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Caracterización de los mecanismos de antagonismo que emplea *Bacillus cereus* seleccionado para el control de *Fusarium verticillioides*

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T E S I S

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Aspirante de:

1.- Se designa al aspirante el tema de tesis titulado:
Caracterización de los mecanismos de antagonismo que emplea *Bacillus cereus* seleccionado para el control de *Fusarium verticillioides*

De manera general el tema abarcará los siguientes aspectos:
Determinar el posible mecanismo microbiano implicado en el efecto inhibitorio contra *F. verticillioides*
Identificar y probar los posibles metabolitos implicados en tal efecto.
Evaluar el efecto de bioprotección en planta contra *Fusarium verticillioides*

2.- Se designan como Directores de Tesis a los Profesores:
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Caracterización de los mecanismos de antagonismo que emplea *Bacillus cereus* seleccionado para el control de *Fusarium verticillioides*

Presentada por el alumno:

Figueroa	López	Alejandro Miguel
Apellido paterno	Apellido materno	Nombre(s)

Con registro:

A	1	2	0	3	1	4

aspirante de:

Doctorado en Ciencias en Biotecnología

Después de intercambiar opiniones los miembros de la Comisión manifestaron ***APROBAR LA TESIS***, en virtud de que satisface los requisitos señalados por las disposiciones reglamentarias vigentes.

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El proyecto de tesis – Caracterización de los mecanismos de antagonismo que emplea *Bacillus cereus* seleccionado para el control de *Fusarium verticillioides* – se realizó en las instalaciones del Departamento de Biotecnología Agrícola del Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR) Unidad Sinaloa del Instituto Politécnico Nacional (IPN), bajo la dirección del Dr. Ignacio Eduardo Maldonado Mendoza (CIIDIR) y de la Dra. Luz Estela González (CIBNOR). El presente trabajo fue financiado por la Fundación Produce Sinaloa (FPS 2013-2015) y la Secretaria de Investigación y Posgrado del IPN (2012-2016). El alumno Alejandro Miguel Figueroa López fue apoyado con una beca para Estudios de Doctorado (No. becario 302070) por el Consejo Nacional de Ciencia y Tecnología (CONACyT).

“Nuestra mayor debilidad radica en renunciar.
La forma más segura de tener éxito es siempre intentarlo una vez más”.

Thomas A. Edison

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Índice

CAPÍTULO I. INTRODUCCIÓN

Sinaloa se ubica como el principal estado productor de maíz en México, produciendo aproximadamente 3, 686, 000 toneladas en el 2014. La producción nacional de maíz ha venido en aumento en los últimos cinco años; de 17, 566,000 toneladas cosechadas en 2011, la producción pasó a 24, 946, 000 toneladas en 2015, situándose Sinaloa con rendimientos de productividad por encima de la media nacional (SIAP-SAGARPA, www.siap.gob.mx).

Entre las enfermedades del maíz más importantes están las pudriciones de mazorcas y tallos. *Fusarium graminearum* y *F. verticillioides* son los patógenos fúngicos más comunes asociados a maíz (Butrón *et al.*, 2015). Morales-Rodríguez (2007) reportó a siete especies de *Fusarium* asociadas a la pudrición de la mazorca en los Valles Altos de México; al igual que en otras partes del mundo, *F. verticillioides* fue la especie más importante. *Fusarium verticillioides* (Saccardo) Neirenberg (Sinónimo, *F. moniliforme* Sheldon; teleomorfo, *Gibberella moniliformis*) es la especie de hongo más común que infecta a maíz causando la pudrición de la mazorca y del tallo (Butrón *et al.*, 2015). *F. verticillioides* es el responsable de importantes pérdidas económicas a nivel mundial desde su aparición en los campos de maíz. La infección del maíz por *F. verticillioides* se puede presentar de diferentes maneras, una de éstas puede ser sistémica a través de las semillas, en el tallo y raíces, causando la pudrición de la totalidad de la planta (Nelson, 1992). Además, este hongo produce un grupo de micotoxinas llamadas fumonisinas, las cuales contaminan el maíz y los productos obtenidos de este cereal y que son causantes de daños a la salud humana (Picot *et al.*, 2012).

La rentabilidad del cultivo se basa en el monocultivo intensivo en el estado de Sinaloa, a consecuencia de esto se han propiciado las condiciones necesarias para la proliferación de enfermedades que representan un alto riesgo para el cultivo. La junta local de Sanidad Vegetal del valle del Fuerte (JLSVVF) realizó un monitoreo en lotes del norte de Sinaloa que mostraban los sintomas caracteristicos de Fusariosis enfocado a determinar el agente causal de esta sintomatología, esto fue durante los ciclos otoño-invierno (OI) 2006-2007 y primavera-verano (PV) 2007. Se identificó a *F. oxysporum* mediante claves taxonómicas como el agente casual, siendo detectado en 84% de las

parcelas en el ciclo OI y 70 % de las parcelas en el ciclo PV. La severidad reportada fue mayor en el ciclo PV donde el 32 % de las plantas fueron dañadas en comparación con el 13 % de plantas dañadas en el ciclo OI. La sintomatología que presentaron las plantas afectadas fue pudrición en el tallo, marchitez, amarillamiento en hojas inferiores, poco crecimiento y otros. Estos hallazgos sugieren que el patógeno se encuentra ampliamente distribuido en el norte de Sinaloa, en los municipios de Ahome, El fuerte y Choix (Quintero-Benítez y Apodaca-Sánchez, 2008). En respuesta a esto, la JLSVVF sugirió que esta enfermedad iba en aumento y se tenían que tomar medidas de prevención y emplear estategias de control proponiendo un manejo integral mediante rotación de cultivos, fungicidas, hibridos resistentes y el uso de microoganismos antagonistas (Quintero-Benítez y Apodaca-Sánchez, 2008).

En un estudio previo en nuestro grupo se describieron 161 aislados de los cuales 117 han sido identificados a la fecha como *F. verticillioides* (*Fv*) asociados a la presencia de fusariosis en maíz, a partir de semillas y raíz y se describieron otras tres especies *F. nygamai*, *F. andiyazi* y *F. thapsinum* (Leyva-Madrigal *et al.*, 2015). Con esto se sugiere que en Sinaloa, a la fusariosis se asocian diferentes especies de *Fusarium* que afectan la producción de maíz en Sinaloa. En conjunto, los datos muestran que en la fusariosis más de una especie de *Fusarium* puede intervenir y ubicarse éstas de manera diferencial en la planta infectando raíz (*Fv*), tallo (*Fo*) o mazorca (*Fv*) (Leyva-Madrigal *et al.*, 2015).

En trabajo previo de nuestro grupo de trabajo se identificaron molecularmente tres aislados como *F. verticillioides* (se utilizaron 5 marcadores moleculares los cuales fueron: el ITS del ADN ribosomal, el gen de la calmodulina (Ver y CI), β-tubulina e histona-3) (Leyva-Madrigal *et al.*, 2015; Figueroa-López *et al.*, 2016). Uno de los tres aislados fúngicos fue seleccionado para pruebas de antagonismo de potenciales bacterias antagónicas a este hongo. La selección se realizó en un ensayo en líquido evaluando la capacidad antagónica hacia este hongo a partir de 11, 520 aislados correspondientes a una colección de bacterias proveniente de la rizósfera del maíz y se seleccionaron 622 aislados como posibles antagonistas. Despues de varias pruebas de selección, se obtuvieron 14 aislados bacterianos que fueron analizados en los híbridos de maíz Cebú y Garañón (Asgrow) (Figueroa-López *et al.*, 2016). A partir de éstas pruebas se seleccionaron tres aislados pertenecientes al género *Bacillus,* siendo el

aislado *Bacillus cereus sensu lato* B25 el que mejor funcionó en las variedades de maiz analizadas tanto en estudios de laboratorio e invernadero, como en campo (Lizárraga-Sánchez *et al.*, 2015; Figueroa-López *et al.*, 2016). Este aislado presentó diferentes mecanismos de promoción de crecimiento analizados en el laboratorio como la producción de agentes quelantes (sideróforos), enzimas proteolíticas, enzimas celulolíticas y la más importante relacionada con la inhibicion de hongos patógenos, enzimas quitinolíticas o quitinasas (Figueroa-López *et al.*, 2016).

La producción de enzimas hidrolíticas lo hace un potencial agente de bioncontrol contra hongos patógenos (Bressan and Fontes-Figueiredo, 2010). Estas enzimas son empleadas por una gran variedad de bacterias rizosféricas y representan una via por la cual las plantas se ven beneficiadas debido a la inhibición de hongos patógenos o por la inducción de resistencia sistémica (Slimene *et al.*, 2015). Las quitinasas han ganado interés recientemente y se utilizan para diferentes aplicaciones biotecnológicas (Karthik *et al.*, 2014). La búsqueda y el uso de estas enzimas va en aumento y existe un gran interés por incrementar su producción, los métodos de biología molecular estan siendo explotados para tener una fuente de quitinasas estable mediante el mejoramiento de microorganismos al introducirle modificaciones en su contenido genético para que las produzcan en mayor proporción (Karthik *et al.*, 2014). Desde hace décadas se ha venido trabajando en tratar de incrementar la capacidad de los microorganismos para producir estas enzimas. Una amplia variedad de sistemas de expresión se ha desarrollado para la producción y clonación de genes pertenecientes a otros organismos (Felse and Panda, 1999). La tecnología del ADN recombinante permitió elaborar construcciones aislando la secuencia codificante de las quitinasas e insertarlas en bacterias reingenieradas para una eficiente expresión con alto rendimiento en la cantidad de proteína producida para evaluar su actividad antifúngica (Pan *et al.*, 2006). Estas enzimas se han utilizado para modificar plantas y sobre-expresar estas enzimas quitinolíticas, insertándoles genes de quitinasas pertenecientes a hongos micoparasíticos y hacerlas tolerantes o completamente resistentes a patógenos fúngicos (Lorito *et al.*, 1998).

En este trabajo se estudia el posible papel de las quitinasas de *Bacillus cereus* B25 en la inhibición de *Fusarium verticillioides* como principal mecanismo de antagonismo. Se analiza mediante técnicas moleculares los niveles de transcrito de los dos genes

implicados en la producción de esta enzima inducidos con lisado fúngico y quitina coloidal, así como también la actividad quitinolítica extracelular. Se realizó también la caracterización parcial de estas dos enzimas mediante su producción recombinante en *E. coli* y el patrón de colonización de *Bacillus cereus* B25 en plantas de raíces de maíz.

El conocer el mecanismo de esta bacteria con el cual ejerce efecto antagónico sobre este patógeno, aportará información que se puede utilizar a futuro como uno de los criterios a considerar para la formulación de un producto agrobiológico que contenga una cepa con las características necesarias para combatir este patógeno en maíz. Adicionalmente, éste conocimiento puede llevarnos a formulaciones a partir de ésta cepa que puedan emplearse para el control de éste u otros hongos fitopatógenos en otros cultivos además de maíz.

CAPÍTULO II. ANTECEDENTES

El maíz y la fusariosis

El maíz es un cultivo que se originó en México de donde se extendió a todo el mundo como uno de los principales cultivos alimenticios. Esta especie es ahora cultivada ampliamente en el mundo (Morris, 2002). Aunque es cosmopolita, es uno de los alimentos básicos de muchos países de América latina, y se consume en Europa oriental y el Sureste de Asia incluyendo China (Christensen, 2002).

En México, el maíz es parte de la alimentación diaria, constituye un insumo para la ganadería y para la obtención de numerosos productos industriales, por lo que, desde el punto de vista alimentario, económico, político y social, es el cultivo agrícola más importante (SIAP 2015). México posee el séptimo lugar en producción de maíz, precedido por EUA, China, Brasil, Union Europea, Ucrania y Argentina. Hablando en términos nacionales, el estado que lidera la producción de maíz es Sinaloa, seguido por Jalisco, Michoacán, Estado de México, Guanajuato, Chihuahua y Guerrero (SIAP-SIACON, 2015.

Entre las enfermedades del maíz más importantes están las pudriciones de mazorcas y tallos. Se han estudiado en paises como Irán, Suiza y México, los agentes causales de estas pudriciones; en Irán se reporta *F. verticillioides* (*Fv*), *F. proliferatum* (*Fp*), *F. fujikuroi, F. nygamai* (*Fn*); siendo *Fv* y *Fp* los más abundantes en maíz (Mohammadi *et al.*, 2016); en Suiza se reporta la incidencia de *F. graminearum*, *F. verticillioides*, *F. proliferatum* y *F. subglutinans* (Dorn *et al.*, 2011); y en México se reportó a cuatro especies de *Fusarium* asociadas a la pudrición de maíz, *F. nygamai, andiyazi, F. thapsinum y F. verticillioides*; asociados a diferentes órganos de la planta (Leyva-Madrigal *et al.*, 2015).

Fusarium verticillioides es el responsable de importantes pérdidas económicas a nivel mundial desde su aparición en los campos de maíz. La infección del maíz por el hongo puede ocurrir sistémicamente a través de la semilla, tallo y raíces, causando la pudrición de toda la planta (Nelson, 1992). Además, este hongo produce un grupo de micotoxinas llamadas fumonisinas, las cuales contaminan el maíz y los productos

obtenidos de este cereal. El consumo de estas micotoxinas causa efectos nocivos en los animales y en la salud humana (Mohammadi *et al.*, 2016).

Fusarium verticillioides (Saccardo) Nirenberg (sinónimo: *F. moniliforme* Sheldon; teleomorfo *Gibberella moniliformis*) pertenece a la sección Liseola, específicamente al complejo de *Fusarium fujikuroi* (Sawada) Wollenweber (Geiser *et al.*, 2013). Es capaz de causar pudrición en tallos y mazorcas, también puede contaminar las semillas de cultivos como el trigo, arroz, avena y sorgo.

La enfermedad se inicia cuando el hongo logra penetrar a la planta con ayuda de insectos o por simple daño mecánico en las raíces, cuando crecen las raíces secundarias. Durante el proceso de invasión a la planta, el hongo puede sintetizar fumonisinas, una familia de micotoxinas, que frecuentemente contaminan el grano del maíz y están asociadas a un gran número de enfermedades en animales, incluyendo el cáncer (Luna-Olvera, 2000). El hongo puede encontrarse en el suelo o simplemente ser acarreado hasta la parte aérea de la planta por insectos vectores o acción del viento. Éste produce pudrición de las raíces y tallos una vez que entra en el tejido de la planta. Cuando la planta cumple su ciclo, los residuos de las plantas infectadas pueden ser depositados el suelo y sirven como inóculo para el próximo cultivo (Figura 1).

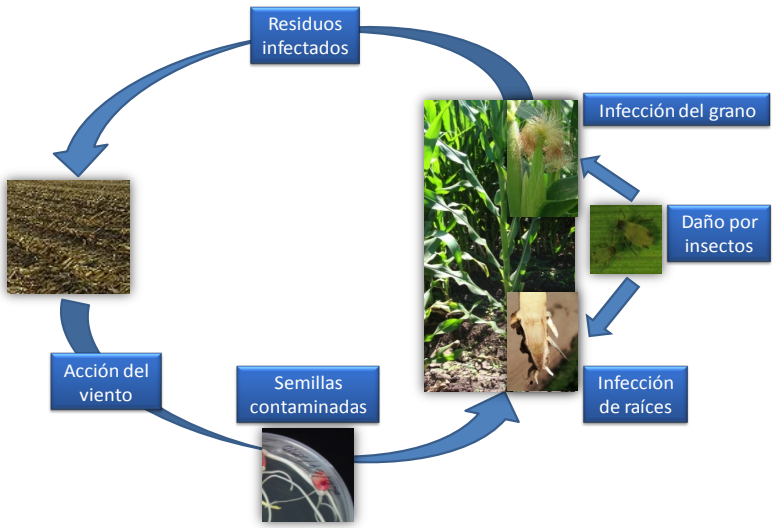

Figura 1. Ciclo de la fusariosis del maíz causada por *Fusarium verticillioides.*

El ciclo biológico de *F. verticillioides* es complejo ya que este hongo es un patógeno no obligado que carece de un hospedero específico. Sus distintas fases de vida

están conformadas por un estado saprofítico y otro parasítico. Durante la primera etapa, *F. verticillioides* obtiene los nutrientes de los tejidos vegetales muertos, produciendo estructuras infectivas como los macroconidios y los microconidios (Figura 2). Los microconidios en *F. verticillioides* consisten en células aisladas, mientras que los macroconidios son células grandes, septadas y menos abundantes (Sutton *et al.*, 1998). En su estado parasítico, después de la extensiva colonización intracelular, el hongo destruye el tejido del cual se alimenta, liberando altas concentraciones de fumonisinas (Luna-Olvera, 2000; Oren *et al.*, 2003).

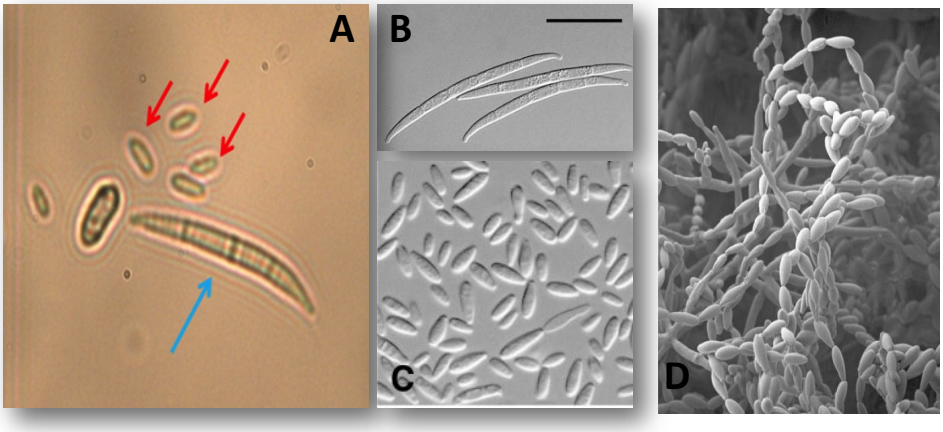

Figura 2. Estructuras infectivas del hongo *F. verticillioides*. A) Las flechas rojas indican los microconidios y la flecha azul muestra un macroconidio (400X). Fotografía de Figueroa-López. B) macroconidios, C) microconidios. Barra de escala = 25 µm. Fotografías B y C obtenidas de Leslie y Summerell, 2006, D) Microconidios en cadena característica de *Fusarium verticillioides*. Fotografía obtenida de Duncan and Howard (2009).

La muerte de las plantas de maíz no es común durante el estado parasítico, pero causa pérdidas económicas. Este fitopatógeno, además de sobrevivir en restos orgánicos de cultivos anteriores (Cotton and Munkvold, 1998), también se transmite a través de semillas (Bacon *et al.*, 1992). En el suelo, *F. verticillioides* regularmente no produce clamidosporas, sino hifas de pared engrosadas que aparentemente prolongan su persistencia (Nelson *et al.*, 1983).

Un estudio reciente en nuestro grupo de trabajo encontró que las plantas de maíz en cultivos de la región se encuentran infectadas por *F. verticillioides*, *F. nygamai*, *F.*

andiyazi y *F. thapsinum*. Se encontró tambien una distribución de las especies en diferentes órganos de la planta; *F. verticillioides* fue el más frecuente en las semillas mientras que *F. nygamai* predominó en las raíces. Se reporta tambien infecciones mixtas entre *F. verticillioides/F. thapsinum* y *F. verticillioides/F. nygamai* en semillas y raíces respectivamente. Los ensayos de patogenicidad de los aislados revelaron que estas cuatro especies pueden infectar maíz y causar diferentes niveles de severidad de la enfermedad. Este es el primer reporte de *F. nygamai y F. thapsinum* infectando maíz en México (Leyva-Madrigal *et al.*, 2015).

Problemas para el control de la fusariosis; agroquímicos y resistencia genética

Las causas de la fusariosis en maíz está relacionada a diversos factores, principalmente la persistencia, estos patógenos pueden sobrevivir en residuos infectados de cultivos anteriores actuando como un reservorio (Cotton and Munkvold, 1998), sirviendo estos residuos como fuente de inóculo para los cultivos siguientes en especial si se trata de labranza de conservación (Vogelgsang *et al.*, 2011). Otro factor es la resistencia a fungicidas, las cepas infectivas de *Fusarium* pueden desarrollar características genéticas que les permita adquirir resistencia a fungicidas (Chen *et al.*, 2014). El mecanismo de resistencia a fungicidas se puede dar de varias formas: 1) se puede modificar el sitio de unión donde actua el fungicida reduciendo la unión de este; 2) se puede sintetizar una enzima alternativa que es capaz de sustituir la enzima afectada por el fungicida; 3) sobreproducción de los compuestos que afectan el fungicida; 4) hacer más eficiente el sistema de eflujo del fungicida desde el interior de las células o 5) reducir la toma de este; 6) y/o romper o degradar la molécula del fungicida (Zhonghua and Michailides, 2005). También se menciona que no existe algún compuesto químico fungicida capaz de eliminar el complejo de las especies de *Fusarium* en Europa, hasta el momento, las buenas prácticas agronómicas para controlar *Fusarium* en maíz han tenido un éxito limitado para controlar la infección y la acumulación de micotoxinas (Dorn *et al.*, 2011). Posiblemente esta sea una de las razones por la cual es complicado erradicarla, la resistencia es un punto clave en la limitación de la vida útil y eficacia de los fungicidas, también es importante para el entendimiento de los procesos moleculares de cómo los hongos adquieren resistencia a cierto grupo de compuestos químicos (Avenot and Michailides, 2010).

El mejoramiento genético del maíz ahora se vuelve una alternativa viable para la búsqueda de híbridos resistentes a la fusariosis y reducción en la acumulación de sus toxinas (Dorn *et al.*, 2011). Se han desarrollado varias líneas de maíz que presentan resistencia a la infección de *Fusarium vercillioides* y otros patógenos que afectan este cultivo (Löffler *et al.*, 2010; Balconi *et al.*, 2014). Williams y Windham (2009) estudiaron la acumulación de fumonisinas y aflatoxinas usando germoplasma de maíz resístente a la acumulación de aflatoxinas, encontraron que en las lineas resultantes Mp715, Mp717 y GA209 se encontraron niveles reducidos de fumonisinas y aflatoxinas; estas líneas fueron empleadas para generar híbridos resistentes. Small *et al*., (2011) evaluaron 24 lineas parentales como recurso para obtener líneas que mostraran resistencia a la infección con *F. verticillioides* en África del Sur. Las pruebas se realizaron en invernaderos mostrando menos del 5% de incidencia, las líneas resistentes fueron CML390, CML 444, CML 182, VO 617Y-2 y RO 549 W. En el trabajo, se sugieren que las líneas CML444 y CML 390 son potencial recurso para ser utilizadas en programas de mejoramiento de maíz.

El mapeo genético es otra estrategia que se ha venido desarrollando a nivel mundial para abordar la problemática de la pudrición de mazorca por *Fusarium*. La búsqueda de marcadores moleculares ligados a genes de resistencia para *Fusarium*, especialmente a *F. graminearum*, ha permitido identificar 11 QTLs (Quantitative trait loci) para la resistencia a pudrición de mazorca seguido de la inoculación en los estigmas y 18 QTLs después de la inoculación en el grano. En este estudio estos alelos provenían de una línea parental resistente (CO387) (Ali *et al.*, 2005).

Reicentemente, en un estudio de asociación genómica (GWAS: Genomic-wide association study) se identificaron SNPs (single-nucleotide polymorphisms) asociados con la resistencia a pudrición de mazorca causada por *Fusarium* en maíz, encontrando tres SNPs en 273 lineas parentales pertenecientes a la Universidad de Carolina del Norte en Estados Unidos de America (Zila *et al.*, 2013). Posteriormente otro análisis similar en 1687 lineas parentales de maíz se encontraron ahora siete SNPs relacionados con la resistencia a la pudrición de mazorca causada por *Fusarium* (Zila *et al.*, 2014). La identificación de variantes alélicas específicas en el maíz contribuye al mejoramiento del maíz con características de resistencia a patógenos fúngicos y en específico a *Fusarium*.

En México se han orientado estudios hacia la búsqueda de resistencia de los híbridos a enfermedades como la pudrición de mazorca causada por *Fusarium* y *Diplodia*. Algunos genotipos de maíz al ser evaluados en distintas condiciones climáticas en regiones diferentes del país, presentan rendimientos variables, así como un comportamiento diferente en lo que respecta a la respuesta a la enfermedad. Durante este proceso se identificó un híbrido de cruza simple entre las líneas CML-271 y CML-310 del Centro Internacional para el Mejoramiento del Maíz y Trigo (CIMMYT) que tiene resistencia a pudrición de mazorca causada por los hongos de los géneros *Diplodia* y *Fusarium*; dicho material fue competitivo en el rendimiento con los híbridos comerciales. Se enfatizó en este estudio que la variabilidad de los patógenos en cada región es determinante para la selección de híbridos resistentes (Betanzos *et al.*, 2009).

Control biológico de Fusarium spp. en maíz

El uso de microorganismos como potenciales agentes de control biológico en la agricultura resulta una alternativa promisoria para combatir enfermedades de plantas en cultivos de interés (de Souza *et al.*, 2015). En la agricultura actual, se busca la sostenibilidad de la productividad agrícola, aunque el uso de agroquímicos ha permitido obtener incrementos substanciales en la producción agrícola; no obstante, sus efectos adversos impactan significativamente la sostenibilidad de esta actividad, además los patógenos pueden adquirir resistencia a estos químicos (Chen *et al.*, 2014).

En la actualidad, se busca implementar técnicas de control biológico de enfermedades, ó de organismos-plaga, para diferentes cultivos a nivel regional. El control biológico de plantas puede ser una alternativa promisoria para el manejo de plagas y enfermedades; además de limitar el uso de pesticidas sintéticos (Nagórska *et al.*, 2007). Se han reportado que algunas bacterias, tienen un efecto indirecto en las raíces de las plantas, al secretar metabolitos como lo son los antibióticos, sideróforos y ácido cianhídrico, que inhiben el desarrollo de organismos fitopatógenos (de Souza *et al.*, 2015).

Se han realizado estudios para implementar métodos que ayuden a los agricultores a combatir el problema de la fusariosis en el cultivo del maíz, pues durante los últimos años las enfermedades causadas por *Fusarium* spp. se han incrementado. Al evaluar la efectividad biológica *in vitro* de bacterias como *P. fluorescens* y *Burkholderia* sp. contra *F. verticillioides in vitro*, se determinó que éstos inhibieron de 38-68% el crecimiento del fitopatógeno. En pruebas *in vivo* estas bacterias disminuyeron los síntomas de la enfermedad 67-88% cuando las plantas de maíz se inocularon con estas bacterias y posteriormente se sembraron en suelo infestado con el hongo (Hernández-Rodríguez *et al.*, 2008)

Bacillus amyloliquefaciens y *Mycobacterium oleovorans* son bacterias que inhiben el crecimiento de *F. verticillioides*, además de disminuir la concentración de fumonisinas en los granos del maíz (Pereira *et al.*, 2007). En estudios *in vitro, Azotobacter armeniacus* y *Arthrobacter globiformis* inhibieron en 80 a 100% y 71 a 80% al mismo hongo, respectivamente (Cavaglieri *et al.*, 2004). *Bacillus subtilis* también inhibe el desarrollo *in vitro* de *F. verticillioides* en 28-78% y reduce la producción de fumonisinas entre 29 y 50% (Cavaglieri *et al.*, 2005).

A menudo es difícil entender exactamente cómo los agentes de biocontrol controlan a los patógenos debido a que pueden emplear una amplia variedad de mecanismos de defensa (Shali *et al.*, 2010).

La rizósfera como fuente para la obtención de microorganismos para el control biológico de enfermedades vegetales

El término rizósfera fue introducido por Hiltner en 1904, y se define como el volumen de suelo inmediato a la raíz en el cual se estimula el crecimiento de microorganismos (Sorensen, 1997). La rizósfera es de mucho interés ya que es un hábitat en el cual se llevan a cabo diversos procesos biológicos e interacciones (Schroth and Hancock, 1982). En la rizósfera existe una amplia gama de compuestos orgánicos, tales como exudados de raíces de bajo peso molecular, secreciones, muscigeles y lisados celulares. Estos compuestos propician que las raíces actúen como una fuente de carbono orgánico, para los microorganismos que crecen en la rizósfera; por ello la densidad de las poblaciones de microorganismos es considerablemente más alta en la

rizósfera que en el suelo lejano a la raíz (Walker *et al.*, 2003). Se ha estimado que cerca del 30% de los fotosintatos producidos por la planta son secretados como exudados de las raíces (Baetz and Martinoia, 2014).

Los exudados de las plantas en la rizósfera, contienen aminoácidos y azúcares, los que proveen una fuente rica de energía y nutrientes para las bacterias, resultando en poblaciones mayores en esta zona que en otras partes del suelo. La mayoría de los microorganismos se encuentran dentro de los 50 μm de superficie radical y las poblaciones dentro de los 10 μm de superficie pueden alcanzar 1.2 x 10^8 células por centímetro cúbico ó 10^9-10^{12} células por gramo de suelo. A pesar del elevado número de bacterias en la rizósfera, solo un 7-15% de la superficie radical es colonizado (Pinton *et al.*, 2001). La diversidad de microorganismos es dinámica con un cambio frecuente en la estructura de la comunidad y la abundancia de especies (Vranova *et al.*, 2013). Un grupo importante de estos microorganismos que ejercen efectos benéficos en el crecimiento de plantas mediante la colonización de las raíces fueron denominados como bacterias promotoras del crecimiento vegetal (BPCVs; o sus siglas en inglés PGPRs: plant growth-promoting rhizobacteria) (Kloepper, 1978).

Microorganismos de la rizósfera: bacterias promotoras del crecimiento vegetal (BPCVs)

La mejora en el crecimiento de las plantas por efecto de las bacterias promotoras de crecimiento se mide como un incremento en la germinación, el vigor, biomasa, proliferación del sistema radical y rendimiento en varias especies de plantas. Desde su reconocimiento como un subconjunto importante de microorganismos colonizadores de raíz, se han identificado bacterias promotoras de crecimiento en diferentes sistemas de cultivo y zonas agroecológicas (Vessey, 2003; Zahir *et al.*, 2003; Ping and Boland, 2004).

Las BPCVs han ganado atención como un grupo importante de bacterias benéficas para la agricultura además de las bacterias simbióticas fijadoras de nitrógeno. La protección a patógenos de suelo que ejercen algunas BPCVs al ser inoculadas en plántulas fue observada inseparablemente de la actividad promotora de crecimiento (Manjula and Podile, 2001; Guo *et al.*, 2004). Sin embargo, el uso de las BPCVs como bio-fertilizantes se ha redescubierto con el incremento de la importancia de los cultivos

orgánicos con la aplicación mínima de insumos (Teplitski *et al.*, 2000). El conocimiento de los mecanismos implicados en la acción de las BPCVs y de su genética y de su actividad bioquímica y fisiológica, ha hecho posible el uso de las BPCVs, tanto para estimular el crecimiento de las plantas como para el control de enfermedades (Podile and Kishore, 2006).

Cepas de géneros como *Aeromonas*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Klebsiella*, *Pseudomonas* y *Serratia*, han sido identificadas como BPCVs (Podile and Kishore, 2006). La diversidad de las BPCVs en la rizósfera varia ampliamente según el tipo de planta y de suelo y nutrientes disponibles (Tilak *et al.*, 2005). Entre la diversidad de BPCVs identificadas, *Pseudomonas* y *Bacillus* spp, tienen una amplia distribución y son los géneros más estudiados; *Azospirillum*, un género de bacterias fijadoras de N_2 es un grupo importante de BPCVs, ya que el tratamiento con la mayoría de cepas y especies de este género afecta positivamente la biomasa de raíz y de parte aérea (Bashan *et al.*, 2004).

Bacterias endofíticas de la rizósfera

Exploraciones iniciales para la identificación de las BPCVs se concentraron en bacterias de la rizósfera, y experimentos posteriores demostraron la aplicación potencial de los endófitos de raíz como BPCVs (Vessey, 2003). Esto sugiere que la mayoría de las bacterias endofíticas tienen su origen en la rizósfera, y posteriormente invaden los tejidos de raíz y colonizan las células de córtex (Kuklinsky-Sobral *et al.*, 2004). Las rizobacterias que se establecen dentro de las raíces de las plantas, formando asociaciones más íntimas, son endofitas. En estas se incluyen una amplia gama de bacterias que forman asociaciones semejantes a la simbiosis rizobias-leguminosas; los microorganismos endófitos estimulan la promoción de crecimiento de modo directo o indirecto. Entonces, una bacteria endófita se define como aquella bacteria que coloniza el tejido interno de la planta sin mostrar un efecto negativo a su hospedero (Schulz and Boyle, 2006), y se calcula que cerca de 300 000 especies de plantas son hospederas de uno o más endófitos (Ryan *et al.*, 2008).

Las bacterias endofitas o endofíticas colonizan un nicho ecológico similar al de los fitopatógenos, esto las hace una alternativa promisoria para el control biológico (Berg *et al.*, 2005). En efecto, existen reportes mostrando que los microorganismos endofíticos pueden tener capacidad de control de patógenos de plantas (Duijff *et al.*, 1997), insectos (Azevedo *et al.*, 2000) y nemátodos (Hallmann *et al.*, 1998). Estas bacterias son capaces de disminuir o prevenir los efectos nocivos de ciertos organismos patógenos. Los efectos benéficos de estas bacterias en sus hospederos parecen ocurrir a través de mecanismos similares a los descritos para las bacterias asociadas a la rizósfera (Ryan *et al.*, 2008). El origen de las enfermedades fúngicas, bacterianas y virales en algunos casos, incluso el daño causado por insectos y nemátodos puede ser reducido después de la inoculación previa con organismos endófitos (Berg *et al.*, 2005).

Mecanismos de acción de las bacterias endofíticas

Dentro de los mecanismos de antagonismo a hongos fitopatógenos está el parasitismo, en el cual los hongos patógenos son inhibidos por bacterias mediante la producción de enzimas hidrolíticas que degradan la pared celular. Ésta es una estructura con gran plasticidad, que da la forma a la célula, controla la permeabilidad y la protege de los cambios osmóticos. Además de estas funciones, constituye el lugar de interacción con el medio externo, localizándose en ella las adhesinas y un gran número de receptores de naturaleza proteica que tras su activación, desencadenan una compleja cascada de señales en el interior de la célula (Pontón, 2008).

La quitina y β-1,3-glucanos son los principales componentes estructurales de la pared celular de los hongos. Las quitinasas y las β-1,3-glucanasas secretadas por los antagonistas se han sugerido como las enzimas claves en el proceso lítico durante la acción micoparasítica (Lorito *et al.*, 1994). Como principal componente estructural de la pared fúngica, al interaccionar con otros organimos es espacialmente el primer lugar de contacto en la interaccion, teniendo un papel clave en el proceso de la inhibición fúngica (Chaffin *et al.*, 1998; Nimrichter *et al.*, 2005; Pontón *et al.*, 2001).

La quitina es esencial en el desarrollo de los hongos, su ausencia causaría efectos negativos en su crecimeinto y morfología. Dada su importancia como compontente

estructural, se considera un sitio clave para atacar a los hongos mediante compuestos antifúngicos (Heitman, 2005).

Quitina

La quitina es un polímero lineal de β-(1-4)-N-acetilglucosamina (GlcNAc) que le sigue a la celulosa en abundancia. Forma parte del exoesqueleto de artrópodos y la pared celular de los hongos filamentosos. Está constituida por unidades de N-acetil-glucosamina con enlaces β-1,4-glicosídicos (Carrillo, 2003). La degradación por la quitinasa produce poca cantidad de N-acetil-glucosamina y preferentemente forma oligómeros (quitobiosa, quitotriosa) (Figura 3). Los oligómeros son convertidos en monómeros por la β-N-acetil-glucosaminidasa (Carrillo, 2003).

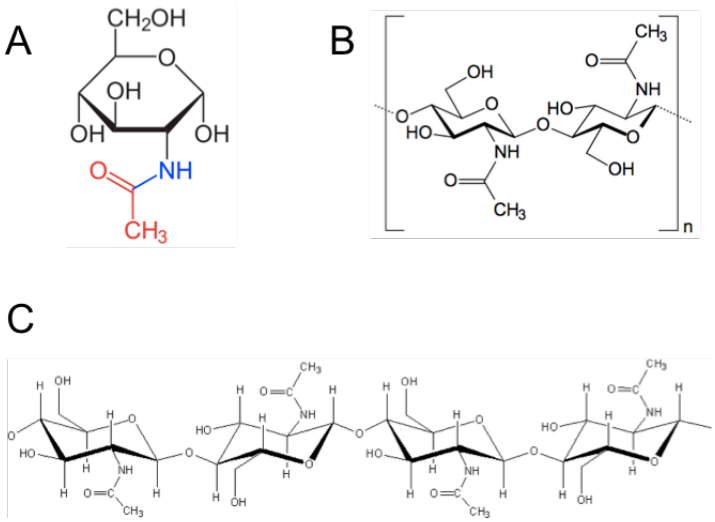

Figura 3. Estructura química de la quitina. A) Molécula de glucosamina, monómero de quitina; B) Molécula de quitobiosa, producto de la desintegración de la molécula de quitina; C) Polímero lineal de quitina.

La biosíntesis de la quitina ha sido estudiada en una gran variedad de organismos. La enzima para la síntesis es llamada quitina sintetasa. Se han reportado tres quitina sintetasas en *Saccharomyces cerevisiae*: CS I, CS II y CS III. Son diferentes entre sí en términos de función y actividad catalítica. La mayor parte de la quitina es sintetizada por la CS III (Bulawa, 1993). Las quitina sintetasas de hongos se agrupan en dos familias y cinco clases (Roncero, 2002). En insectos existen dos genes de quitina

sintetasa (Merzendorfer, 2006). En nemátodos hay dos genes de quitina sintetasas que se expresan diferencialmente (Veronico *et al.*, 2001). Por lo tanto, los organismos pueden tener diferentes enzimas quitina sintetasas que llevan a cabo funciones diferentes, estas usan UDP-*N*-acetilglucosamina como sustrato para producir fibras de quitina (Cohen, 2001). Los genomas de algunos clorovirus contienen algunos genes de quitina sintetasa (*cs*). Al introducir el gen de clorovirus CVK2 en células de *Chlorella*, una alga que no posee genes para sintetizar quitina, ésta adquiere la capacidad de producir quitina (Kawasaki *et al.*, 2002).

Quitinasas

Las quitinasas, son enzimas que hidrolizan quitina, se presentan en un amplio rango de organismos incluyendo los virus (Hawtin *et al.*, 1995), bacterias (Wen *et al.*, 2002), hongos (Gruber *et al.*, 2011), insectos (Sakuda *et al.*, 1987), plantas superiores (Kasprzewska, 2003) y mamíferos (Bussink *et al.*, 2007). La mayoría de los organismos (bacterias, insectos y plantas) poseen familias de varios genes de quitinasas con funciones distintas, en la digestión, muda de cutícula, y diferenciación celular. Basadas en la similitud de la secuencia de aminoácidos, las enzimas quitinolíticas están agrupadas dentro de las familias 18, 19 y 20 de las glicosil-hidrolasas (Henrissat and Bairoch, 1993). La familia 18 es diversa en términos evolutivos, ya que engloba quitinasas de organismos como bacterias, hongos, virus, animales y algunas plantas. La familia 19 comprende quitinasas presentes en plantas y en *Streptomyces.* La familia 20 incluye a la β-N-acetil-hexosaminidasa que se encuentra en *Streptomyces* y humanos.

Las quitinasas se clasifican en dos grandes categorías de acuerdo al lugar en donde se lleva cabo la reacción de hidrólisis en el sustrato: endo- y exo-quitinasas. Las endo-quitinasas son enzimas que rompen en sitios internos, lo cual genera multímeros de bajo peso molecular de GlcNAc, como la quitotriosa y quitobiosa. Las exo-quitinasas a su vez se dividen en dos sub-categorías, las quitobiosidasas y las β-1-4 N-glucosaminosidasas (Dahiya *et al.*, 2006).

Las quitobiosidasas catalizan la liberación de diacetilquitobiosa comenzando con los extremos terminales no reducidos de las microfibras de la quitina, y las β-1,4 N-acetilglucosaminidasas se unen a productos oligoméricos de endoquitinasas y

quitobiosidasas, generando monómeros de GlcNAc. Las quitinasas se clasifican también de acuerdo al grupo de organismos que las producen, partiendo para ello de las secuencias de los dominios catalíticos. Así tenemos que en plantas, las quitinasas se clasifican en cinco clases y se representan con los números romanos I, II, III, IV y V. En los hongos se clasifican en grupos, siendo estos el A y B, por último en las bacterias, las quitinasas están claramente separadas en tres subfamilias que son A, B y C (Dahiya *et al.* 2006).

Por otro lado, la expresión de los genes de quitinasas en microorganismos es controlada por medio de un sistema inductor/represor, en el cual, la quitina u otros productos de la degradación actúan como inductores. En hongos, se han observado que la glucosa y otras fuentes de carbono funcionan como represoras del sistema. La expresión de los genes ech42 y chit33 de *Trichoderma harzianum* es inducida por fragmentos de paredes celulares, por quitina coloidal o por la ausencia de carbono, por otro lado, la presencia de altas concentraciones de glucosa o de glicerol inhibe su expresión (Carsolio *et al.*, 1994; Limón *et al.*, 1995). La transcripción del gen ech42 puede ser inducido por otros medio como el estrés fisiológico, bajas temperaturas y por una alta presión osmótica (Felse y Panda 1999).

Papel de las quitinasas en los organismos

Las quitinasas juegan un papel importante y muy diverso en diferentes organismos. En invertebrados, las quitinasas usualmente son parte del tracto digestivo. En insectos y crustáceos, las quitinasas están asociadas a la necesidad de la degradación parcial de la cutícula que necesitan para realizar la muda. En plantas, las quitinasas están involucradas en la resistencia contra patógenos debido a su actividad antifúngica (Taira *et al.*, 2002). En los hongos se cree que juegan un papel auto-lítico, nutricional y morfogenético. Y en virus, están involucradas en patogénesis (Patil *et al.*, 2000). En bacterias, las quitinasas son elementales en la nutrición y parasitismo. Además de las posibles aplicaciones anteriores, se pueden usar para la producción de quito-oligosacáridos, los cuales se les ha encontrado función como agentes antibacteriales, inductores de lisozima e inmuno-mejoradores (Wen *et al.*, 2002). También estas

enzimas pueden ser usadas en la agricultura para controlar patógenos de plantas (Karasuda *et al.*, 2003).

Las quitinasas en el control biológico de hongos fitopatógenos por BPCVs

Una alternativa para los fungicidas es el uso de bacterias benéficas. Además de las *Pseudomonas*, el género *Bacillus* es probablemente el más ampliamente estudiado y comercializado como agente de biocontrol (Paulitz and Bélanger, 2001). Algunas de las características de este género asociadas a los mecanismos de biocontrol incluyen la producción de diversos compuestos entre ellos están las enzimas líticas que contribuyen a la degradación de paredes celulares fúngicas (Paulitz and Bélanger, 2001). Especies de *Bacillus* han mostrado ser efectivos y son comercialmente aplicados como agentes de biocontrol contra hongos, nemátodos y plagas (Jiang *et al.*, 2006).

Las quitinasas purificadas de *B. subtilis* AF1 (Manjula *et al.*, 2004), *Serratia marcescens* (Ordentlich *et al.*, 1988; Kishore *et al.*, 2005) y *S. plymuthica* son consideradas como muy eficaces y altamente antifúngicas (Frankowski *et al.*, 2001). Dentro del complejo de metabolitos que produce *B. subtilis* también puede secretar una serie de proteínas tales como bacisubina (41.9 kDa) (Liu *et al.*, 2007), endo-1-4-β-glucanasa (46.60 kDa), bacilomicina D sintetasa C (309.04 kDa) (NCBI) que ya están reportadas para esta cepa (Li *et al.*, 2009; Xie *et al.*, 1998) y estas proteínas secretadas tienen efecto inhibitorio contra diferentes patógenos de plantas como *Fusarium oxysporum*, *Rhizoctonia solani*, *F. moniliforme* y *Sclerotinia sclerotiorum* (Li *et al.*, 2009).

Bacillus cereus es una bacteria que causa enfermedades intestinales a humanos, y posiblemente algunas de las proteasas que produce estén implicadas en sus procesos de infección (Orhan *et al.*, 2005). Pero de igual forma puede producir proteínas con actividad antifúngica, las cuales poseen un efecto inhibitorio sobre patógenos de plantas como *F. oxysporum*, *F. solani* y *P. ultimum*. El efecto observado en este estudio se lo atribuyeron a una proteasa secretada por *B. cereus* (Chang *et al.*, 2010). Además de producir proteasas anti-fúngicas *B. cereus* tiene la capacidad de producir una enzima llamada quitosanasa D11 la cual recibe este nombre porque se identificó de la cepa *B.*

cereus D11 la cual puede inhibir el desarrollo micelial de *Rhizoctonia solani* (Gao *et al.*, 2008).

Diferentes especies del género *Bacillus* pueden producir quitinasas, entre estas se encuentran *B. amyloliquefaciens* (Sabry, 1992), *Bacillus subtilis* que produce una quitinasa llamada NPU001 que inhibe el crecimiento del hongo *Fusarium oxysporum* (Chang *et al.*, 2010); *Bacillus cereus* produce enzimas hidrolíticas antifúngicas, como quitinasas, quitosanasas y proteasas, cuando crece en un medio de cultivo adicionado con cascara de camarón en polvo. Fue capaz de inhibir el crecimiento de *F. oxysporum*, *F.solani* y *Pythium ultimum* (Chang *et al.*, 2007). El peso molecular de las quitinasas producidas por el género *Bacillus* varia entre 35 y 89 kDa (Chang *et al.*, 2003). Mitsutomi *et al.* (1995) purificaron dos quitinasas de la cepa *Streptomyces griseus* HUT6037, ambas quitinasas presentan un peso molecular de 27 kDa y tienen una temperatura óptima de 55ºC. El agente de biocontrol *S. lydicus* WYEC108, es capaz de lisar las oosporas de *Phythium ultimum* y de igual forma causar daño en la estructura de la hifas. WYEC108 produce altos niveles de quitinasa cuando se encuentra creciendo con quitina obtenida de la pared celular de este hongo adicionada al medio de cultivo (Yuan and Crawford, 1995). El-Sayed et al. (2000) identificaron dos quitinasas, A y B, purificadas del sobrenadante de un cultivo de *Streptomyces albocinaceus* S-22, estas enzimas mostraron actividad antifúngica lisando la pared celular. Ambas enzimas tienen un peso molecular de 43 y 45 kDa y presentaron actividad óptima a una temperatura y pH de 40ºC y 5.6 respectivamente.

Los microorganismos quitinolíticos han demostrado ser eficientes para controlar enfermedades causadas por patógenos fúngicos (Nagpure *et al.*, 2013). Unos de los mecanismos responsables de la inhibición de hongos patógenos es la producción de enzimas líticas extracelulares, y es también uno de los mecanismos de biocontrol más importantes para controlar enfermedades de plantas (Gohel *et al.*, 2006).

BPCVs endofíticas y control biológico de hongos fitopatógenos

Las interacciones benéficas entre microorganismo-planta que promueven la salud y el desarrollo de éstas son de gran interés (Ryan *et al.*, 2008). Dentro de estas relaciones se encuentra una en especial que trataremos en este trabajo; los organismos endófitos son

un ejemplo de una interacción mutualista y se pueden describir como aquellos que pueden colonizar los tejidos internos de las plantas sin mostrar una señal aparente o efecto negativo hacia su hospedero (Schulz and Boyle, 2006). Se considera que cerca de 300 000 especies de plantas que existen en la tierra son hospederas de uno o más microorganismos endófitos y solo pocas de estas plantas han sido estudiadas para analizar sus organismos endófitos (Strobel *et al.*, 2004). Consecuentemente, la oportunidad de encontrar nuevos microorganismos endófitos de plantas en los diversos ecosistemas es considerable (Ryan et al., 2008). Muchos de los organismos endófitos colonizan diferentes compartimentos en la planta, en el apoplasto, incluyendo espacios intercelulares y haces vasculares; algunos colonizan órganos reproductivos de la planta, por ejemplo flores, frutas y semillas (Ryan et al., 2008).

Las bacterias endófitas colonizan un nicho ecológico similar al de los patógenos por que las hace adecuadas como agentes de biocontrol (Berg *et al.*, 2005). La colonización al interior de la planta inicia generalmente con el establecimiento de las bacterias en las raíces, seguido de la colonización en la rizósfera y posteriormente el anclaje al rizoplano (Balsanelli et al., 2010). Los sitios preferidos de adhesión de las bacterias y posterior entrada es la zona apical de la raíz (sitio activo de penetración), y la zona de raices basales con pequeñas grietas causadas por la emergencia de raíces laterales (zona de penetración pasiva) (Zachow et al., 2010). En la zona de penetración activa las bacterias endófitas deben contar con una batería de enzimas capaces de hidrolizar la pared celular de las raíces (Compant et al., 2005). Después de establecerse en la planta, los organismos endófitos pueden influenciar positivamente el crecimiento de la planta y su resistencia a diferentes condiciones de estrés (Ryan et al., 2008). En el presente trabajo se aborda el empleo de la bacteria *Bacillus cereus sensu lato* cepa B25 para el control biológico del hongo Fusarium verticillioides y se propone que ésta es capaz de controlar *Fusarium verticillioides* en maíz, debido a que ambos microorganismos compiten por el mismo nicho dentro de la planta como se aborda en el capítulo VI.

CAPÍTULO III. HIPÓTESIS Y OBJETIVOS

HIPÓTESIS

Las quitinasas secretadas por *Bacillus cereus* tendrán actividad antagónica y participarán en la supresión del crecimiento de *Fusarium verticillioides,* el agente causal de la pudrición de raíz, tallo y mazorca en cultivos de maíz.

OBJETIVO GENERAL

Demostrar que las quitinasas producidas por *B. cereus* B25 participan en la inhibición contra *Fusarium verticillioides.*

OBJETIVOS ESPECÍFICOS

1. Evaluar los niveles de expresión de los genes de quitinasas ChiA y ChiB de *Bacillus cereus B*25 es respuesta a quitina coloidal en un ensayo de antagonismo *in vitro* contra *Fusarium verticillioides*.

2. Evaluar las quitinasas recombinantes ChiA y ChiB de *Bacillus cereus* para demostrar su actividad anti fúngica y enzimática.

3. Determinar el patrón de colonización de raíz de maíz por *Bacillus cereus* mediante la técnica de hibridación fluorescente *in situ* con el fin de mostrar la localización de esta bacteria en el tejido vascular de la raíz.

CAPÍTULO IV. INDUCTION OF *Bacillus cereus* CHITINASES AS A RESPONSE TO LYSATES OF *Fusarium verticillioides*

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Abstract

This study was carried out to evaluate the relative expression and enzymatic activity of Bacillus cereus sensu lato B25 chitinases in response to a *Fusarium verticillioides* (*Fv*) lysate. Phylogenetic studies revealed that B25 chitinases are closely related to chitinases from other *Bacillus* species clustering with type A and B chitinases from *B. thuringiensis* and *B. cereus*. The transcript levels of both ChiA and ChiB chitinases showed peaks of accumulation at 72 and 24 h, respectively, after the addition of either fungal lysate or colloidal chitin. Furthermore, an induction of exochitinase and endochitinase activity was detected in the supernatant of B25 after addition of colloidal chitin and fungal lysate and putatively attributed to ChiA and ChiB, respectively. These enzymatic activities were induced after 12 h and remained constant up to 72 h after the addition of treatments. Combined, the responses of ChiA and ChiB to Fv lysate suggest that these chitinases may partake in the antagonistic mechanism that B25 exerts upon *Fv*, possibly resulting in fungal growth inhibition.

Introduction

Maize has a central role in the Mexican agriculture, and its importance as a crop is reflected in the large land area dedicated to its cultivation. This crop is affected by multiple pathogens, of which one of the most common is *Fusarium verticillioides* (*Fv*), triggering the development of *Fusarium* stalk, ear and root rot (SERR) with significant economic losses (Lizárraga-Sánchez *et al.*, 2015).

Chitin is the main structural component of the fungal cell wall, a homopolymer of β-1,4 N-acetyl-D-glucosamine (GlcNAc), the second-most abundant polysaccharide in nature after cellulose (Nagpure *et al.*, 2013). Fungi such as *Trichoderma* and bacteria from the genera *Bacillus*, *Serratia* and *Alteromonas* can hydrolyze chitin by means of chitinase production making them valuable for biotechnological control approaches, due to their potentially important role in the inhibition of pathogenic fungi (Ashwini and Srividya, 2014).

Chitinases from different *Bacillus* spp. exhibit antifungal activity against several phytopathogenic fungi (Liang *et al.*, 2014). *Bacillus cereus* strain CH2 has been reported to suppress *Verticillium* spore growth (Li *et al.*, 2008), whereas chitinases from the strain YQ 308 inhibit the growth of phytopathogenic fungi such as *F. oxysporum*, *F. solani* and *Pythium ultimum* (Chang *et al.*, 2003).

Recently, we screened a collection of 11,520 bacterial isolates from the rhizosphere of maize in order to identify a biological control agent for *Fusarium* SERR. From this work, three *Bacillus* isolates (*B. megaterium*, *Bacillus* sp. and *B. cereus sensu lato*) showing promising potential as antagonists against *Fv*, the causative agent of *Fusarium* SERR of maize were selected both *in vitro* and *in planta* (Figueroa-López *et al.*, 2016). Additional field trials have shown that strain B25 was the most effective bacterium at decreasing the incidence and severity of *Fusarium* stalk rot (FSR) and *Fusarium* ear rot (FER), as well as fumonisin levels in grain (Lizárraga-Sánchez *et al.*, 2015). Moreover, *in vitro* assays indicate that B25 produces siderophores and exhibits protease, glucanase and chitinase activities (Figueroa-López *et al.*, 2016). Based on these findings, coupled with the report that chitinase activity has an antifungal effect (Nagpure *et al.*, 2013). we hypothesized that B25 chitinases are part of the antagonistic mechanisms used by this strain against *Fv*. Some properties and characteristic on *Bacillus* chitinases have been reported. Nevertheless, the relative expression has not been studied in *B. cereus* chitinases. This work investigates the effect of *Fv* lysates on B25 ChiA and ChiB transcript levels and their corresponding enzymatic activities.

Materials and Methods

Organisms and culture conditions

The bacterial strain was stored at −70 °C in Luria Bertani (LB) broth (Sigma, Cat. No. L3022, St. Louis, MO, USA) with glycerol (15 %, v/v), activated on LB agar and cultured overnight at 25 °C for 24 h. The following day, one single colony was taken and placed on LB broth to be cultured for 24 h at 30 ºC and a bacterial suspension containing 1 x 10^6 c.f.u./ml was used in the experiments.

Fungal isolate *Fv* P03 was previously molecularly identified (Leyva-Madrigal *et al.*, 2014). This fungal strain is maintained as a frozen stock (-70 ºC) in potato dextrose broth (BD, Cat. No. 25492, Le Pont de Claix, France) containing 15% of glycerol.

Preparation of colloidal chitin and fungal lysate (chitin sources)

Colloidal chitin (CC) was prepared from commercial chitin powder (Sigma Aldrich, Cat. No. C7170, St. Louis, MO, USA) according to Wiwat *et al.* (1999). An *Fv* lysate was obtained from a 7-day old culture grown at 25 ºC in potato dextrose agar (BD, Cat. No. 213400, Le Pont de Claix, France). Mycelia were collected with a stainless steel spreader of bacteria in distilled water, dried in an oven at 50 ºC for 2 days, and subsequently ground up in a mortar (Anitha and Rabeeth, 2010). Heat treatment does not damage chitin composition of the fungal cell wall (Deguchi *et al.*, 2015). Thus, in order to obtain sterile fungal lysate (FL) this was autoclaved at 121 ºC for 15 min and stored at 4 °C until use.

Chitinase induction assay

B25 chitinase induction assays consisted of the addition of two chitin sources as chitinase inducers: colloidal chitin (CC) and fungal lysate (FL); the B25 strain without inducers or chitin served as the control. The induction experiment was performed in 500 ml flasks at 30 ºC for 72 h containing 100 ml of medium with the following components in g/l: 2 tryptone, 0.5 yeast extract, 1 NaCl, 0.125 KH_2PO_4, 0.125 K_2HPO_4, 0.05 calcium acetate and 0.05 magnesium acetate (Sato and Araki, 2007). The B25 strain was grown for 8 h in the medium described above, after this the chitinase inducers (CC and FL) were added at a concentration of 0.1 % w/v. At different time points (0, 12, 24,

72 h), 1 ml samples from each flask (three flasks per inducer used) were taken with a micropipette and placed in a 1.5 ml Eppendorf tube and centrifuged at 2,000 g for 5 min to separate bacterial cells, used for molecular analyses, from the culture supernatant employed for enzymatic activity assays. This experiment was performed by triplicate.

Quantitative PCR (qPCR)

Cell pellets from 1 ml of cell culture were collected and 300 µl of lysis buffer (0.03 M Tris-HCl, 0.01 M EDTA and 20 g/l lysozyme) were added and incubated for 30 min at 37 ºC. Total RNA was isolated using TRIzol® Reagent (Thermo Fisher Scientific, Cat. No. 15596-026, Waltham, MA, USA), according to the manufacturer's instructions. RQ1 DNAse (PROMEGA, Cat. No. M6101, Fitchburg, WI, USA) was used to avoid DNA contamination. First-strand cDNA was prepared from total RNA using random hexamers with SuperScript™ III reverse transcriptase (Thermo Fisher Scientific, Cat. No. 18080-044, Waltham, MA, USA), following the manufacturer's instructions. Reagents and qPCR conditions were prepared as described in (Cervantes-Gámez *et al.*, 2015). All qPCR reactions were performed in a Rotor Gene-Q Real time PCR system instrument (Qiagen, Cat. No. 9001550, Hilden, Ger.) using SYBR Green Master Mix (Qiagen, Cat. No. 204074, Hilden Ger.). For PCR amplification, the thermocycler was programmed for 40 cycles at 95 °C for 5 s and 60 °C at 10 s, after an initial denaturation at 95 ºC for 5 min. Dissociation curves were performed at the end of each run to confirm single amplifications. The 30S ribosomal protein 21(rpsU) was used for data normalization (Table 1) (Reiter *et al.*, 2011). Two primer pairs were designed for each gene, based on the B25 chitinase nucleotide sequences allowing for amplification of two different nucleotide regions (Table 1). The comparative threshold cycle method $2^{-\Delta\Delta Ct}$ was used to analyze relative mRNA expression, as previously reported (Cervantes-Gámez *et al.*, 2015). In this method, the expression of the chitinase gene was normalized according to rpsU gene expression across all treatment conditions. Subsequently, the normalized expression of each treatment was compared to that of the control condition. The result was used to determine the relative expression (i.e. the $2^{-\Delta\Delta Ct}$ value).

Table 1. Oligonucleotides used for qPCR

Gene	Position	Oligo sequence 5'→3'	Reference
Chi A	64f	CCTTTCCAAGCACAAGCAG	This study
	166r	TCCCATTTTGGTGAAACGTC	
Chi A	557f	GCATGGCTCCTGAAACAGC	This study
	692r	CTACCAGCGTTGTAGTGTTG	
Chi B	391f	TCAGGGACAACTTGGGAAG	This study
	513r	CCAAGTCCAGCCACCAAC	
Chi B	1561f	GCTGGAGAAGAGAAATGGAG	This study
	1673r	GATTTATTTCCAGCAGCATC	
rpsU		GTCTTTGGAGGATGCACTTCG	(Reiter *et al.*, 2011)
		GCTTTCTTGCCGCTTCAGAT	

Chitinase activity

The substrate-specific chitinase activity was determined using a chitinase assay kit (Sigma Aldrich, Cat. No. C7170, St. Louis, MO, USA). One unit of chitinase activity was defined as the amount of enzyme required to release 1 μmol of 4-methylumbelliferone from the substrate per minute at pH 5.0 and 37 ºC. Each type of enzymatic activity was assayed using three biological replicates per sampling point in two independent experiments.

Phylogenetic analysis

Sequences were obtained from the B25 genome sequencing analysis conducted in our laboratory (unpublished results). Chitinase sequences were deposited in GenBank at the NCBI (National Center for Biotechnology Information) under accession numbers KR809875 (ChiA) and KR809876 (ChiB). Nucleotide sequences of the B25 chitinases were compared in GenBank using the BLAST-N and BLAST-X algorithms. MEGA 6.06 (Tamura *et al.*, 2011) was used for alignment and phylogenetic analysis. Deduced amino acid sequences were aligned using the MUSCLE alignment program (Edgar, 2004). The phylogenetic tree was constructed using the Whelan and Goldman (WAG) model and the maximum likelihood (ML) method. Tree topology support was assessed by 1000 bootstrap replicates.

Statistical analysis

The results were analyzed using SAS software version 9 (SAS Institute Inc., Cary, NC, USA). Chitinase activity data were subjected to a repeated-measure analysis of variance (ANOVA, PROC MIXED procedure), to analyze the effects of treatment, time and their interaction on the measured variable. Data were fitted to different covariance structures and the best fit was used for further analysis. Heterogeneous autoregressive structure was assumed for endochitinase activity and Toeplitz with two bands structure for exochitinase activity. Tukey's adjusted least-square-means test was used to assess the differences between treatments ($P<0.05$). All tests were carried out using triplicate samples and were performed at least twice.

Results and Discussion

Sequence analysis of B25 chitinases

The ChiA and ChiB chitinases from B25 share similar features with other chitinases reported from *B. cereus* CH (Mabuchi and Araki, 2001) and *B. thuringiensis* serovar *sotto* (Zhong *et al.*, 2005). The B25 ChiA gene contains a 1083 nucleotide-long open reading frame (ORF) that encodes a 360 amino acids peptide, with a calculated molecular mass of 39.4 kDa and a theoretical isoelectric point of 7.36 (Acc. No. KR809875) (Figure 1). The ChiB gene contains a 2025 nucleotide-long ORF encoding a 674 amino acids peptide, with a calculated molecular mass of 74.2 kDa and a theoretical isoelectric point of 5.88 (Acc. No. KR809876) (Figure 2). A putative Shine-Dalgarno sequence (AGGAG) located 8-9 bp upstream of the ATG initiation codon was previously predicted (Huang *et al.*, 2005).

Our analysis of the ChiA and ChiB sequences revealed the presence of predicted signal peptides (SignalP 4.0) at their N-terminal regions (27 and 32 amino acids, respectively), providing evidence that these are secreted proteins. In addition, both ChiA and ChiB contain within their active sites three essential conserved amino acid residues in a DxDxE motif; this motif is highly conserved in a variety of chitinases (Yamabhai *et al.*, 2008) (Figure 1 and 2). The catalytic domain of ChiA shows homology with type A chitinases from *B. cereus* (Sato and Araki, 2007) and *B. thuringiensis* (Murawska *et al.*, 2013) (Figure 1).

Multiple sequence alignment revealed several amino acid substitutions that characterize the ChiB sequence: in position 13, the leucine observed in type B chitinases from other *Bacillus* strains is replaced by an isoleucine; and the Asp-190

within the ChiB active site differs from the other four *Bacillus* chitinases, which all contain Glu-190 (Figure 2). The ChiB catalytic domain is categorized as belonging to the family of 18-glycosyl hydrolases on the basis of amino acid sequence (Henrissat and Bairoch, 1993). Similar to other *Bacillus* chitinases, the ChiB protein contains a fibronectin type-III like domain (FnIII) and a cellulose-binding domain in the C-terminal region (Figure 2) (Driss *et al.*, 2005). These sequence and domain analyses confirm the categorization of B25 ChiA and ChiB as type A (exochitinase activity) and B (endochitinase activity) chitinases, respectively.

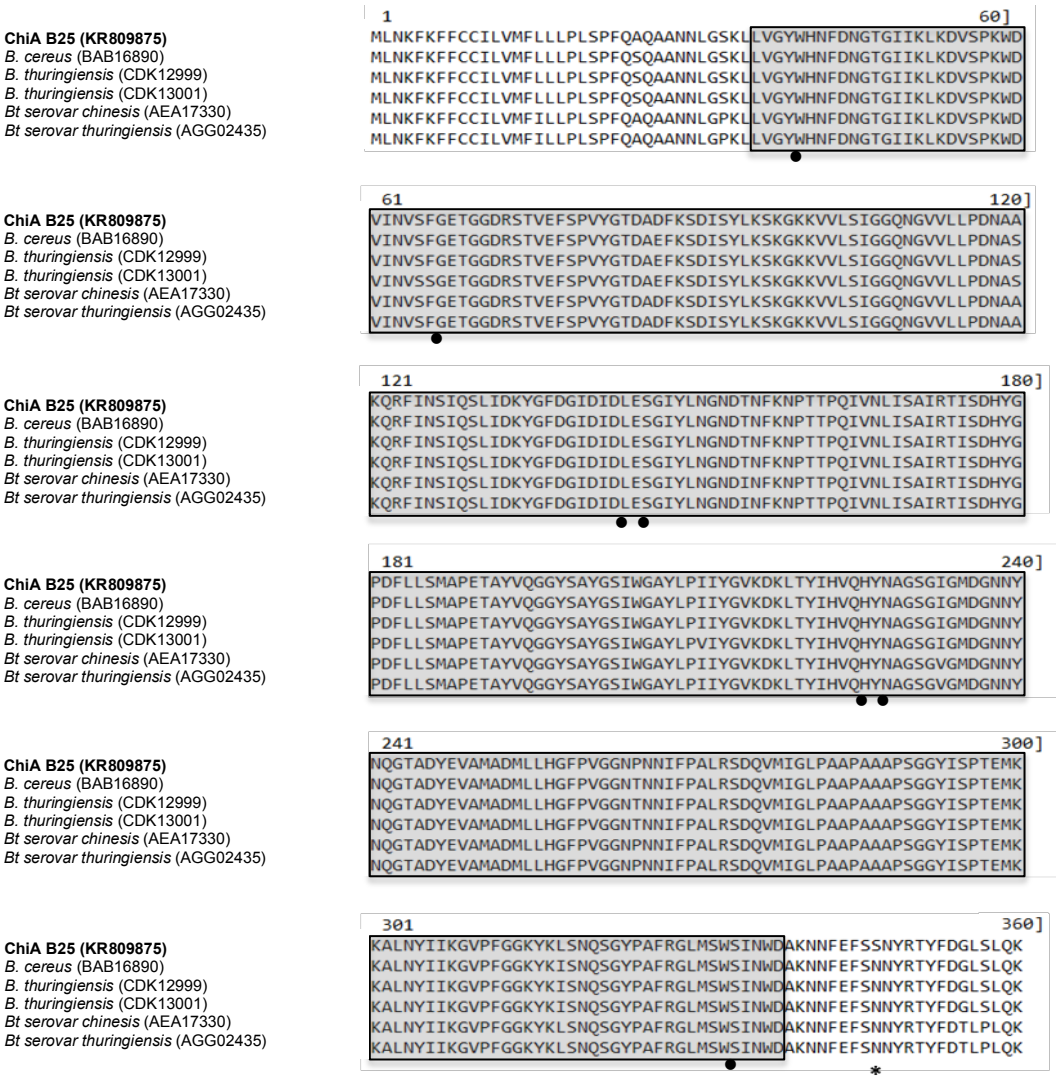

Figure 1. Multiple sequence alignment of the deduced amino acid sequence of ChiA chitinase of *Bacillus cereus sensu lato* B25 (bold case). An amino acid change detected in the *B. cereus* B25 chitinase respect to the other included sequences is indicated with an asterisk. A gray box indicates the GH18 glycosyl hydrolase domain. Amino acids of the active site are indicated with dots.

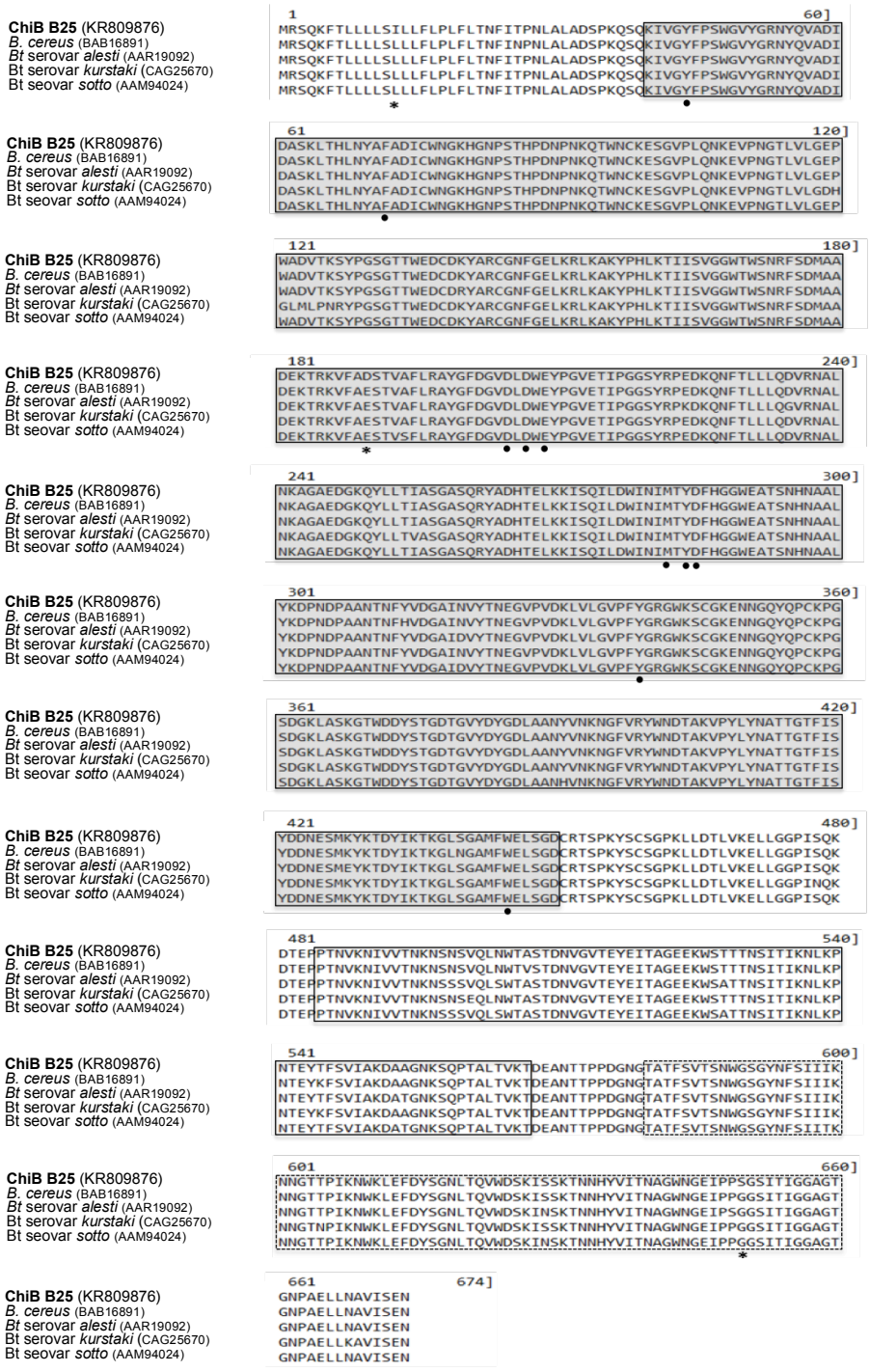

Figure 2. Multiple sequence alignment of the deduced amino acid sequence of ChiB chitinase of *Bacillus cereus sensu lato* B25 (bold case). Amino acid changes detected in the ChiB chitinase respect to other chitinase sequences included are indicated with asterisks. A gray box indicates the GH18 glycosyl hydrolase domain. An open box indicates the fibronectin type III domain. The cellulose-binding domain is represented by a dotted box. Amino acids of the active site are indicated with black dots.

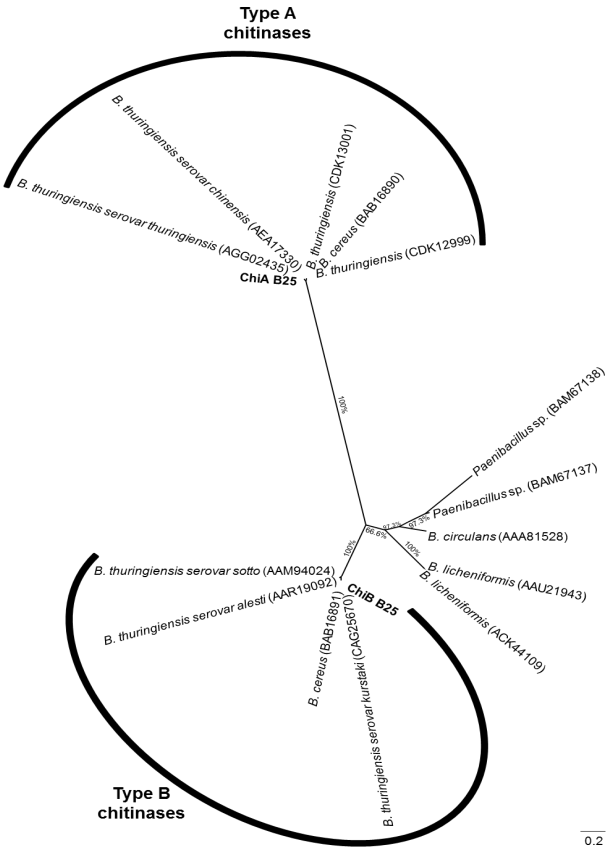

Figure 3. Maximum likelihood tree (log likelihood = -9212.82) based on complete amino acid sequences of type A and B chitinases from different *Bacillus* species. The tree was constructed with Mega 6.0 (bootstraps = 1000), using the Whelan and Goldman (WAG) substitution model with gamma distribution (+G). Chitinase sequences from *B. cereus* B25 are shown in boldface. The corresponding sequences of *Paenibacillus* sp. were used as an out-group. Database accession numbers of the sequences are provided in parentheses. Bootstrap values are shown as percentages. The scale bar indicates the expected number of amino acid substitutions per unit branch length.

Phylogenetic relationship of ChiA and ChiB

Phylogenetic analysis of the B25 ChiA and ChiB sequences indicates that they cluster with type A and type B chitinases, respectively, and are most closely related to chitinases from *B. thuringiensis* and *B. cereus* (Figure 3). These results complement our findings from the sequence and domain analyses of ChiA and ChiB (Figure 1 and 2). Specifically, *B. thuringiensis* and *B. cereus* belong to the *B. cereus* group (also composed of *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis*), although it is difficult to differentiate the identity of these species. *Bacillus thuringiensis* produces crystal proteins during sporulation, and this feature is used to phenotypically distinguish it from *B. cereus* (Rasko *et al.*, 2005). Overall, several studies have revealed that these species are quite similar genetically, and may even

constitute a single species (Zwick *et al.*, 2012), resulting in the term *B. cereus sensu lato* to describe members of this species complex.

ChiA and ChiB transcript levels increase in response to the inducers colloidal chitin (CC) and fungal lysate (FL)

Chitinases play an important role in fungal pathogen control, and several studies have shown that application of fungal cell walls to bacteria induces bacterial chitinases (Anitha and Rabeeth, 2010). The relative expression of the B25 ChiA and ChiB genes was evaluated by quantitative PCR, in order to investigate their responses when challenged with colloidal chitin and fungal lysate. Colloidal chitin was used as an induction control of chitinases transcription (Liu *et al.*, 2011). Both chitinases transcripts were detected from zero time, this supports their constitutive expression as reported before for other *B. cereus* strains (Sato and Araki, 2007). In the presence of fungal lysate, ChiA transcript levels increased along time and a peak of induction at 72 h with 7.3-fold change, whereas in colloidal chitin the induction was 4.2-fold change relative to the rpsU control gene (Figure 4A). ChiB gene expression was induced by colloidal chitin and fungal lysate; an induction peak was found at 24 h, showing the highest induction when the fungal lysate was added with 8.6-fold change (Figure 4B).

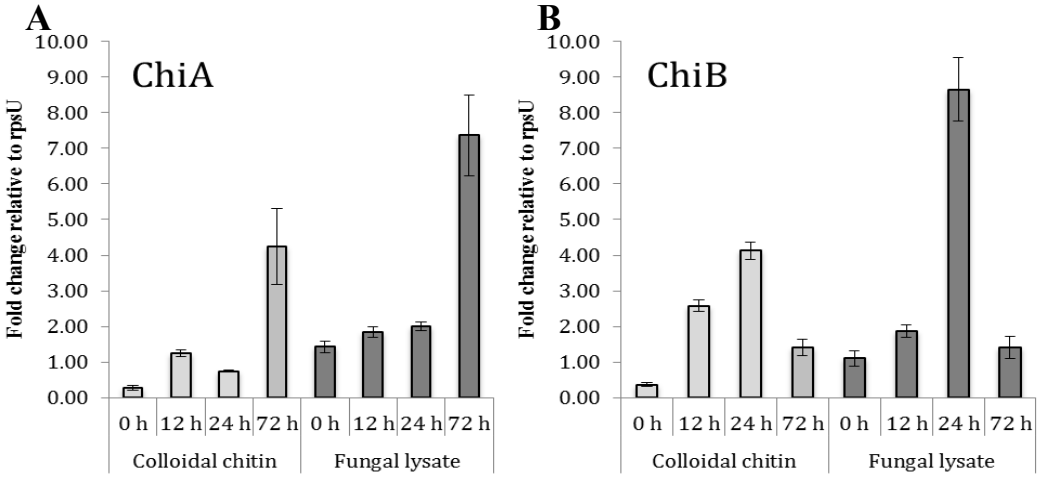

Figure 4. Differential expression of *B. cereus sensu lato* B25 ChiA and ChiB chitinases, induced by colloidal chitin and fungal lysate. The relative expression of ChiA and ChiB under these treatments (in comparison to rpsU in the control condition at each time) is presented in A) and B), respectively. Error bars indicate the standard deviation.

The sequential increase in ChiB (24 h) and ChiA (72 h) transcript levels suggests that both genes might act together to degrade chitin from the fungal lysate in a

time-coordinated manner. It has been reported that gene expression in *B. cereus* CH chitinases is induced by a variety of chitin oligomers since 12 h of induction (Sato and Araki, 2007). However, little information is available on how chitinase transcript levels change in response to phytopathogenic fungal lysates. Our results demonstrate that both colloidal chitin and fungal lysate are good inducers of B25 ChiA and ChiB expression. Furthermore, these responses may be part of the mechanism that enables degradation of chitin in the fungal cell wall.

Extracellular chitinase activity

Next, we investigated the presence of extracellular chitinase activity in the supernatant culture media. We assumed that when the bacterium is grown in liquid medium added with colloidal chitin or fungal lysate, the supernatant of the culture media will contain ChiA and ChiB proteins, since both chitinases contain signal peptides that could allow for their extracellular allocation. We then quantitated the different types of chitinase activity using various fluorochromic substrates that can distinguish diverse endo- and exochitinase activities. A significant increase in both exo- (4-fold increase on average) and endochitinase (2-fold increase on average) activities was observed for both colloidal chitin and fungal lysate treatments with respect to the control condition (Table 2, significant treatment effect; Table 3). This increase was detected at 12 h and remained constant throughout time (Table 2, significant time effect; Table 3), as reported by Sato and Araki (2007). No significant differences were observed for the endo- and exochitinase activities after 12 h of induction at any other time between colloidal chitin and fungal lysate (Table 3).

Table 2. Summary of repeated measure analysis of variance (ANOVA) for chitinase activity of *Bacillus cereus sensu lato* B25 at four different times.

Effect	DF[a]	F[b]	P[c]
Endochitinase activity			
Treatment	2, 6	302.92	<0.0001
Time	3, 18	1215.04	<0.0001
Treatment *Time	6, 18	190.34	<0.0001
Exochitinase activity			
Treatment	2, 3	1164.75	<0.0001
Time	3, 9	1768.73	<0.0001
Treatment *Time	6, 9	131.49	<0.0001

[a] Numerator, denominator degrees of freedom (Proc Mixed, SAS).[b] Fisher test. [c] Probability.

On the other hand, we did not find a direct correlation between transcripts accumulation of ChiA and ChiB and chitinase activity. Exochitinase activity was

detected at the starting point of the experiment when the colloidal chitin and fungal lysate were added. Other secreted chitinases from *B. cereus* and *B. thuringiensis* (Wang *et al.*, 2001) sharing high homology (98%) with ChiA from this report (Data not shown) also act as exochitinases (Li *et al.*, 2008). We only can suggest that ChiA may act as an exochitinase based on its similarity to other ChiA proteins. The peak of induction for the ChiB gene was observed at 24 h (Figure 4B). Using a combination of gene cloning and expression analysis, Chen *et al.* (2009) demonstrated that the activity of a *Bacillus cereus* ChiB gene sharing a 97% amino acid sequence similarity with the ChiB gene from this study. Other chitinases similar to ChiB have been characterized as endochitinases from *Bacillus cereus* and *B. thuringiensis* (Casados-Vázquez *et al.*, 2015). It is possible to suggest B25 ChiB may act as an endochitinase.

The lysis process of insoluble chitin consists of three main steps: (1) cleavage of the polymer into water-soluble oligomers; (2) splitting of these oligomers into dimers; and (3) cleavage of dimers into monomers (Beier and Bertilsson, 2013). We suggest that ChiB could possibly act as an endochitinase that generates chitin dimers and/or oligomers; after their release, these products would then become substrates for exochitinases such as ChiA, which could degrade them into monosaccharides. This agrees with enzymatic activity measurements showing the induction of endo- and exochitinase activities after 12 h of culture under colloidal chitin and fungal lysate treatments (Table 3). Since transcripts for both chitinases are present from the beginning of the experiment (Figures 4A, B) it is possible to suggest that: 1) ChiA and ChiB transcripts level might be sufficient to cause an accumulation in the ChiA and ChiB protein amount and an increase in their enzymatic activity (Table 3); 2) the presence of the ChiA and ChiB proteins since the beginning of the experiment (Table 3) can cause the accumulation in time of chitin oligomers that may induce enzyme activity as reported by Sato and Araki (2007); 3) pre-made ChiA and ChiB proteins are only activated by the addition of colloidal chitin or fungal lysate (Nielsen *et al.*, 2011). The assay for the exochitinase activity with 4-Methylumbelliferyl N-acetil-β-D-glucosaminide was carried out, but this activity was no detected in the culture medium.

Table 3. Chitinase activity measured in supernatants samples of induction experiment. Exochitinase activity using 4-Methylumbelliferyl N,N-´diacetyl-β-D-chitobioside as the substrate. Endochitinase activity using 4-Methylumbelliferyl β-D-N,N´, N´´-triacetylchitotriose as the substrate.

	Chitinase activity (U/ml)							
Treatment	Endochitinase				Exochitinase			
	0 h	12 h	24 h	72 h	0 h	12 h	24 h	72 h
Control	6.6±1.24 a, A	20.6 ±0.72 a, B	18.8±0.62 a, B	12.7±0.01 a, B	33.8±0.59 a, A	119±4.47 a, B	119.9±1.92 a, B	121.9±0.74 a, B
Colloidal chitin	4.5±0.30 ab, A	79.2±5.32 b, B	75.1±7.93 b, B	79.9±1.93 b, B	34.9±0.94 a, A	284±3.58 b, B	270.1±0.04 b, B	272.4±6.34 b, B
Fungal lysate	2.5±0.15 b, A	84.5±2.6 b, BD	72.8±0.58 b, C	80.8±10.1 b, CD	32.6±0.91 a, A	274.4±9.14 b, B	280.1±13.91 b, B	262.8±2.74 b, B

Different lower case letters in the same column indicate differences ($P<0.05$) between treatments at a given time. Different upper case letters in the same line indicate differences ($P<0.05$) between times in a given treatment. U: the amount of enzyme needed to release 1 µmol 4-methylumbelliferone from the substrate per minute at pH 5.0 and 37 °C.

The relative expression peaks for ChiB at 24 h and ChiA at 72 h could be related to the increase on chitin oligosaccharides generated by ChiB subsequently used by ChiA: however we cannot currently substantiate this since we found no direct correlation between the relative expression of chitinase and enzymatic activity. Recent advances in post-translational studies of the regulatory processing in mRNA and proteins, found that the abundance of protein may or not correlate with the mRNA levels due the RNA is less stable than proteins (Vogel and Marcotte, 2012). The mRNA half-life of ChiA and ChiB in *B. cereus* have not been studied but in *B. subtilis* is about 7 min (Hambraeus *et al.*, 2003), while in *B. licheniformis* the half-life of the chitinase protein has been calculated as long as 20 days when grown at 37 ºC (Nguyen *et al.*, 2012).

Conclusion

We identified two chitinases in the genome of *Bacillus cereus sensu lato* B25, ChiA and ChiB, which were putatively identified as exo- and endochitinase respectively, by sequence analysis and comparison to other sequences previously reported for other *Bacillus* species. Both chitinases were induced by colloidal chitin and fungal lysate, showing the possible role of these enzymes on fungal inhibition as a part of a broad range of mechanisms that the bacterium employs to inhibit fungal growth. The lack of correlation between the expression and enzymatic activity results may be due to the different mechanisms of RNA and protein processing. To confirm these findings,

cloning, expression, purification and enzymatic characterization of these two genes are currently being addressed in our laboratory.

CAPÍTULO V. CLONING, EXPRESSION AND PURIFICATION OF ChiA AND ChiB *Bacillus cereus* B25 CHITINASES

Manuscript to be submmited in Microbial Biotechnology

Abstract

Chitin, a β-(1,4)-linked polymer of N-acetyl D-glucosamine (GlcNAc), is widely distributed as a structural polysaccharide and can be found in fungal cell walls and the exoskeletons of arthropods. Microorganisms from very diverse genera have been reported to produce chitinases including the *Bacillus* species. Chitinases have received increased attention in recent years due to their wide range of biotechnological applications. *Bacillus cereus* B25 posseses two chitinases (ChiA and ChiB) as mentioned in the previous chapter and the aim of this study was the expression and purification of both recombinant proteins for further caracterization. The chitinase B gene from *Bacillus cereus sensu lato* B25 was cloned in *Escherichia coli* DH5α and expressed on *Escherichia coli* BL21 Star™ (DE3) using a Gateway technology. Previously the chitinase was pre-characterized as an endochitinase based on finding and sequence analysis. The purification of recombinant protein ChiA could not be accomplished, possible due to the formation of inclusion bodies in the recombinant bacteria. The expression and purification of recombinant protein ChiB allowed evaluating its enzymatic activity and confirming the results observed in previous results. The chitinase assay with specific substrates showed endochitinase activity as expected, but the susbtrate for chitobiosidase activity (exochitinase) was hydrolyzed too; suggesting that the recChiB have two kinds of chitinase activities. The elucidation of chitinase activity provides us with more clues about how *Bacillus cereus sensu lato strain* B25 degrades chitin, the recChiB was able to hydrolyze chitotriose and chitobiose, this is a very interesting feature and it may constitute a possible major mechanism to affect fungal growth.

Introduction

Chitin, a β-(1,4)-linked polymer of N-acetyl D-glucosamine (GlcNAc), is widely distributed as a structural polysaccharide and can be found in fungal cell walls and the exoskeletons of arthropods (Lenardon *et al.*, 2010). Although it is not present in plants, chitin is the most abundant organic compound following cellulose (Kurita, 2000). Chitin is structurally identical to cellulose, with the exception that its acetamide group (-NHCOCH3,-NAc) is replaced by a hydroxyl (–OH) group at C2 (Kurita, 2000). Glycosyl hydrolases cleave the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Davies and Henrissat, 1995). This catalytic glycosyl hydrolase domain is common to all chitinases (EC 3.2.1.14) which fragment chitin by breaking the β-1,4-glycosidic bonds between N-acetyl-glucosamine chitin residues (Li and Greene, 2010). Chitinases play very diverse roles in the organisms. For example, they have been implicated in resistance against plant fungal pathogens due to their inducible nature and antifungal activities *in vitro* (Taira *et al.*, 2002); they are also thought to have autolytic, nutritional, and morphogenetic roles in fungi (Reetarani *et al.*, 2000). Chitinases can be classified into two major categories: endo- and exochitinases. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating soluble low molecular weight oligomers of N-acetylglucosamine (*e.g.* chitotetraose, chitotriose and the dimer di-acetylchitobiose) (Cohen-Kupiec and Chet, 1998). Exochitinases are divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of di-acetylchitobiose starting from the non-reducing end of the chitin microfibrils; and 1-4-β-N-acetylglucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GIcNAc (Cohen-Kupiec and Chet, 1998). Microorganisms from very diverse genera have been reported to produce chitinases (Sharma *et al.*, 2011), and can be used in free or immobilized form against fungi. Chitinolytic microorganisms such as bacteria are a great source for obtaining various chitinases, due to their low production cost and the availability of raw materials (Sharma *et al.*, 2011). Chitinases have received increased attention in recent years due to their wide range of biotechnological applications. This includes uses during fungi protoplast preparation (Yabuki *et al.*, 1984); as a protective agent against fungal

phytopathogens (Shali *et al.*, 2010; Taira *et al.*, 2002); and as biologically active substances in the production of oligosaccharides (Usui *et al.*, 1990).

Chitinase-producing microorganisms are reported to be effective in controlling crop plant diseases caused by different fungal pathogens (Nagpure *et al.*, 2013). A variety of mechanisms exist for plant disease control, including the production of extracellular lytic enzymes that inhibit pathogenic fungi. Specifically, the ability of certain bacteria (especially Actinomycetes) to parasitize and degrade spores of fungal plant pathogens has been well established (Gohel *et al.*, 2006). Although cell wall-degrading enzymes produced by biocontrol strains of bacteria have been documented, there is little direct evidence for their presence and activity in the rhizosphere (Chang *et al.*, 2010).

Several chitinase-producing bacterial species have been reported with wide practical applications, notably including *Ewingella americana* (Inglis and Peberdy, 1997), *Massilia timonae* (Adrangi *et al.*, 2010), *Microbispora* sp. V2 (Nawani *et al.*, 2002), *Micrococcus* sp. AG48 (Annamalai *et al.*, 2010), *Monascus purpureus* (Wang *et al.*, 2002), *Paenibacillus* sp. D1 (Singh and Chhatpar, 2011), *Paecilomyces variotii* (Nguyen *et al.*, 2009), *Pseudomonas aeruginosa* K-187 (Wang and Chang, 1997), *Pseudomonas* sp. TKU008 (Wang *et al.*, 2010), *Ralstonia* sp A-471 (Ueda *et al.*, 2005), and *Serratia marcescens* NK1 (Nawani *et al.*, 2002).

Bacillus cereus B25 posseses two chitinases (ChiA and ChiB) as mentioned in the previous chapter and the aim of this study was the expression and purification of both recombinant proteins for further caracterization. Previously in chapter IV, we assayed the *Bacillus cereus sensu lato* B25 on culture media containing colloidal chitin and fungal lysate to induce the expression of both genes and then measured the chitinase activity on culture medium (Chapter IV. Table 3). At the same time the secuence analyses of both chitinases was carried out, showing that ChiA has the same protein domains present in the type A chitinases of several *Bacillus* species, this indicated us that chitinase A will be an exochitinase as it is reported. In the similar way, the ChiB showed the same domain structure of the type B chitinases of *Bacillus* species reported as endochitinases.

Based on these observations and the evidence of the presence of two chitinase activities found in supernatant of medium where *Bacillus cereus sensu lato* B25 was grown (exochitinase activity using 4-Methylumbelliferyl N,N-´diacetyl-β-D-chitobioside as a substrate, and endochitinase activity using 4-Methylumbelliferyl β-D-N,N´, N´´-triacetylchitotriose as a substrate.), we hypothesized that each of the two

genes contained one type of enzymatic activity and we postulated ChiA to have exochitinase activity and ChiB endochitinase activity.

Materials and Methods

DNA isolation and plasmid constructions

Genomic DNA of *Bacillus cereus* B25 was isolated using the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's instructions. The primers for the PCR amplifications were designed according to the chitinase sequences (GenBank accession numbers: KR809875 for ChiA and KR809876 for ChiB) of *Bacillus cereus* B25 obtained from its genome sequencing data (Douriet-Gámez *et al.*, 2016) including a TOPO adapter for directional cloning; for ChiA amplification G2ChiA-TP-F (5´-CACC ATG TTA AAC AAG TTC AAA TTT TTT TGT TGT ATT TTA- 3´) and G2ChiA-R (5´- TTA TTT TTG CAA GGA AAG ACC ATC-3´) primers were used; for the ChiB gene ChiBTP-F (5´CACC ATG AGG CTC CAA AAA TTC ACA CTG CTA TTA CTA 3`) and ChiBTP-R (5´ CTA GTT TTC GCT AAT GGA TGC ATT TAC AAG TTC 3´) were used. The conditions for a standard PCR reaction were as follows Buffer 1X, 1.5 mM $MgCl_2$, 0.5 mM of each primer, 500 μM each deoxynuleotide triphospate (dNTP), and 1 U of Platinum Taq DNA polymerase (ThermoFisher Scientific) in a volume of 25μl. PCR amplification was performed using the following program: 1 cycle at 94 ºC for 5 min, 30 cycles at 94 ºC for 30 s, 60 ºC for 20 s and 72 ºC for 2 min and an aditional final step at 72 ºC at 10 min. The amplified gene products were visualized using ethidium bromide after agarose gel electrophoresis and purified using QIAquick Gel Extraction Kit (QIAGEN). The products were ligated into pGEM-T easy vector (Promega; Madison, WI, USA) following the manufacturer's instructions. The plasmids were tranformed in *E. coli* DH5α chemical competent cells, positive colonies were verified by restriction (NcoI-EcoRI for ChiA/pGEM-T and NcoI for ChiB/pGEM-T) and colony PCR; plasmid was isolated by QIAprep Spin Miniprep kit (QIAGEN). For the expression of both genes they were amplified with Accuprime™ Pfx Polymerase (ThermoFisher Scientific) using the ChiA-ChiB/pGEM-T plasmid as a DNA template to generate blunt-end PCR products and they were purified using QIAquick PCR purification kit (QIAGEN), the products were ligated on pENTR™/D-TOPO® and transformed on OneShot®TOP10 Chemical competents *E.coli* cells

(ThermoFisher Scientific), positive colonies were verified by restriction analysis (AseI for ChiA-pENTR/D-TOPO and NheI-EcoRV for ChiB-pENTR/D-TOPO) and sequencing. The plasmids were isolated and used to carry out an LR recombination reaction (ThermoFisher Scientific) to insert the genes into Gateway pDEST17 expression vector. This vector has a 6xHis tag fusion needed for the purification of the recombinant protein. At this step an expression clone was generated that was transformed in *E. coli* BL21 Star^{TM} (DE3) chemical competent cells.

Expression of recombinant chitinases

The expression of chitinases from *Bacillus cereus* B25 was caried out as follows: one tube with 5 ml of LB broth containing 100 μg/ml of carbenicillin was inoculated with a single colony containing the expression vector pDEST17/ChiB and incubated on a rotary shaker at 200 rpm/min at 37ºC until they reached an OD_{600} of 0.6-1. One tube for ChiA and ChiB. The LB culture of ChiA and ChiB were splitted to inoculate two flasks with 2.5 ml for each transformant (two for ChiA and two for ChiB) on a 15 ml of fresh LB broth containing 100 μg/ml of carbenicillin. One of the cultures were added L-arabinose at the final concentration of 0.2 % w/v. One flask contains L-arabinose (induced) and one flask serve as control condition (uninduced) for each ChiA and ChiB. In a control used to supress the expression, glucose was added at the final concentration of 0.2 % w/v. The expression experiment was incubated at 37 ºC for 6 h in a rotary shaker at 200 rpm/min. Cells were harvested by centrifugation at 14 000 rpm for 5 min and freezed at -20 until use. For the protein analysis the cell pellets were suspended in 350 μl on lysis buffer (20 mg/ml lyzosyme; 10 mM Tris-HCl, pH8) and cell debris was colected by centrifugation at 8000 rpm for 2 min, which was used for subsequent protein purification by immobilized metal affinity columns. The protein purification was performed according to the Ni-NTA Spin kit manufacturer instructions (Qiagen, Cat. No. 31314). The column was equilibrated with Buffer B according to the purification protocol and was done under denaturing conditions. After the protein has been added it was washed three times with Buffer C from the kit and the protein was eluted with Buffer E. In the purification procces all the flowtrough wash steps were recovery and the two final elution steps were stored at -20 ºC. the major purified protein concentration were recovery in the final elution step in 200 μl final volume. All the flowtrough samples were analized by SDS-PAGE at 12 % (80v for 2 h). The purified enzyme was stored at 4ºC for subsequent characterization.

Chitinase activity

The substrate-specific chitinase activity was determined using a chitinase assay kit (Sigma Aldrich, Cat. No. C7170, St. Louis, MO, USA). One unit of chitinase activity was defined as the amount of enzyme required to releasing 1 μmol of 4-methylumbelliferone from the substrate per minute at pH 5.0 and 37 ºC. Each recombinant chitinase activity (endo and exo) was assayed using three replicates.

Zymogram to evaluate chitinase activity

The zymography was performed as follows; standard SDS-PAGE (12% acrylamide) was performed adding to the gel colloidal chitin at a final concentration of 0.3%. The SDS-PAGE gel was run in a vertical chamber during 2h at 80v, the polyacrilamide gel was incubated on sodium acetate buffer (0.1 M, pH 6, Triton X100 5%) for 1 h and subsequently incubated overnight on a rocking table at 37ºC on sodium acetate buffer (0.1 M, pH 6). Chitinase activity bands were visualized by Congo red staining for 20-30 min at room temperature and then unstained until the excess of Congo red was eliminated with 1M of NaCl. Chitinase activity was positive when a clear band was developed with the dye.

Results

Expression of recChiB and recChiA

The ChiB gene coding for one chitinase from *Bacillus cereus* B25 was successfully cloned into the pDEST17 expression vector and inserted in *E. coli* BL21. This expression vector contains the ChiB gene fused to a 6xHis tag used for the purification of the recombinant protein. After 4 h of induction of recombinant ChiB, the cells were harvested, lyzed and analized by SDS-PAGE to verify production of recChiB.

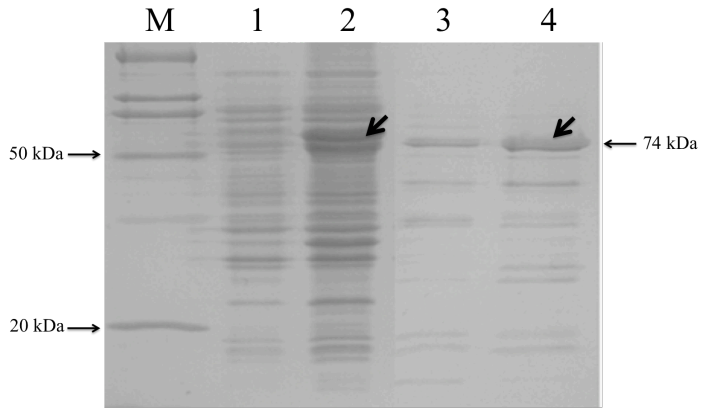

Figure 1. SDS-PAGE analysis of ChiB recombinant protein. M, marker proteins (Benchmark protein ladder); 1, insoluble fraction uninduced; 2 insoluble fraction induced; 3, soluble fraction uninduced and 4, soluble fraction induced. In all lines 5 µl of sample were added.

The insoluble fraction seemed more appropiate to use for the purification step, as shown in the figure 1, since a major band corresponding to a peptide with the reported molecular mass for ChiB (~74 kDa) (Huang *et al.*, 2005) was induced (Figure 1, lane 2).

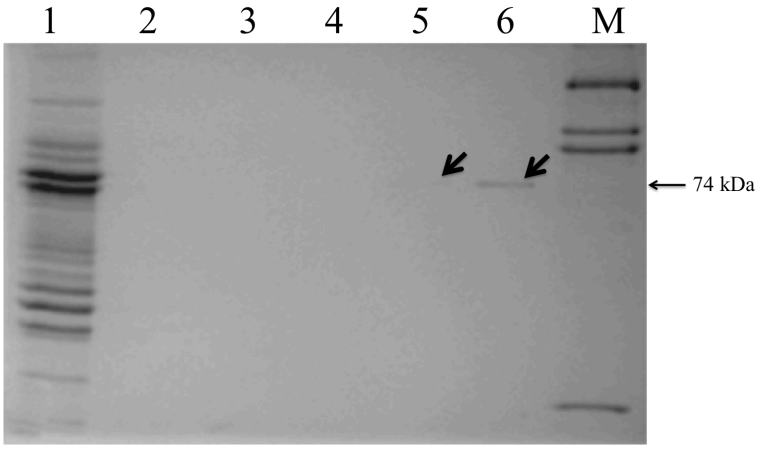

Figure 2. SDS-PAGE analysis of purified recombinant chitinase ChiB. Lane 1, crude extract of induced cells; lane 2, first wash with buffer C; lane 3, second wash with buffer C; lane 4, third wash with buffer C; lane 5, first elution; lane 6, second elution of recombinant protein. M, molecular marker. In all lines 5 µl of sample were loaded.

The purification of the recombinant ChiB (recChiB) was performed as shown in figure 2, the purified His-tagged chitinase B showed one single band in the elutions steps 5 and 6. The purified protein shows a ~74 kDa molecular weight band which corresponds to the reported size of *Bacillus* ChiB (Huang *et al.*, 2005).

The purification of recChiA was not succesful, we atributed the failure to purify it to the formation of inclusion bodies on the recombinant strain of *E. coli*. When the *E.coli* is used for the expression of recombinant proteins the accumulation of the foreign pepdtide can form aggregates of recombinant proteins called inclusion bodies, this inclusion bodies can be active or inactive (Martínez-Alonso *et al.*, 2009). We tried measuring chitinase activity of the cell pellets without any success. Further work will be required to purify and characterize ChiA.

Substrate specificity and chitinase activity of recChiB

The activity of recChiB was evaluated with various fluorescent chitinase susbstrates as part of the characterization procedure. We tested three different fluorogenic susbtrates to determine one of the specific chitinase activities: 1) N-acetil glucosaminidase (exochitinase) (4-Methylumbelliferyl N-acetil-β-D-glucosaminide), 2) chitobiosidase (exochitinase) (4-Methylumbelliferyl N, N'-diacetil-β-D-chitobioside) and 3) endochitinase (4-metilumbeliferil β-D-N, N ', N''- triacetylchitotriose).

Our initial hyphotesis was that the enzymatic activity for this chitinase was that of an endochitinase, based on the phylogenetic analysis with the ChiB aminoacid sequence and the existence of similar domains (see previous chapter) with those of chitinase B of *B. cereus* (Chen *et al.*, 2009).

As expected, purified recChiB was able to degrade 4-metilumbeliferil β-D-N, N ', N''- triacetylchitotriose a substrate that demonstrates its activity as one endochitinase (Figure 3A). Unexpectedly, the recChiB was also able to hydrolyze 4-methylumbelliferyl N, N'-diacetil-β-D-chitobioside exhibiting additionally chitobiosidase (exochitinase) activity (Figure 3B). As observed in the previous Chapter IV when chitinase activity was tested for exochitinase (glucosaminidase activity) with the substrate 4-methylumbelliferyl N-acetil-β-D-glucosaminide on the crude culture media where *Bacillus cereus* B25 was grown this exochitinase activity was not detected. In order to confirm this finding, this type of exochitinase activity was assayed in recChiB again confirming the absence of this enzymatic activity in this recombinant protein (Figure 3C).

The results obtained suggest that recChiB possesses two diferent chitinase activities [endochitinase and chitobiosidase (exochitinase)]; the presence of the two chitinase activities in one single chitinase has been previously reported for other chitinases like ChiA of *Serratia marcescens* BJL200 (Brurberg *et al.*, 1996) and ChiA74 of *Bacillus thuringiensis* (Casados-Vázquez *et al.*, 2015).

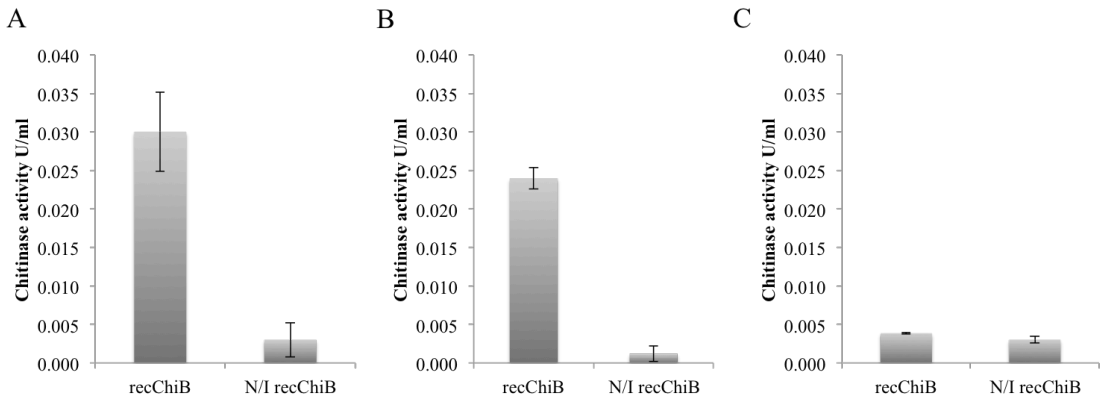

Figure 3. Chitinase activity of recChiB on specific substrates. A) Endochitinase acitivity tested on 4-metilumbeliferil β-D N, N ', N"- triacetylchitotriose. B) Exochitinase activity tested on 4-Methylumbelliferyl N, N'-diacetil-β-D-chitobioside. C) Exohitinase activity tested on 4-Methylumbelliferyl N-acetil-β-D-glucosaminide. The abbreviation recChiB means purified recombinant protein of ChiB gene; N/I recChiB means cell debris of non-induced cells containg the expression vector with ChiB gene. U: the amount of enzyme needed to release 1 µmol 4-methylumbelliferone from the substrate per minute at pH 5.0 and 37 °C.

Zymogram analysis

Chitinase activity of the purified recChiB was confirmed using zymograms. The chitinase activity was observed in the supernanant after lysate induced cells containing the recChiB gene, and was observed in lane 1 of the figure 4. The cells debris showed chitinase activity and the presence of other proteins; and the purified protein show a single cleared band, suggesting that chitinase activity from this recombinant protein is degrading the coloidal chitin in the polyacrylamide gel.

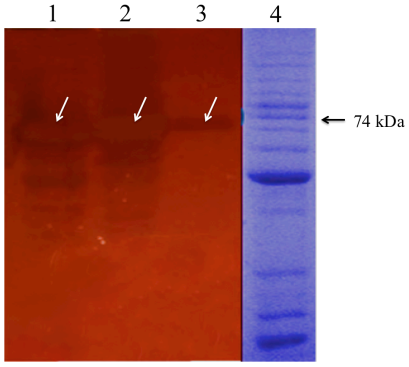

Figure 4. Zymogram analysis of chitinase activity of recChiB. Lane 1, supernanant of recChiB without purification. Lane 2, cell debris of recChiB without purification. Lane 3, Final eluate of the purification process. Lane 4, molecular marker (BenchMark protein ladder).

Conclusion

Here we showed the production of recombinant chitinase ChiB in *E. coli* using an expression vector based on Gateway technology, and we demonstrated the ability of this protein to exhibit a dual chitinase activity: endo- and exo-chitinase activity.

The elucidation of chitinase activity provides us with more clues about how *Bacillus cereus sensu lato strain* B25 degrades chitin, the recChiB was able to hydrolyze chitotriose and chitobiose, this is a very interesting feature and it constitutes a possible major mechanism that may affect fungal growth as discussed in the following chapter.

The recChiA could not be purified, another strategy will be needed to achieve this goal. Future work will be directed to make this happen, since it is necessary to evaluate ChiA activity to elucidate the complete mechanism used by *B. cereus sensu lato* strain B25 to degrade chitin.

CAPÍTULO VI. COLONIZATION PATTERN OF *Bacillus cereus* B25 ON MAIZE ROOTS, EVIDENCE FOR ENDOPHYTISM AND A PROPOSED ROLE OF THIS BACTERIUM IN THE CONTROL OF *Fusarium verticillioides* IN MAIZE**.**

Manuscript to be submmited in Microbial Biotechnology

Abstract

Biological control is becoming increasingly relevant, in line with concerns for more sustainable agricultural practices that can help reduce environmental and health impacts caused by agrochemicals. Our understanding of the mechanisms employed by many biological control agents is still expanding, making this an opportune time to investigate novel theories of interaction between plants and microorganisms relevant to biocontrol. The focus of this chapter is on fungal diseases, which are a serious threat to many different crops. Chitin, a structural polysaccharide found in fungal cell walls, is one of the first points of interaction between the fungus and its plant host. Chitinases have been implicated in plant resistance against fungal pathogens, due to their inducible nature and antifungal effect. Plant chitinases function as a surveillance mechanism to help monitor the presence of fungal invaders. This is achieved through inhibiting fungal growth directly or indirectly, by releasing small signaling molecules (elicitors) from the fungal cell wall that alert the plant of the fungal invader presence and initiate the plant defense responses. Some fungi, such as the maize pathogen *Fusarium verticillioides*, evade this surveillance mechanism by producing effector proteins that act upon plant chitinases, allowing the fungus to escape the plant's defenses. Chitinolytic bacteria thus exhibit great potential for the control of fungal phytopathogens. A tripartite interaction exists between host plant, fungal invader and biological control agent (*e.g.* endophytic chitinolytic bacteria). We propose that bacterial chitinases may be used to counteract the mechanism that allows fungi to evade plant defenses. This situation could give the upper hand to plants, allowing them to survive and overcome the fungal invasion. Potential means to corroborate this proposed mechanism by future research are discussed.

Introduction

The extensive use of biocontrol strategies aimed at fungal phytopathogens has been held back by the absence of sufficient information regarding the mechanisms by which microorganisms such as bacteria exert their control over phytopathogens (Shali *et al.*, 2010). In addition, it is often difficult to fully comprehend how these biocontrol agents work against plant pathogens, as they employ a wide variety of strategies (Shali *et al.*, 2010). Recent research has focused on using biological methods to protect crops from the invasion and spread of pathogens, an approach that is mainly promoted by current worldwide food safety regulations and food export/import requirements.

Pathogen management using antagonistic microorganisms or their secondary metabolites is now considered to be a viable disease control strategy (Kim *et al.*, 2011). Nevertheless, in many cases, identifying the contributing mechanisms requires a systematic approach to directly evaluate individual traits and their roles within the overall operating mechanisms.

Fv is a fungal pathogen that causes ear, stem and root rots in maize and other crops. In maize seeds, *Fv* produces fumonisins, a type of mycotoxin that is toxic to horses, swine, poultry, and has been correlated with human toxicity (Bullerman, 1996; Marasas *et al.*, 2004; Riley *et al.*, 1993). Fumonisins are commonly found on maize, most likely due to the endophytic nature of mycotoxin-producing strains of *Fv*. Therefore, it is important to develop control strategies that could attack both the endophytic and saprophytic fungal stages. The endophytic stage serves both as a source of mycotoxin production and as an infection source (Bacon and Hinton, 2011). The endophytic stage also makes fungicide control extremely difficult (Bacon and Hinton, 2011).

During a pathogen attack, the plant-pathogen interaction induces a variety of plant defense mechanisms including cell wall strengthening (Bradley *et al.*, 1992), *de novo* production of antimicrobial compounds (Gupta *et al.*, 2010), and rapid localized cell death (Alvarez, 2000). For example, plants encode a variety of chitinases that are involved in defense-mediated responses against fungal pathogen invasion; these enzymes then cleave chitin oligosaccharides from fungal cell walls, which are then detected by plant receptor proteins that induce or "elicit" a defense response (Shibuya and Minami, 2001). This defense mechanism is known as pathogen-associated

molecular patterns (PAMP), and it triggers general plant defense responses referred to as PAMP-triggered immunity (PTI) (Boyd *et al.*, 2013). These plant defense responses are activated following the recognition of PAMPs by the plant (Boyd *et al.*, 2013), and they have been well documented in different plant species including *Oriza sativa* L. (Kaku *et al.*, 2006) and *Arabidopsis thaliana* (Miya *et al.*, 2007).

Although fungal disease resistance has been successfully achieved by transgenic overexpression of plant chitinases (see Cletus *et al.* (2013) for a current review), recent evidence suggests that this strategy is not compatible for all fungal diseases. Fungal pathogens are able to suppress the different components of PTI via "effector" proteins delivered into the plant (Boyd *et al.*, 2013).

In a study to evaluate maize chitinases in a protein extract from maize seed was found that some fungi altered the chitinolytic activity; western blots of protein extracts from maize seeds parasitized by the ear-rot pathogen *Bipolaris zeicola* lack two major chitinase activity bands that are present in seed protein extracts of non-inoculated ears. Combining protein extracts from *B. zeicola*-parasitized seeds with those from non-inoculated seeds results in the loss of these two chitinase activity bands; it was therefore hypothesized that this is due to a specific molecular interaction between a fungal effector and a targeted maize chitinase (Naumann *et al.*, 2009). Maize seeds contain abundant amounts of two chitinases: ChitA and ChitB (Huynh *et al.*, 1992). Maize chitinases are modified by effector proteins from *B. zeicola* and are referred to as Bz-cmp (*Bipolaris zeicola*-chitin modified protein). These proteins affect the size of chitinases, resulting in an apparent reduction in their molecular weight. This is accompanied by the loss of ChitA's chitin-binding ability whereas the chitinase enzymatic activity remains intact, indicating that Bz-cmp modifies the chitin binding domain of ChitA (Naumann *et al.*, 2009). These proteins have also been found in maize seed inoculated with *Stenorcarpella maydis* (Naumann and Wicklow, 2010), *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. subglutinans* and *Fv* (Naumann *et al.*, 2011). Subsequent amino acid sequence analysis in a maize - *Fv* interaction revealed that Fv-cmp cleaves maize ChitA in a region that is conserved in other class IV plant chitinases, suggesting that this effector protein may truncate chitinases from other plants (Naumann and Wicklow, 2010). To explain these observations, two mechanisms have been proposed: either the modification of the maize chitinases directly suppresses fungal growth inhibition, or it suppresses the induction of defense responses (Naumann *et al.*, 2011). This last mechanism could help explain how *Fv* successfully colonizes

maize plants.

The present study focuses on the maize–*Fv* interaction and the role that endophytic bacteria may play in the control of this fungal disease. Here we discuss a possible mechanism for fungal growth inhibition in plant-bacteria-fungus tripartite associations. Importantly, we propose a possible role for endophytic bacterial chitinases from the *Bacillus cereu sensu lato* strain B25 in the control of *Fv* infection in maize.

Materials and Methods

Sequence alignment of maize and B25 chitinases

Sequences of B25 were taken from the sequenced genome of this bacterium (Douriet-Gámez et al., submitted) and plant chitinase sequences were taken from Naumman et al., (2011). Alignment of the sequences was conducted using the software MUSCLE (Edgar, 2004).

Colonization experiment in maize plants

This experiment was set up in magenta boxes. Each experimental unit consisted of three seeds and fungal inoculum in 200 grams of sterile sand as substrate containing $1x10^5$ *Fusarium verticillioides* conidia per gram of substrate. Boxes were incubated at 16 hours light at 25 °C and 8 hours darkness at 20 °C.After two weeks, the plants were harvested and roots were taken for further analysis.

For bacterial colonization using *B. cereus sensu lato* B25 similar conditions were employed except that the substrate was completely sterile and free of fungal inoculum.

Confocal laser microscopy

Images were taken from separate experiments using plants inoculated with only *B. cereus* or *F. verticillioides*. Red fluorescence counter-staining was made with rhodamine B, a dye which stains plant cell walls (Blachutzik *et al.*, 2012). Green fluorescence of both microorganisms is caused by the staining in the root tissues by staining with the wheat germ agglutinin (WGA) coupled to the fluorophore Alexa-Fluor 488 nm™. WGA is a lectin, which binds to chitin residues of fungal cell walls or to peptidoglycan cell walls in Gram positive bacteria, such as *B. cereus*. The lectin is coupled to the fluorophore Alexa Fluor which is excited at 548 nm and emits

fluorescence at 488 nm. Images were obtained using a Leica TCS SP8 confocal laser microscope using a white laser and capturing the green fluorescence emitted at 488nm and the red counter staining with rhodamine B by exciting it at 553 nm and registering the emission at 627 nm.

Results and discusion

Fungal effector proteins can modify class IV chitinases of plants, but they cannot affect bacterial chitinases

Fungal effector proteins can modify class IV chitinases of plants, but they cannot affect bacterial chitinases since the amino acid sequence in their chitin binding protein domain is different (Table 1). Sequence alignment of the Fv-cmp target region of chitinases from *Bacillus cereus* and maize indicate that the cleavage site catalyzed by Fv-cmp is nonexistent in the bacterial sequences (Table 1).

This suggests that *B. cereus* and possibly other chitinase-producing bacterial strains are good potential candidates for biocontrol of *Fv*. In particular, the chitin binding ability of these proteins will not be affected by Fv-cmp. Additionally, bacterial chitinases may inhibit fungal growth of this pathogen, even though maize chitinases will be inactivated. This may represent a novel protection mechanism by chitinolytic bacteria, which could enable them to counteract the fungal invasion mechanism.

Table 1. Sequence alignment of chitinases from *Bacillus cereus* and maize. Modified from Naumman *et al*. (2011).

Chitinase	**Chitin binding domain** *Fv*-cmp target region														**Accession**	**Reference**
ChitA-LH82	D	D	Y	C	G	D	G	C	Q	S	G	P	C	R	ACX37090	(Naumann and Wicklow, 2010)
ChitA-B37	D	A	Y	C	G	D	G	C	Q	S	G	P	C	R	AAA33444	(Huynh *et al.*, 1992)
ChitB	D	E	Y	C	G	D	G	C	Q	S	G	P	C	R	AAA33445	(Huynh *et al.*, 1992)
ChiA	L	K	D	V	S	P	K	W	D	V	I	N	V	S	KR809875	(Douriet-Gámez *et al.*, 2016)
ChiB	L	K	D	V	S	P	K	W	D	V	I	N	V	S	KR809876	(Douriet-Gámez *et al.*, 2016)

An alternative fungal defense mechanism could involve *Bacillus* spp., whose occurrence as endophytes has been reported from different plants including pigeon pea

(Rajendran *et al.*, 2008), wheat, kudzu (Selvakumar *et al.*, 2008), and soybean nodules (Bai *et al.*, 2002). *B. cereus* has been reported as a plant endophyte of such species as grapevine (West *et al.*, 2010), wheat (Wang *et al.*, 2011), coffee (Shiomi *et al.*, 2006) and maize (Orole and Adejumo, 2011). Once bacteria possessing fungal phytopathogen antagonistic mechanisms penetrate the interior of a plant, they will likely be employed in the same way as non-endophytic bacteria growing outside of the plant. Intercellular spaces and xylem vessels are the most commonly reported locations for endophytic bacteria (Reinhold-Hurek and Hurek, 1998). By sharing similar niches as vascular fungal phytopathogens, endophytes can potentially be exploited for biotechnological use in fungal disease control (Hallmann *et al.*, 1997).

Our review of the literature leads us to lead us to postulate and explore the endophytic nature of the *B. cereus sensu lato* strain B25.

B25 and *F. verticillioides* are both endophytic

Preliminary results from our research group suggest that a chitinolytic *B. cereus* strain (Link to Video) and *Fv* both colonize the vascular vessels of the maize root, thus sharing a similar niche (Figure 1). Confocal laser microscopy has enabled us to reveal the presence of both organisms growing within the root vessels, suggesting that they grow endophytically. This observation could explain why this *B. cereus* strain demonstrates potential as an effective *Fv* control agent *in planta* (Cordero-Ramírez, 2014; Leyva-Madrigal *et al.*, 2015) and in maize field tests (data not show) when applied as seed coverage at planting time. Specifically, *B. cereus* establishes and persists in vascular vessels within the plant host. If the plant root is attacked by *Fv*, the pathogen will eventually arrive at the root vascular vessels after breaking through plant defense mechanisms (*e.g.* plant chitinases). Upon entry into the vascular tissue, the fungus will be confronted by *B. cereus*.

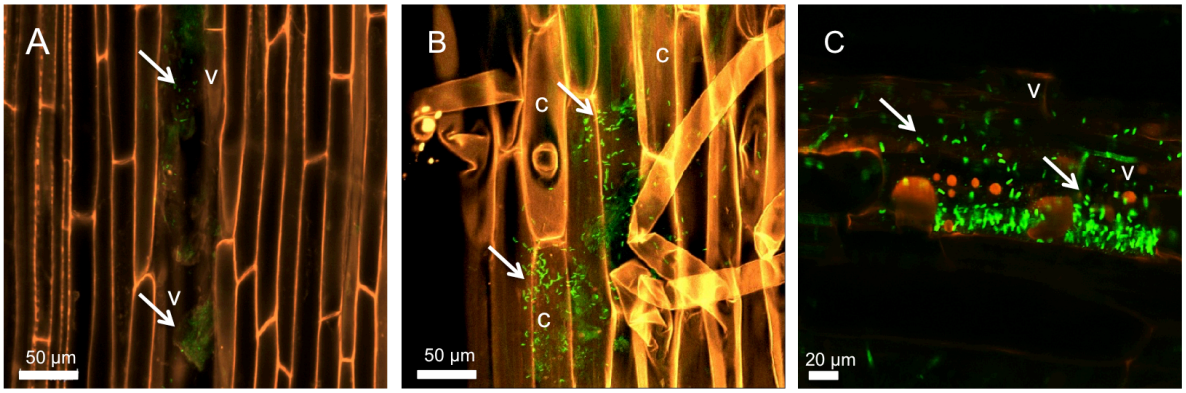

Figure 1. Confocal laser scanning microscopy showing a similar endophytic location in vascular vessels in maize roots of *Bacillus cereus Bc*25 and *F. verticillioides*. A) *Bacillus cereus* colonization of vascular vessels, arrows show bacteria's cell. B) *Bacillus cereus* colonization on cortical cells, C) microphotograph showing the vascular localization of *F. verticillioides*. Letter "c" in the image indicates the cortex cells and letter "v" indicate the vascular cells.

***Bacillus cereus sensu lato* B25 may exert its biological control against *F. verticillioides* due to its endophytic nature**

By sharing similar niches inside the host plant, chitinase-producing endophytic *B. cereus* may be able to control *Fv* in maize. *Fv* and other fungi colonize maize successfully because they overcome the plant defense mechanisms, as reviewed above. This fungus develops through vascular vessels that produce conidia, spreading infection in the plant through the stem, ears and roots (Figure 2A). If bacteria colonizing the vascular vessels are present in the same niche as *Fv*, a scenario can be envisioned in which bacterial chitinases may be released in response to *Fv* and thereby inhibit fungal development. This should result in the effective control of *Fv* since the fungus cannot recognize and cleave the chitin binding domain of *B. cereus* chitinases, due to differences in the amino acidic sequence of the plant chitin binding domain (Figure 2B).

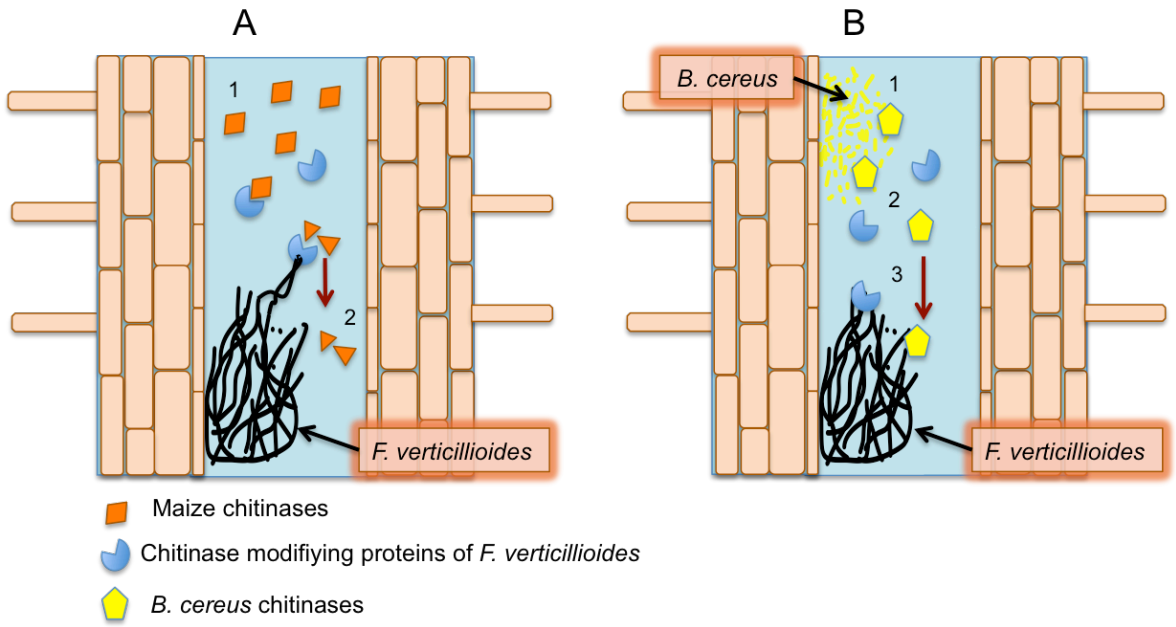

Figure 2. Proposed mechanism of *F. verticillioides* (*Fv*) biological control exerted by *Bacillus cereus* chitinase activity. A) Plant infected with *Fv*. 1) Sensing of the fungal infection and release of chitinases by plant cells 2) Modification of plant chitinases due to the action of chitinase effector proteins (*Fv*-cmp) released by *Fv* resulting in the lack of binding to chitin residues of the fungus. B) Plant infected with *Fv* and colonized endophytically with the biological control agent *B. cereus*. 1. Release of bacterial chitinases in response to infection of *Fv*. 2. Chitinase effector proteins (*Fv*-cmp) from *Fv* cannot affect the bacterial chitinases. 3. Bacterial chitinases can bind to chitin and inhibit *Fusarium* development by degrading the fungal cell wall.

B25 produces chitinases as part of its antagonistic arsenal of mechanisms, which differ from the endogenous plant versions and thus will not be annihilated by the fungus Fv-cmp effector proteins. Release of chitin oligosaccharides may possibly act as elicitors of the plant response, provoking a dual attack by the bacteria and the plant, inhibiting fungus growth. This plausible scenario will require confirmation in the near future.

Conclusion

To this end, our laboratory is currently investigating *B. cereus* chitinases at the molecular level and conducting confocal scanning laser microscopy analyses to confirm the endophytic colocalization pattern of the bacterium and the fungus within the

vascular vessels of maize roots. Other experiments are needed to confirm these observations, such as immunolocalization of B25 chitinases and Fv-cmp in the maize roots.

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