected to ANOVA in repeated measures (α=0.05) under a mixed model where fixed effects were treatments and random effects were experimental sites. Additionally, to compare between the two BSD epidemiological intervals, the area under disease progress curve (AUDPC) and apparent infection rate (r) were subjected to ANOVA (α=0.05) under a tri-factorial design where factors were treatments (4), sites (3) and epidemic stages or intervals (2). Tukey's means comparisons (α=0.05) were done in cases of ANOVA statistical significance. Matrices from presence/absence DGGE ribotypes on

each microbial group were subjected to cluster analysis and dendrograms were constructed to associate similar groups of ribotypes by calculation of Jaccard's similarity indices and Ward's distance. All analyses were performed using the software R© (Ver. 2.14.0, The R Foundation for Statistical Computing).

Results

Culture supernatant chitinase activity and stability as a function of variable pH and temperature

After reaction of CFFSUR-B12 culture supernatant with the chromogenic substrate PNG at different combinations of pH (4, 7 and 9) and temperature (4, 10, 20, 29 and 37°C), chitinase activity increased to widely differing extents with increasing temperature from 4 to 37ºC at all of the reaction pHs tested (Fig. 1). Highest chitinase activities were at combination of 29 and 37°C with pH 4 and 9, while at pH 7 activity was significantly lower (Fig. 1). After 12 h of incubation at different temperatures and pH values, chitinase residual activity from culture supernatant was maintained high and constant in the pH range of 4 to 8 but decreased 48% at pH 9, the most alkaline pH tested. Chitinases were termostable in the range of 4-37°C, but residual activity gradually decreased at higher temperatures until total inactivation occurred at 60°C (Fig. 2).

Effectiveness of CFFSUR-B12 spore suspension and culture supernatant on BSD severity

The repeated measures analysis revealed that 15 day-interval sprayings of CFFSUR-B12 spore suspension on recently flowered banana plants (clone “Francés”), reduced BSD severity indices (II) over time as compared to mancozeb and water-treated plants, but was similar to CFFSUR-B12 culture supernatant treated-plants (MS_{error}=1.02, d.f.=56, α=0.05).

No differences between treatments were found in repeated measures analysis of either total leaves per plant ($F_{3, 56}$=1.01, *P*=0.39) or BSD youngest leaves spotted with grade 1 in Stover's scale as modified by Gauhl ($F_{3, 56}$=0.77, *P*=0.52) (Table 2).

Cumulative BSD Infection Indices (II) observed in experimental plants after treatment with CFFSUR-B12 spore suspension and culture supernatant, clearly showed two stages of epidemiological growth (Fig. 3): the first stage had a BSD infection rate "r" of 0.029 that was significantly higher than the second stage infection rate of 0.019 (MS_{error}=0.00004, d.f.=96, α=0.05). Nevertheless, all treatments presented similar BSD infection rates in both stages ($F_{3, 96}$=1.17, *P*=0.32). Moreover, the overall area under disease progress curve (AUDPC) of 1652.92 from the second stage was significantly higher than the area of 707.82 from first epidemiological stage (MS_{error}=111671.0, d.f.=96, α=0.05). CFFSUR-B12 spore-treated banana plants showed the lowest AUDPC value (965.17), which was similar to the area from culture supernatant (1109.93) but statistically different from mancozeb (1355.33) and water-treated plants (1291.04) (MS_{error}=111671.0, d.f.=96, α=0.05) (Fig. 4). We also found that CFFSUR-B12 spores elicited a delay in BSD disease progress (Δt) of 15 and 19 d, while culture supernatant caused a delay of 9 and 12 d in the first and second stage, respectively, when compared to mancozeb. Similar BSD delays were obtained when biocontrol agents were compared to water-treated plants (control). Comparisons between CFFSUR-B12 biocontrol agents showed that spore suspension caused a BSD delay of 7 and 8 d at first and second epidemiological stages, respectively, in comparison to culture supernatant.

Effects of biocontrol agents on diversity of banana root and leaf associated bacteria

Shannon-Wiener (H') indices subjected to repeated measures analysis showed that sprayings of CFFSUR-B12 spores and culture supernatant on banana plants did not affect

culturable bacterial diversity from the rhizosphere ($F_{3, 8}$=0.29, P=0.83) or phylloplane ($F_{3, 8}$=0.22, P=0.88). Nevertheless, diversity indices changed over time in both rhizosphere ($F_{3, 33}$=18.56, P<0.05) and leaf ($F_{3, 33}$=4.51, P<0.05) (Table 3).

Any of the treatments sprayed in this assay substantially changed diversity of non-culturable fungal, bacterial and actinomycete communities, represented as DGGE ribotypes profiles (Fig. 5).

Ribotype dendrograms (not shown) constructed by Jaccard's similitude indices and Ward's distance grouping method showed that 88% of fungal ribotypes (34) were unaffected after treatment sprayings. Only one ribotype was not detected after the third spraying of CFFSUR-B12 spores, while two ribotypes were lost and one was added after the first and third culture supernatant sprayings, respectively. In the case of mancozeb and water treatments (control), one fungal ribotype was lost but another appeared after three sprayings (Fig. 5a).

Eighty-nine % of bacterial ribotypes (31) were unaltered after all treatment sprayings. Three additions, detected at the end of the assay, were observed after one CFFSUR-B12 spore sprayings. After three mancozeb sprayings two bacterial ribotypes disappeared (Fig. 5b).

Finally, 88% of actinomycetal ribotypes (34) remained unaffected after treatment sprayings. After the first spraying of CFFSUR-B12 spores, four bands were lost but a new ribotype appeared. Also, after the first culture supernatant and mancozeb sprayings, three new ribotypes were detected and one was lost. In control band profiles, there were two actinomycetal ribotype additions after the first spraying (Fig. 5c).

Discussion

In this research we found that *Streptomyces galilaeus* CFFSUR-B12 spore suspensions sprayed on recently-flowered banana plants (clone "Francés") were highly effective in reducing Black Sigatoka Disease (BSD) infection indices (II), in decreasing the area under disease progress curve (AUDPC) and in delaying BSD progress (Δt) up to 19 days with respect to mancozeb and water (control) treatments under field conditions. Moreover, the microbial biocontrol agent showed no detrimental effects on the diversity of banana rhizosphere and leaf associated microbial communities.

The overall BSD epidemiological progress observed on experimental plants during field assays described a logistic growth, where the availability of healthy and new leaf tissue to infect on recently-flowered banana plants affected the epidemic course and caused a significant decrease in infection rate (r) during second BSD epidemiological stage (Van der Plank 1963; Whitney 1976; Zadoks and Schein 1979).

The lack of control of *M. fijiensis* on mancozeb-treated plants, which were similar to controls, was shown by these plants having the highest II and AUDPC values. This leads us to hypothesize that continuous spraying of this fungicide on banana plantations has induced some insensitivity and likely some degree of resistance development. Although mancozeb is known to have a multisite mode of action against fungi and a low probability to provoke resistance, unlike monosite-action fungicides (Romero and Sutton 1997, 1998), some degree of insensitivity to mancozeb has been observed in *M. fijiensis* isolates from Southern Mexico and Brazil (Aguilar-Barragan et al. 2014; Gomes et al. 2014).

In contrast, CFFSUR-B12 biocontrol agents (spores and supernatant) effectively reduced BSD inoculum density when sprayed on banana plants. It has been stated that mycoparasite biocontrol agents, such as strain CFFSUR-B12, act directly against inoculum disease

(Kranz 2003). It is likely that the intimate contact between biocontrol agents and *M. fijiensis* spores on the banana phylloplane inhibited ascospore germination and/or germ tube elongation, as we previously observed *in vitro* during dual confrontation assays (Moreno-Castillo et al. 2016). Apparently, bacterial spore suspensions could not stop already- established BSD infections, since we have observed in microcosm assays that CFFSUR-B12 does not become established as a foliar endophyte on banana plants.

When we compared CFFSUR-B12 spore suspension and culture supernatant with the effect of mancozeb on BSD progress, we found that spores delayed up to 19 days while supernatant delayed up to 12 days. This a favorable trait of the biocontrol agents because they may substitute, reduce or complement mancozeb sprayings on comercial banana plantations (Swiontek et al. 2014). Reduction of only one mancozeb spraying could represent savings of about 211 000 USD for banana growers by eliminating the application of about 30 000 l of Dithane® 600 (18 t of active ingredient) on 12 000 ha grown with banana in the Soconusco region of Chiapas, Mexico.

Likewise, CFFSUR-B12 spore-treated plants were harvested with 8.4 leaves per plant and with grade one of BSD on leaf number 5.93, parameters which reach the quality standards requested by international trading and export companies. Higher numbers of total and healthy leaves per plant at harvest helps to avoid premature ripening during transport and extends banana shelf life (Stover 1972).

The strain CFFSUR-B12 is a potential biocontrol agent because, in addition to producing highly active and stable extracellular chitinases, the actinomycete also produces antifungal metabolites and other lytic enzymes that acted together to reduce BSD severity on treated banana plants, as previously demonstrated during *in vitro* assays against *M. fijiensis* (Moreno-Castillo et al. 2016). Members of *Streptomyces* are widely recognized for

360 producing about seven thousand secondary metabolites that may inhibit or alter
361 physiological processes in fungi and other microorganisms (Chater et al. 2010), besides
362 producing spores that may allow them to survive for longer under adverse phylloplane
363 conditions (Andrews 1992).
364 Research on biocontrol of BSD by the use of chitinolytic bacteria under field conditions is
365 scarce. González et al. (1996a, b) isolated and evaluated strains A23 and R1 of *Serratia*
366 *marcescens*, *S. entomophyla* A100 and *Bacillus cereus* A30. Gutierrez-Monsalve et al.
367 (2015) tested a microbial fungicide based on spores and extracellular metabolites produced
368 by *Bacillus subtilis* EACB0015 and Fu et al. (2010) evaluated strain B106 of *Bacillus*
369 *subtilis* for biocontrol of Yellow Sigatoka disease. To our best knowledge, our work is the
370 first report of a *Streptomyces* strain as an effective biocontrol agent of BSD under field
371 conditions.
372 We found no deleterious effect of CFFSUR-B12 spores and culture supernatant on the
373 diversity of banana leaf and rhizosphere associated microbial communities, since ribotype
374 fluctuation (additions and losses) before and after spraying of treatments was attributable to
375 microbial interactions, irrigation or fertilization, among others, but not to an actual effect
376 from treatments sprayed on banana plants. These findings allow us to ensure that CFFSUR-
377 B12 biocontrol agents are harmless and innocuous to banana microbial populations. This is
378 supported by literature showing that biocontrol agents used for management of
379 phytopathogens are known to have low non-target or environmental effects (Follett and
380 Duan 2000; Suckling and Sforza 2014). Root dip-inoculation of strawberry plants with
381 spore suspensions of *Serratia phymuthica* HRO-C48 or *Streptomyces* sp. HRO-71 caused
382 only minor and transient effects on microbioal rhizospheric communities (Scherwinski et
383 al. 2007). Nevertheless, site-specific research is required before a biocontrol agent is

384 introduced in a new environment or pathosystem (Thomas and Willis 1998; Trabelsi and
385 Mhamdi 2013). Lack of noticeable effects of treatments (including mancozeb) on banana-
386 associated microbiota was probably due to a low deposition of treatments in the
387 rhizosphere, since most of the sprayed volume was retained in banana plants foliage during
388 spraying. Nevertheless, we either observed detrimental effects on culturable bacterial
389 communities associated to banana phylloplane, which suggests a high adaptation to inhabit
390 this environment. Deleterious effects of fungicides (including mancozeb) and insecticides
391 on soil microbiota community structure have been demonstrated, but pesticide applications
392 were made directly to the soil (Ferreira et al. 2009; Imfeld and Vuilleumier 2012; Sigler
393 and Turco 2002).
394 Future research must now be directed to corroborate CFFSUR-B12 as a biocontrol agent of
395 the BSD on a wider-area-scale, as well as designing a protocol for the semi-industrial
396 production of spore suspension and formulation tests.
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Table 1. Primers and temperature conditions used for amplification of sub-regions of 16S rDNA (from bacteria and actinomycetes) and 18S rDNA (from fungi).

Group	Primer	Sequence (5'-3')	Temperature conditions [a]
Fungi	NS1	GTAGTCATATGCTTGTCTC	35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s
	GCFung	CGCCCGCCGCGCCCCGCGCC CGGCCCGCCGCCCCCGCCCC ATTCCCCGTTACCCGTTG	
Bacteria	F984GC	CGCCCGGGGCGCGCCCCGGG CGGGGCGGGGGCACGGGGG GAACGCGAAGAACCTTAC	30 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 60 s
	R1378	CGGTGTGTACAAGGCCCGGG AACG	
Actinomycetes	1st step		
	F243	GGATGAGCCCGCGGCCTA	30 cycles of 95°C for 60 s, 63°C for 60 s, and 72°C for 2 min
	R1378	CGGTGTGTACAAGGCCCGGG AACG	
	2nd step		
	F984GC	CGCCCGGGGCGCGCCCCGGG CGGGGCGGGGGCACGGGGG GAACGCGAAGAACCTTAC	30 cycles of 95°C for 60 s, 53°C for 60 s, and 72°C for 60 s
	R1378	CGGTGTGTACAAGGCCCGGG AACG	

[a] All temperature programs included an activation step at 94°C for 5 min and a final primer extension of 72°C for 5 min.

586 **Table 2**. Mean (± standard error) Infection Indices (II) of Black Sigatoka Disease (BSD),
587 total numbers of leaves per plant (*N*) and youngest leaves spotted with BSD (YLS) of
588 recently-flowered banana treated plants at initial spraying (Jan 24th, 2015) and conclusion
589 of field assay (April 9th, 2015). CFFSUR-B12 and CCS are spore suspension ($1x10^9$ c.f.u.
590 ml^{-1}) and culture supernatant of strain CFFSUR-B12, respectively. Dithane® 600 is
591 mancozeb-based fungicide and controls were water-treated plants. Same letters within
592 columns indicate non-significant statistical difference between treatments (P>0.05).

Treatment	II		*N*		YLS	
	initial	final	initial	final	initial	final
CFFSUR-B12	6.44 ± 0.79a	14.28 ± 1.78a	11.73 ± 0.30a	8.40 ± 0.54a	9.93 ± 0.36a	5.93 ± 0.46a
CCS	7.66 ± 0.87a	15.97 ± 1.16ab	11.73 ± 0.25a	7.07 ± 0.54a	9.67 ± 0.29a	4.93 ± 0.54a
Dithane® 600	7.16 ± 0.81a	17.05 ± 1.08b	13.20 ± 0.31a	7.67 ± 0.50a	10.53 ± 0.32a	4.67 ± 0.43a
Control	6.85 ± 0.71a	17.89 ± 1.92b	12.00 ± 0.35a	7.67 ± 0.48a	9.67 ± 0.23a	4.80 ± 0.35a

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Table 3. Mean (± standard error) Shannon-Wiener (H') indices of culturable banana rhizosphere and leaf associated bacteria at different days (d) after initial spraying of treatments. CFFSUR-B12 and CCS are spore suspension ($1x10^9$ c.f.u. ml^{-1}) and culture supernatant of strain CFFSUR-B12, respectively. Dithane® 600 is mancozeb-based fungicide. Within columns and plant part, same letters indicate non-significant statistical difference on time-course evolution of H' (P>0.05).

Plant part	Treatment	Shannon-Wiener (H')			
		Initial	14 d	28 d	42 d
Leaf	CFFSUR-B12	1.12 ± 0.09a	0.75 ± 0.17a	1.32 ± 0.19a	1.11 ± 0.04a
	CCS	0.79 ± 0.14a	0.88 ± 0.20a	1.32 ± 0.12a	1.18 ± 0.18a
	Dithane® 600	0.99 ± 0.22a	0.78 ± 0.16a	0.96 ± 0.18a	1.18 ± 0.18a
	Control	0.66 ± 0.17a	1.10 ± 0.16a	1.26 ± 0.11a	1.10 ± 0.15a
Rhizosphere	CFFSUR-B12	0.79 ± 0.15a	0.83 ± 0.10a	1.04 ± 0.09a	1.60 ± 0.13a
	CCS	0.90 ± 0.26a	0.74 ± 0.17a	1.23 ± 0.08a	1.47 ± 0.14a
	Dithane® 600	0.78 ± 0.13a	0.76 ± 0.11a	1.28 ± 0.15a	1.28 ± 0.22a
	Control	0.75 ± 0.12a	0.70 ± 0.26a	0.64 ± 0.30a	1.71 ± 0.07a

Figure captions

Fig. 1 Chitinolytic activities of CFFSUR-B12 crude culture supernatant incubated at different temperature and pH values. Bars are mean absorbance at 405 nm (± standard error) for triplicate reactions. Within temperatures, bars with the same letters are statistically similar (Tukey α=0.05)

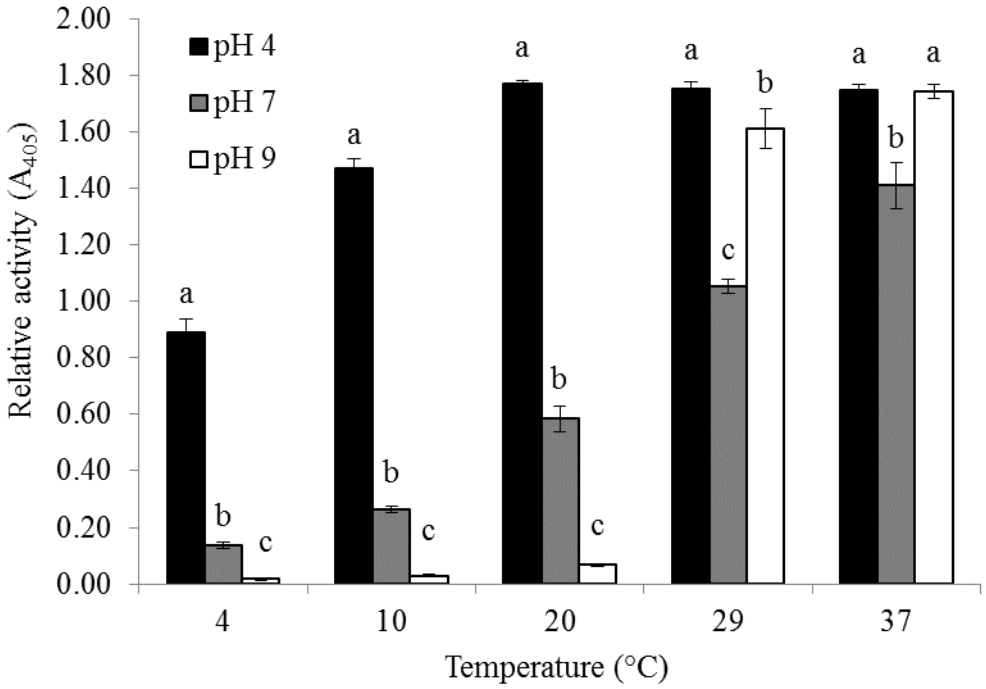
Fig. 2 Residual chitinase activity after incubation of CFFSUR-B12 crude culture supernatant at different temperature and pH levels. Points on each curve are mean absorbance at 405 nm (± standard error) for triplicate reactions. Within each curve, points with the same letters are statistically similar (Tukey α=0.05)

Fig. 3 Time-course evolution of cumulative Black Sigatoka Disease (BSD) infection indices (II) on recently-flowered plants treated under field conditions, from January 24th to April 9th, 2015. Vertical dashed line separates both BSD epidemiological stages and their apparent infection rates (r) are given. CFFSUR-B12 denotes a spore suspension ($1x10^9$ c.f.u. ml^{-1}) and CCS is culture supernatant of strain CFFSUR-B12. Dithane® 600 is fungicide based on mancozeb and control were water-treated plants.

Fig. 4 Area under the disease progress curve (AUDPC) (± standard error) of the Black Sigatoka Disease infection indices of recently-flowered plants treated under field conditions. CFFSUR-B12 and CCS denote a spore suspension ($1x10^9$ c.f.u. ml^{-1}) and culture supernatant of strain CFFSUR-B12, respectively. Dithane® 600 is fungicide based on mancozeb and control were water-treated plants. Bars with same letters are statistically similar (Tukey α=0.05)

Fig. 5 DGGE ribotype profiles from a) fungal, b) bacterial and c) actinomycete communities resident on rhizosphere of recently-flowered banana plants treated before (lanes i) and after one (lanes 1) or three (lanes 3) sprayings of treatments. Upper and

bottom panels correspond to profiles from two repetitions. B12 denotes a spore suspension ($1x10^9$ c.f.u. ml^{-1}) and CCS is the culture supernatant of strain CFFSUR-B12, respectively. D600 is the mancozeb-based fungicide Dithane® 600 and C belongs to water-treated control plants. Solid and dashed squares indicate ribotypes added and lost, respectively.


pH 4
pH 7
pH 9
Relative activity (A_{405})
2.00
1.80
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1.40
1.20
1.00
0.80
0.60
0.40
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0.00
a
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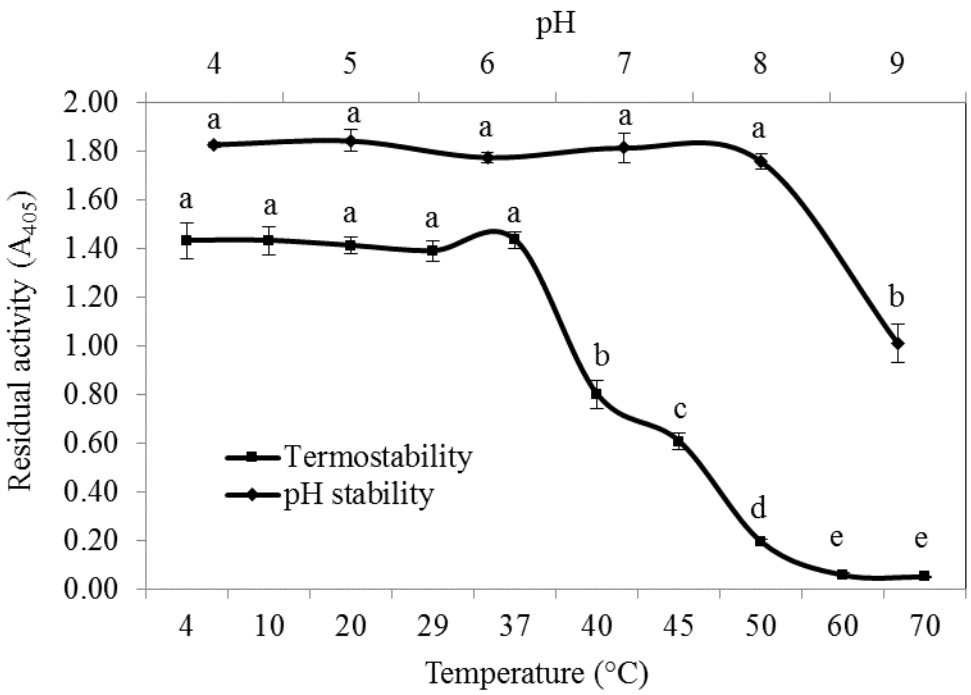
Temperature (°C)

Fig. 1


pH
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Residual activity (A_{405})
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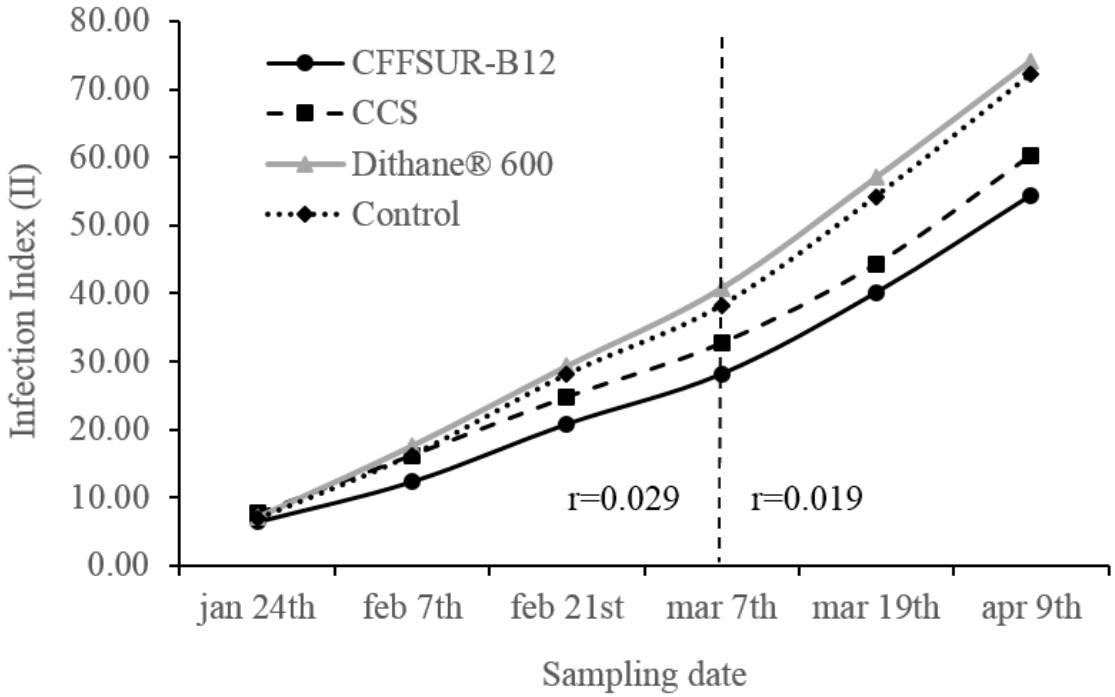
Temperature (°C)

Fig. 2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Infection Index (II)
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CCS
Dithane® 600
Control
r=0.029
r=0.019
jan 24th
feb 7th
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Fig. 3

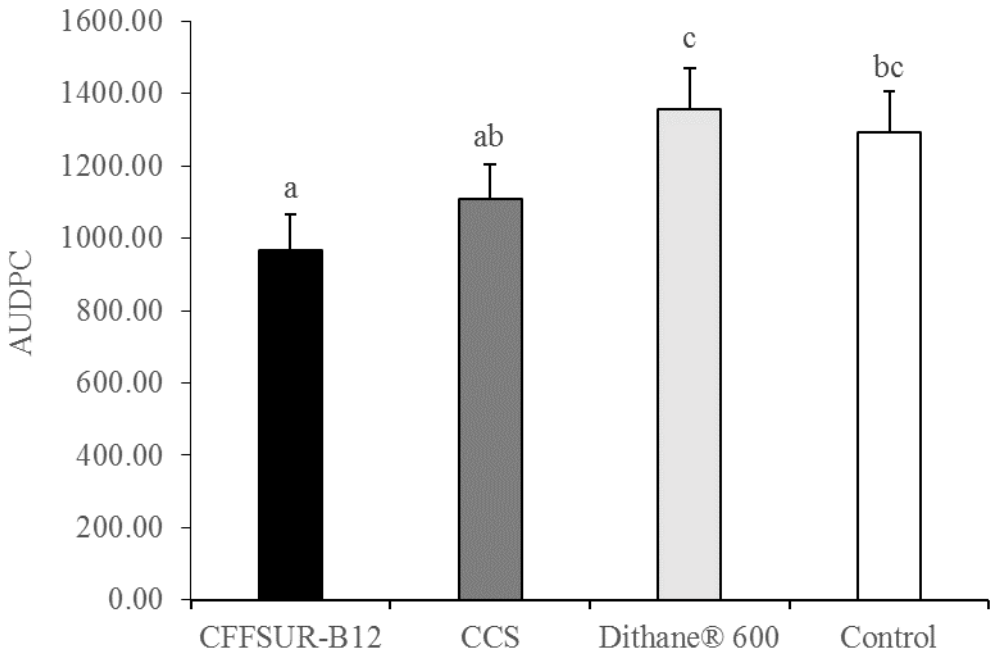
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Fig. 4

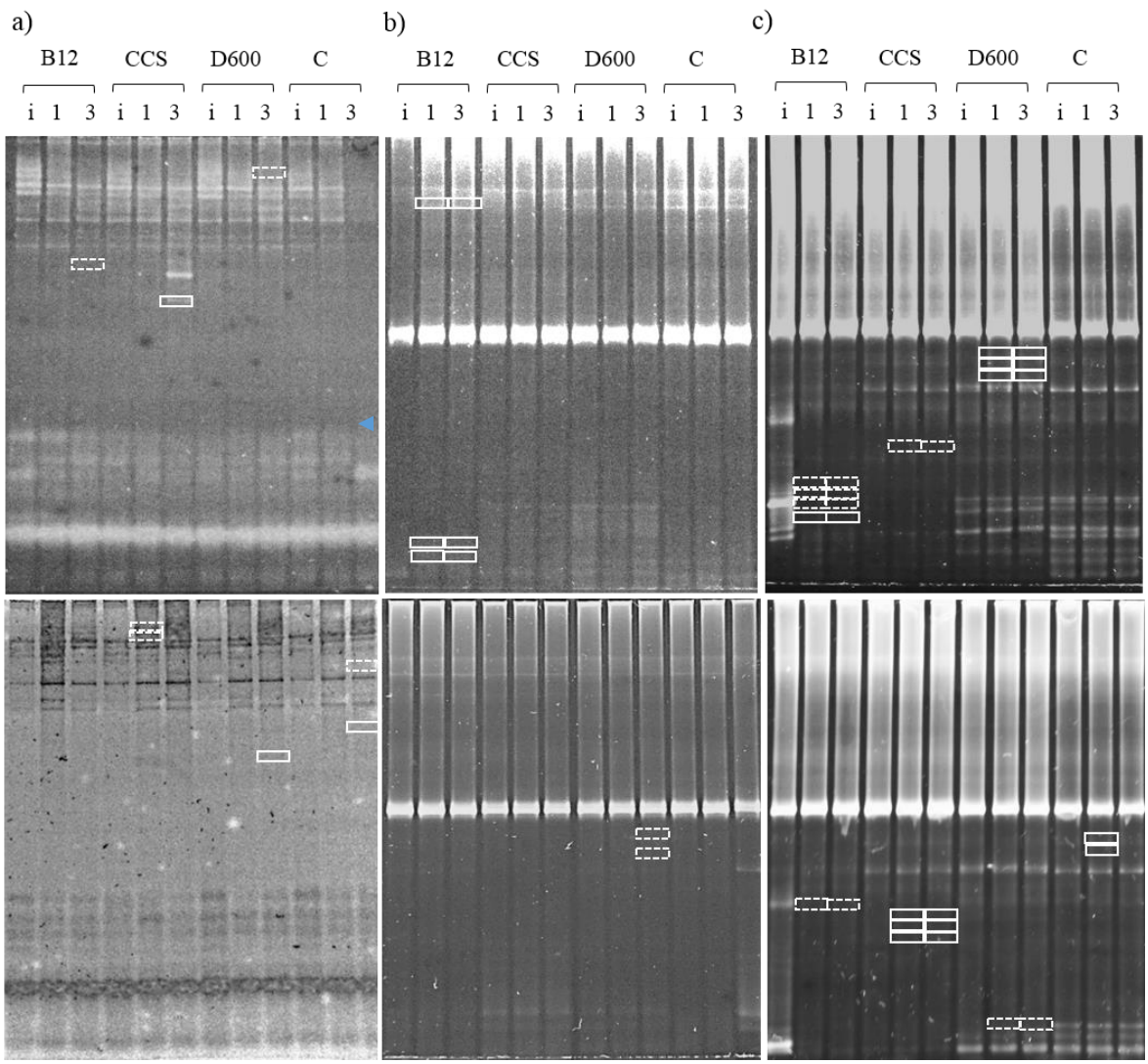

Fig. 5

Acknowledgements

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6.3 Interacción *St. galilaeus* CFFSUR-B12-plantas de banano

Después de inocular CFFSUR-B12 en las hojas de las plantas de banano, no se detectaron diferencias estadísticas significativas entre los tratamientos y el testigo en cuanto a las variables altura de planta ($F_{2,27}$=0.01, *P*=0.98), grosor de tallo ($F_{2,27}$=1.49, *P*=0.24) y número de hojas por planta ($F_{2,27}$=1.04, *P*=0.36), así como tampoco en la tasa de crecimiento ($F_{2,27}$=1.92, *P*=0.16) y de emisión foliar ($F_{2,27}$=0.70, *P*=0.50). Asimismo, después de la inoculación del actinomiceto en las raíces de las plantas, tampoco hubieron diferencias estadísticas en cuanto a la altura de planta ($F_{2,33}$=0.50, *P*=0.61), número de hojas por planta ($F_{2,33}$=0.51, *P*=0.60) y en las tasas de crecimiento ($F_{2,33}$=0.12, *P*=0.88) y de emisión foliar ($F_{2,33}$=1.07, *P*=0.35) (Figura 4).

En los ensayos *in vitro* tampoco hubieron diferencias estadísticas con respecto al testigo en la tasa de incremento en biomasa vegetal ($F_{2,27}$=1.63, *P*=0.21), después de inocular CFFSUR-B12 en hojas o raíces de las vitroplantas de banano (Figura 5).

En el ensayo colorimétrico no fue detectada la presencia de AIA en el medio de cultivo suplementado con triptófano e inoculado con el actinomiceto (Figura 6).

En cuanto al establecimiento como endófitos, CFFSUR-B12 se estableció en muy baja proporción tanto en raíces (3.3 %, Figura 7) como en hojas (160 u.f.c. g^{-1} de tejido), lo cual solamente ocurrió a los 56 días después de la inoculación (ddi). Por otro lado, CFFSUR-B12 permaneció vivo en la rizósfera desde 36 hasta 84 ddi, en niveles de 1-5x10^5 y 9x10^4 u.f.c. g^{-1} de suelo, respectivamente. Posteriormente, solo se detectó a 123 ddi en valores cercanos a 10 u.f.c. g^{-1} de suelo (Figura 8).

Por otra parte, *S. marcescens* CFFSUR-B2 se estableció como endófito radicular en mayor proporción a los 56 ddi (10-30 % de secciones colonizadas), mientras que a 36, 123 y 140 ddi solamente se detectó un establecimiento del 3.3 % (Figura 7). Asimismo, su sobrevivencia en la rizósfera fue únicamente hasta los 36 ddi (Figura 8). Como endófito foliar solamente se detectó a los 36 ddi en niveles de 1 330 u.f.c. g^{-1} de tejido. Posterior a esta fecha, CFFSUR-B2 ya no fue detectada.

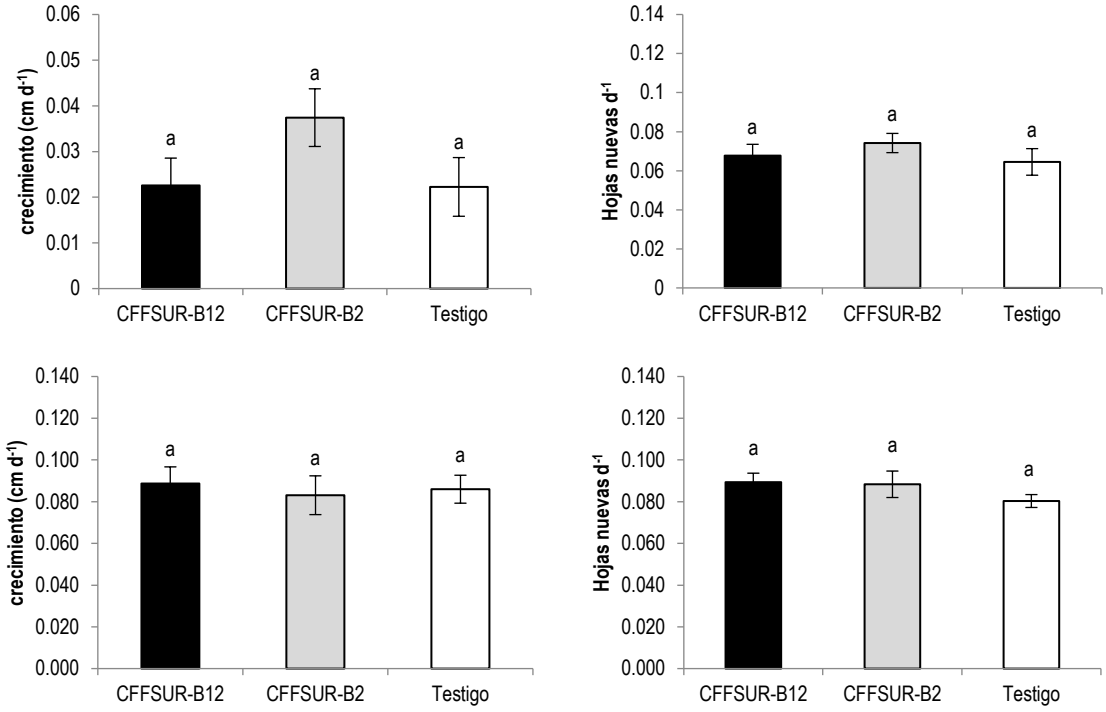

Figura 4. Tasa de crecimiento (cm d^{-1}) y de emisión foliar (hojas nuevas d^{-1}) (± error estándar) de plantas de banano inoculadas vía foliar (paneles superiores) y a la raíz (paneles inferiores) con *Streptomyces galilaeus* CFFSUR-B12 y *Serratia marcescens* CFFSUR-B2. Promedios con la misma letra son estadísticamente iguales (*P*>0.05).

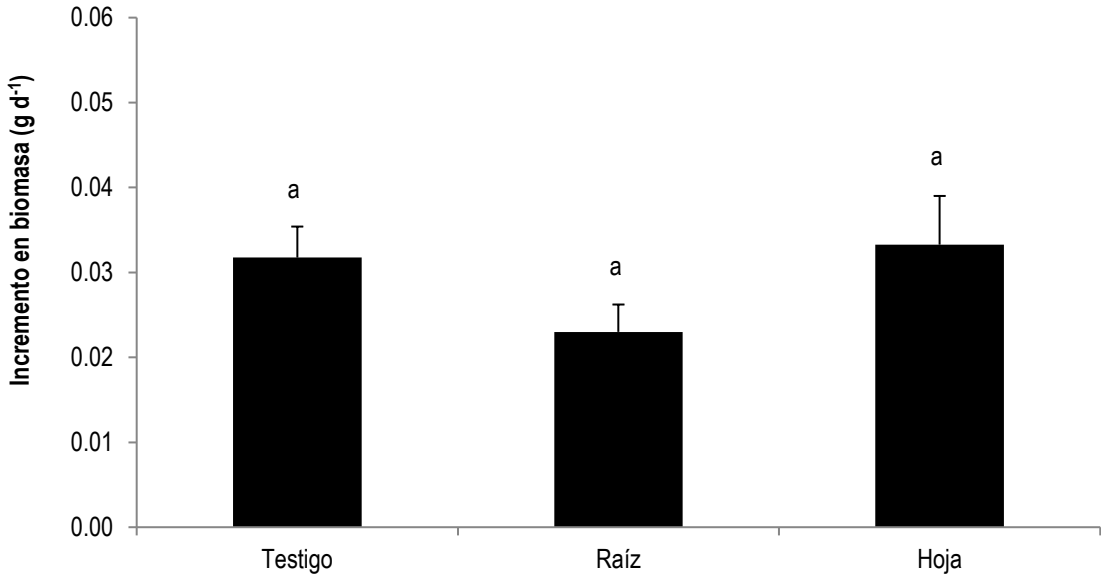

Figura 5. Tasa de incremento en biomasa (g d^{-1}) (± error estándar) de vitroplantas de banano inoculadas *in vitro* a la raíz y hoja con *Streptomyces galilaeus* CFFSUR-B12. Misma letra sobre las barras denotan similitud estadística (*P*>0.05).

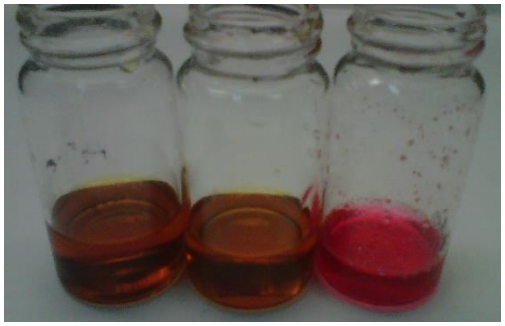

Figura 6. Ensayo colorimétrico para la determinación de Acido 3-Indol Acético (AIA): medio de cultivo inoculado con *Streptomyces galilaeus* CFFSUR-B12 (izquierda), control negativo que corresponde a medio de cultivo no inoculado (centro) y control positivo con AIA estándar (Sigma).

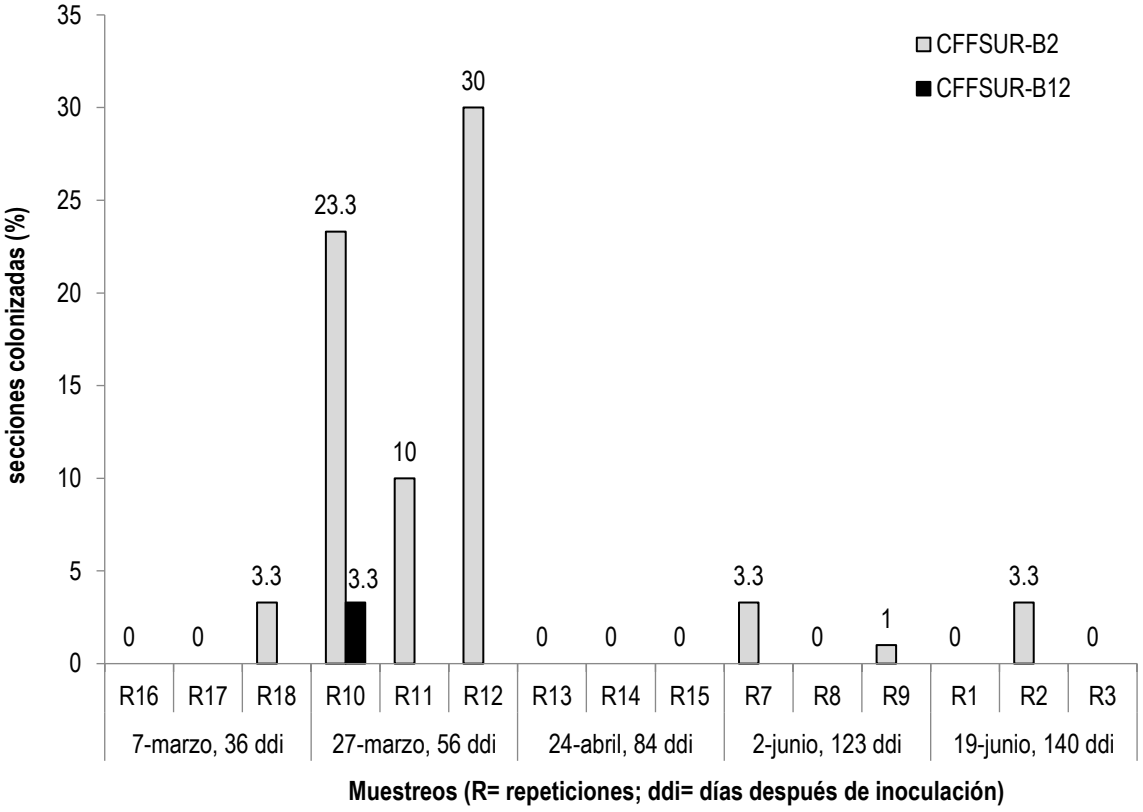

Figura 7. Secciones de raíces de banano colonizadas (%) con las cepas *Streptomyces galilaeus* CFFSUR-B12 y *Serratia marcescens* CFFSUR-B2 a diferentes intervalos de muestreo posteriores a la inoculación radicular con las bacterias.

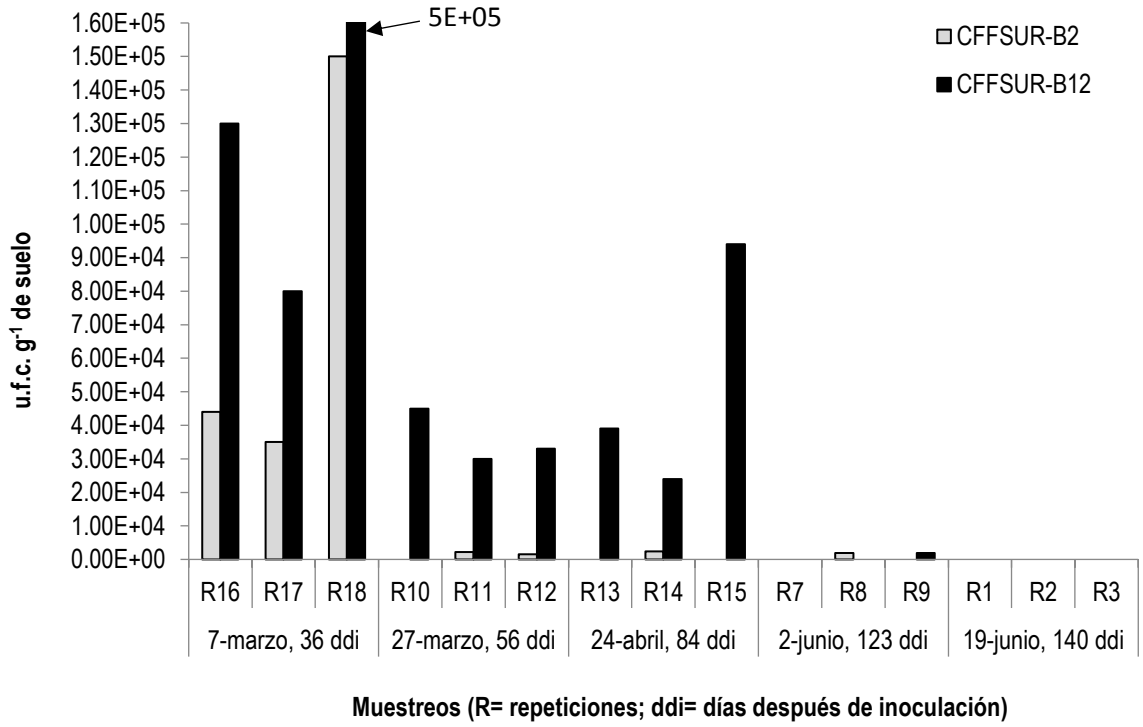

Figura 8. Sobrevivencia de las cepas CFFSUR-B12 y CFFSUR-B2 en la rizósfera de plantas de banano, a diferentes intervalos de muestreo posteriores a la inoculación radicular de las bacterias.

VII. DISCUSION GENERAL

En la presente investigación se evaluaron las interacciones de *Streptomyces galilaeus* CFFSUR-B12 con *Mycosphaerella fijiensis* Morelet y con las plantas de banano, así como también con los microorganismos no blanco asociados a la rizósfera y filósfera de esta musácea, después de ser asperjado y evaluado como posible agente de biocontrol de la Sigatoka Negra del Banano (SNB) en campo.

La importancia del estudio y la caracterización de las quitinasas producidas por los agentes de biocontrol, se fundamenta en que estas enzimas cumplen un rol clave en la interacción antagónica entre los agentes de biocontrol y los hongos fitopatógenos (Herrera-Estrella y Chet 1999), ya que degradan la quitina estructural de las paredes celulares y alteran la permeabilidad o la rigidez de las hifas (Latgé et al. 2007, 2010). En este trabajo se determinó mediante zimogramas que la cepa CFFSUR-B12 produjo cuatro bandas de degradación quitinolítica, dos de ellas con masas moleculares de 20 y 32 kDa que se ubican dentro de los rangos normales reportados para otras cepas de *Streptomyces* y en general para las quitinasas bacterianas (Bhattacharya et al. 2007), mientras que dos quitinasas muy activas y muy grandes (>170 kDa) fueron detectadas e identificadas como la misma proteína. Al comprobar que estas quitinasas (>170 kDa) estaban glicosiladas, fue posible explicar los tamaños tan grandes y la razón por la que además no pudieron ser purificadas, ya que las glicoproteínas requieren de protocolos más específicos de purificación (Gerard 1990). La glicosilación es un tipo de modificación post-traduccional que normalmente ocurre en proteínas, enzimas y otras moléculas que la bacteria exporta hacia su ambiente circundante, que además de incrementar la actividad, permite alargar la vida útil de las enzimas en el exterior celular (Reguera y Leschine 2003; Shylaja y Seshadri 1989). La glicosilación también puede funcionar como una forma de “etiquetar y asignar” la función específica que debe realizar cada una de las distintas isoformas enzimáticas producidas por un organismo (Dahiya et al. 2006). La gran mayoría de los estudios en *Streptomyces* han detectado quitinasas entre 20 y 81 kDa. Sin embargo, en *Streptomyces violaceusniger* MTCC 3959 y en *Streptomyces* sp. 80 se han reportado dos quitinasas de 114.8 kDa (Nagpure et al. 2014) y de 206.8 kDa (Fróes et al. 2012), respectivamente. En la cepa Nima de *Serratia marcescens* también

fueron detectadas dos quitinasas de 180.5 y 180.8 kDa (Ruiz-Sánchez et al. 2005), las cuales probablemente hayan contenido en su estructura algún tipo de modificación post-traduccional. También es posible que las quitinasas >170 kDa de la cepa CFFSUR-B12 sean precursoras de quitinasas más pequeñas, lo cual ocurre mediante un proceso post-traduccional en el que ciertas proteasas "parten" las quitinasas grandes para generar isoformas de menor masa molecular. Este tipo de modificación ha sido observado en quitinasas producidas por *Streptomyces olivaceoviridis* ATCC 11238 (Romaguera et al. 1992) y por *Serratia marcescens* KCTC2171 (Gal et al. 1998).

El género *Streptomyces* es diverso y hay cepas que pueden producir quitinasas muy estables y activas en altos rangos de temperatura y pH. El sobrenadante de *Streptomyces violaceusniger* MTCC 3959 mostró su actividad óptima a 60 °C y mantuvo una estabilidad de 100 % a 50 °C y a pH 9 (Nagpure et al. 2014). La cepa OPC-520 de *Streptomyces thermoviolaceus* produjo una quitinasa con un óptimo de actividad a 80 °C y a un pH 9 (Tsujibo et al. 1993). En el presente estudio, las quitinasas contenidas en el sobrenadante de CFFSUR-B12 solamente mostraron estabilidad y actividad hasta los 37 °C y pH 9, temperatura que comúnmente se presenta en las condiciones de campo donde se cultiva el banano en el Soconusco. Estos resultados nos indican que, al ser aplicado en campo, el sobrenadante de CFFSUR-B12 podría mantener su actividad quitinolítica completa mientras la temperatura ambiental no sobrepase los 37 °C, pues la pérdida total de su actividad quitinasa implicaría una reducción de aproximadamente el 50 % en su actividad antifúngica contra *M. fijiensis* (Moreno-Castillo et al. 2016).

Esto significa que las quitinasas de CFFSUR-B12 cumplen un rol parcial en la inhibición de *M. fijiensis*, tal como ha sido demostrado en cepas de *Streptomyces* contra otros patógenos (Joo 2005; Hagag and Abdallh 2012; Kim et al. 2003). Algunos autores han encontrado que se requiere de la acción simultánea entre enzimas líticas y metabolitos extracelulares para poder inhibir el desarrollo de los hongos (Akutsu et al. 1993; El-Tarabily et al. 2000; Huang et al. 2013; Macagnan et al. 2008; Prapagdee et al. 2008), tal como pudimos observar cuando se separaron y evaluaron individualmente la fracción proteica (>10 kDa) y los metabolitos (<10 kDa) del sobrenadante de CFFSUR-B12 (Moreno-Castillo et al. 2016). Al parecer, las quitinasas cumplen la función de abrir puntos de entrada en las paredes celulares que permiten el ingreso de los metabolitos

hacia el interior de las hifas, en donde causan la disrupción de procesos celulares (Gutiérrez-Román et al. 2015; Quecine et al. 2008; Regev et al. 1996).

Por otra parte, la suspensión de esporas y el sobrenadante de cultivo de CFFSUR-B12 (fracción proteica más metabolitos) fueron los mejores tratamientos en los ensayos de inhibición *in vitro* de *M. fijiensis*. Gutiérrez-Román y colaboradores (2015) demostraron que una suspensión celular de *Serratia marcescens* CFFSUR-B2 inhibió *in vitro* la germinación de ascosporas, la elongación del tubo germinativo y el crecimiento micelial de *M. fijiensis* en niveles superiores al 90 %. Probablemente, el contacto entre bacterias y el hongo haya inducido la producción del arsenal antifúngico de los antagonistas (Trejo-Estrada et al. 1998; Zhao et al. 2013), causando la inhibición del hongo. Por su parte, los sobrenadantes de cultivos bacterianos también han demostrado ser efectivos para inhibir el desarrollo del fitopatógeno: Ceballos et al. (2012) obtuvieron porcentajes de inhibición del crecimiento de *M. fijiensis* superiores al 80 % cuando evaluaron el sobrenadante de cultivo de varias cepas de *Bacillus* spp. Por su parte, Riveros et al. (2003) encontraron que al aplicar *in vitro* los filtrados bacterianos de varias cepas de *Bacillus* spp. y de *Serratia* spp. se obtiene un efecto inhibitorio en la germinación y crecimiento de *M. fijiensis* variable entre el 50 y el 100 %. En condiciones de invernadero, Cruz-Martín et al. (2010) demostraron que al aplicar los sobrenadantes de cultivos de dos cepas de *Bacillus* sp., se retarda el desarrollo de la SNB y se reduce el número de lesiones necróticas en plantas inoculadas con *M. fijiensis* en comparación con las plantas no tratadas.

Posteriormente se encontró que las plantas que fueron asperjadas en campo con la suspensión de esporas de CFFSUR-B12, presentaron los menores índices de SNB, además de retrasar hasta por 19 días el progreso de la enfermedad en comparación con las plantas tratadas con el fungicida mancozeb y agua (testigo). El sobrenadante fue similar al mancozeb en cuanto a control de la SNB, pero aun así, logró retrasar el progreso de la enfermedad por 12 días en comparación con el fungicida. Estos resultados permiten ver que la cepa CFFSUR-B12 tiene alto potencial como agente de biocontrol y como alternativa para ser incluida dentro de los programas de manejo convencional de la SNB en la región del Soconusco.

Dado que los agentes de biocontrol con hábitos micoparásitos (como CFFSUR-B12) tienen efecto directo sobre la densidad de inóculo de la enfermedad (Kranz 2003),

es posible que al entrar en contacto las esporas de CFFSUR-B12 con el inóculo del hongo sobre el filoplano, se haya inhibido la germinación y el desarrollo de las esporas de *M. fijiensis*, tal como se ha demostrado *in vitro* (Moreno-Castillo et al. 2016). Por consiguiente, las plantas tratadas con las esporas bacterianas presentaron los menores niveles de infección por SNB. Sin embargo, se piensa que las esporas bacterianas actuaron como un biofungicida protectante, pues eliminaron el inóculo nuevo pero no detuvieron el desarrollo de las infecciones de SNB ya establecidas dentro del mesófilo. Por otro lado, los altos niveles de enfermedad observados en las plantas tratadas con mancozeb (similares al testigo), sugieren que las aplicaciones permanentes y continuas del fungicida en las plantaciones de banano probablemente estén generando en el hongo algún mecanismo de resistencia a la molécula. En ciertas cepas de *M. fijiensis* provenientes del sureste Mexicano (Aguilar-Barragán et al. 2014) y de Brasil (Gomes et al. 2014) se ha empezado a observar *in vitro* cierto grado de tolerancia y pérdida de sensibilidad al mancozeb.

Aunque se menciona en la literatura que el control biológico tiene bajo impacto ambiental y en organismos no-blanco (Follett y Duan 2000; Suckling y Sforza 2014), en este trabajo se demostró la inocuidad de la cepa CFFSUR-B12 como agente de biocontrol sobre la microbiota asociada a las plantas de banano. Se ha documentado que la aplicación de insecticidas y fungicidas como el mancozeb, causan efectos detrimentales sobre las comunidades microbianas del suelo, aunque en estos trabajos las aspersiones fueron realizadas directamente al suelo (Ferreira et al. 2009; Imfeld y Vuilleumier 2012; Sigler y Turco 2002). Esto nos hace suponer que cuando las plantas de banano fueron asperjadas con los tratamientos, la mayor parte del volumen asperjado fue retenido en el follaje y muy poco alcanzó la rizósfera. Sin embargo, tampoco se afectó la diversidad de las bacterias del filoplano, lo que sugiere que estos microorganismos están adaptados a vivir en las condiciones de manejo altamente intensivo que se da a las plantaciones comerciales de banano.

En referencia a la interacción microorganismo con las plantas de banano, se encontró que CFFSUR-B12 no promovió el crecimiento vegetal ni tampoco se estableció como endófito en hojas o raíces del banano. Probablemente esta condición se haya debido a que el actinomiceto no produce ácido indol-acético, la cual es una fitohormona

y molécula señal clave para establecer “comunicación” entre bacteria y planta. Además, probablemente CFFSUR-B12 no produce la enzima ACC deaminasa que desdobla la molécula precursora del etileno en α-cetobutirato y amonio, permitiendo reducir los niveles de etileno en la planta hospedera (Lindow y Brandl 2003; Hardoim et al. 2008). Se ha encontrado que algunas cepas de *Streptomyces* tienen la capacidad de establecerse como endófitos en una diversidad de plantas (Cao et al. 2004; Nimnoi et al. 2010; Shimizu et al. 2006, 2009; Verma et al. 2009), pueden ser promotoras de crecimiento vegetal (Shimizu 2011) y pueden inducir resistencia vegetal a las enfermedades (Franco et al. 2007; Lehr et al. 2008; Shimizu et al. 2006). Sin embargo, este no fue el caso de nuestra cepa CFFSUR-B12 de *St. galilaeus* en las plantas de banano. Por otra parte, CFFSUR-B12 permaneció latente en la rizósfera de las plantas de banano hasta por 84 días después de la inoculación, dado que *Streptomyces* es un género altamente adaptado a vivir en las condiciones que ofrece el suelo (Kämpfer 2012; Williams y Robinson 1981). Sin embargo, el decremento paulatino de las poblaciones del actinomiceto después de 84 días, probablemente se haya debido a que en la rizósfera no cubrió las demandas nutricionales de la bacteria.

Futuras investigaciones sobre este actinomiceto y sus metabolitos deben ser orientadas hacia 1) la evaluación de las aplicaciones en áreas comerciales de mayor superficie, 2) incorporar paulatinamente los agentes de biocontrol en los programas convencionales de manejo de la SNB, y 3) diseñar un método viable para escalar la producción de los agentes de biocontrol (Gutierrez-Monsalve et al. 2015). Otra posibilidad sería evaluar la cepa CFFSUR-B12 en combinación con otras cepas antagónicas y compatibles que pudieran potenciar su efectividad en el control de la SNB. Asimismo, la formulación del biofungicida es otro punto clave a considerar para poder alargar la vida de anaquel y la sobrevivencia en campo de los agentes de biocontrol (Mohamed y Benali 2010), principalmente en las condiciones altamente adversas del filoplano del banano a escala comercial (Ceballos et al. 2012).

VIII. CONCLUSIONES GENERALES

1.- *Streptomyces galilaeus* CFFSUR-B12 produce tres tipos de quitinasas que se detectaron como cuatro bandas de degradación quitinolítica en zimogramas del sobrenadante de cultivo, las cuales presentaron masas de 20 kDa, 32 kDa y dos bandas de >170 kDa identificadas como la misma proteína por análisis MS/MALDI-TOF, con una modificación post-traduccional de tipo glicosilación.

2.- Las quitinasas extracelulares de *St. galilaeus* CFFSUR-B12 tienen una acción parcial en la interacción antagónica actinomiceto-hongo, porque no inhibieron la germinación de ascosporas, pero si la elongación del tubo germinativo y el crecimiento micelial de *Mycosphaerella fijiensis* en 35 % y 62 %, respectivamente.

3.- La suspensión de esporas y el sobrenadante de cultivo de la cepa CFFSUR-B12 inhibieron la elongación del tubo germinativo y el crecimiento micelial de *M. fijiensis* en valores superiores a 90 %, mientras que la germinación de ascosporas fue inhibida en 56 % y 96 % por las esporas y sobrenadante, respectivamente.

4.- La actividad antifúngica del sobrenadante de cultivo de CFFSUR-B12 sobre *M. fijiensis* se debió a la acción conjunta de su fracción proteica (>10 kDa) y su fracción de metabolitos (<10 kDa).

5.- Bajo condiciones de campo, la suspensión de esporas de CFFSUR-B12 es efectiva para reducir los niveles de infección de la Sigatoka Negra del Banano (SNB) y retrasar la epidemia hasta por 19 días, comparado con la aplicación del agroquímico a base de mancozeb.

6.- El sobrenadante de cultivo de CFFSUR-B12 permite niveles de infección de SNB similares al mancozeb, pero causó un retraso de 12 días en la epidemia.

7.- Las aspersiones de la suspensión de esporas y el sobrenadante de cultivo de *St. galilaeus* CFFSUR-B12 no afectaron la diversidad de las comunidades de hongos, bacterias y actinomicetos asociados a la rizósfera y filósfera del banano.

8.- *St. galilaeus* CFFSUR-B12 no promueve el crecimiento vegetal después de ser inoculado en las raíces y en las hojas de las plantas de banano.

9.- *St. galilaeus* CFFSUR-B12 no se establece como endófito radicular o foliar en las plantas de banano, pero permanece activo y latente en la rizósfera del banano hasta por 84 días después de su inoculación en raíz.

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