UNIVERSIDAD MICHOACANA DE SAN NICOLAS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUIMICO BIOLOGICAS

EVALUACIÓN DE LA ACTIVIDAD DE BIOCONTROL Y LOS MECANISMOS DIRECTOS DE PROMOCIÓN DE CRECIMIENTO VEGETAL EN CEPAS DE *Pseudomonas fluorescens*

TESIS

PARA OBTENER EL GRADO DE

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PRESENTA

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Resumen

En este trabajo se analizaron 300 aislados de la rizósfera de plantas de alfalfa (*Medicago sativa*). Cuatro de esto aislados mostraron capacidad para inhibir el crecimiento de los hongos fitopatógenos, *Botrytis cinerea, Fusarium oxysporum, Rhizoctonia solani, Diaporthe phaseolorum y Colletotrichum lindemutianum*. Mediante la secuenciación del ADN ribosomal 16S estos cuatro aislados fueron identificados como *Pseudomonas fluorescens* UM16, UM240, UM256 y UM270. De acuerdo con los resultados, las cuatro cepas protegen a *M. truncatula* de la infección de *B. cinérea* y promueven su crecimiento mediante compuestos orgánicos difusibles y volátiles. Se identificó en todas las cepas, la presencia de los genes*, phlD, hcnAB y acdS*, que están involucrados en la biosíntesis de 2,4-Diacetilfloroglucinol (DAPG), ácido cianhídrico (HCN) y ACC desaminasa respectivamente. Así como *phzCD* relacionados con la producción de fenazinas, únicamente en *P. fluorescens* UM16 y UM270. Además la producción de ácido indol acético, sideróforos, proteasas, biofilm y la capacidad de solubilizar fosfatos fueron determinadas. El perfil de compuestos orgánicos volátiles, muestra que la mayoría de estos, son compuestos que contienen azufre, lo cual es importante especialmente en plantas que crecen bajo deficiencia de este elemento. Con todo esto se sugiere que existe un gran potencial por parte de estas cuatro cepas para el desarrollo de bioproductos promotores de crecimiento vegetal.

Palabras clave. Pseudomonas, Biocontrol, Promocion de crecimiento vegetal, COVs, CODs.

Abstract.

In this work 300 isolated from the rhizosphere of alfalfa plants (Medicago sativa) were analyzed. Four isolates showed ability to inhibit the growth of phytopathogenic fungi, *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Colletotrichum lindemutianum* and *Diaporthe phaseolorum*. By sequencing of 16S ribosomal DNA these four isolates were identified as *Pseudomonas fluorescens* UM16, UM240, UM256 and UM270. According to the results, four strains protect *M. truncatula* from infection of *B. cinerea* and promote their growth by diffusible and volatile organic compounds. Was identified in all strains, the presence of, *phlD*, *hcnAB* and *acdS genes*, which are involved in the biosynthesis of 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN) and ACC deaminase respectively and *phzCD* related to the production of phenazines, only for *P. fluorescens* UM16 and UM270. Furthermore the production of indole acetic acid, siderophores, proteases, biofilm and the phosphates solubilization capacity were determined. The profile of volatile organic compounds, shows that most of these are compounds containing sulfur, which is important especially in plants grown under deficiency of this element. With all this, it suggests that exist a great potential by these four strains for the development of bio plant growth promoters.

1. Introducción.

La agricultura es uno de los sectores más importantes en México, la producción de más de 60 productos estratégicos según el Servicio de Información Agroalimentaria y Pesquera (SIAP), ayuda a alimentar a la población, al ganado y como materia prima para diferentes procesos, además de una parte sustancial que es exportada a diferentes países del mundo. Michoacán es uno de los estados con mayor superficie sembrada en el país (Fig. 1), cultivos como el aguacate, la zarzamora y la fresa son algunos de los principales en la región.

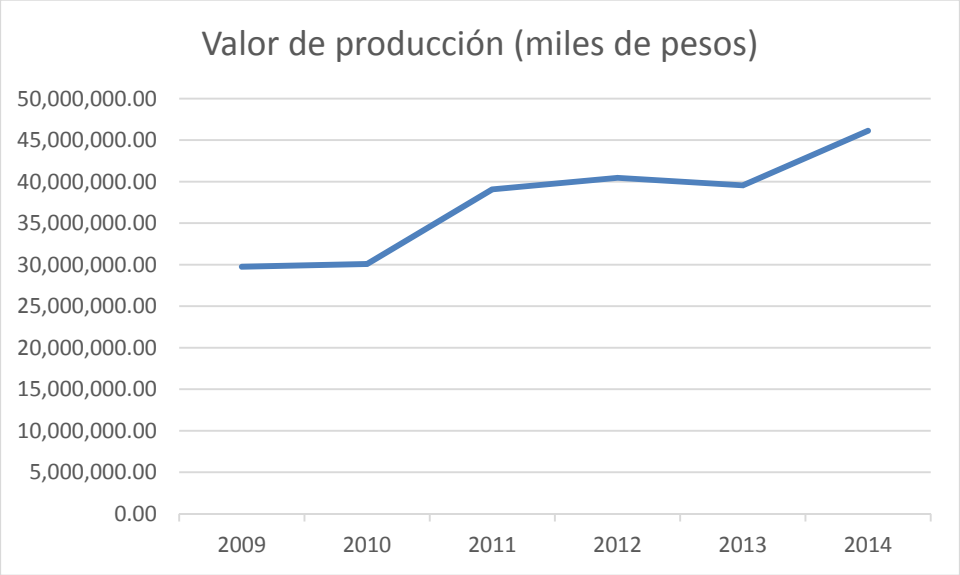

Figura 1. Producción agrícola durante el periodo 2009-2014 del estado de Michoacán de Ocampo, incluyendo ciclos y perennes en modalidad de riego y temporal (Datos oficiales SAGARPA).

De acuerdo con la FAO, los alimentos deben garantizar no causar ningún daño al consumidor a la vez que aporten los nutrientes necesarios para un óptimo rendimiento, pero uno de los principales problemas para cubrir esta necesidad son las grandes pérdidas agrícolas provocadas por una enorme variedad de plagas.

Por años, el uso de fertilizantes para aumentar la producción y de plaguicidas para evitar estas pérdidas fue una gran solución contra el hambre sin embargo el uso indiscriminado de ellos representa un potencial peligro para la salud (Tabla 1).

Tabla 1. Aspectos negativos del uso excesivo de agroquímicos.

País	**Compuesto**	**Resultados**	**Referencia**
Brasil	Insecticidas organoclorados (OCP)	Residuos de insecticidas OC en la biota (Pescados, camarones, ostras y mejillones) del lago Paranoá.	Caldas et al. 1999
Polonia	Pesticidas organonitrogenados, organofosforados y organoclorados	Las concentraciones de plaguicidas en muestras de precipitación revelaron fluctuaciones estacionales, con mayores concentraciones observadas durante los períodos de aplicación.	Grynkiewicz et al. 2003
India	Pesticidas organoclorados	Identificados a partir de muestras de agua potable. Alta concentración de metil paratión y endosulfán, observadas en muestras de agua recogidas de río	Agarwal et al. 2015
Cuba	Fertilizante nitrogenado	Medidas por encima de 10 mg de N03/l, contenido máximo que pueden alcanzar las aguas naturales.	Vargas et al. 1999.
México (“Zona muerta"	Nitrógeno y fosforo	Pérdida de hábitat, la muerte de peces, las floraciones de algas tóxicas, y la hipoxia.	Greenhalgh y Faeth, 2001.

del Golfo)			
Polonia	concentraciones de cloruros	Elevados niveles de cloruros de las aguas residuales	Kolebuk et al. 2015.

Uno de los esfuerzos de la comunidad científica es el de reducir, o en su caso eliminar, el uso de fertilizantes y pesticidas en la agricultura. De esta manera, se ha tratado de desarrollar bioinoculantes con capacidades de biocontrol y de forma ideal, con características promotoras del crecimiento vegetal. Por ejemplo, el uso de agentes bacterianos como componente principal de los bioinoculantes, los cuales no dañan el medio ambiente ni la salud humana y animal. En los siguientes párrafos trataremos de explicar cómo funcionan, sus mecanismos directos e indirectos de promoción del crecimiento y protección vegetal, así como las ventajas que pueden tener comparado con el uso de agroquímicos. Lo anterior nos llevará hacia una agricultura sustentable y productiva.

1.1 La rizósfera y sus componentes.

La Rizósfera es la tierra adyacente a las raíces de las plantas, donde se concentra una mayor cantidad de microorganismos. El término, rizósfera, fue acuñado por primera vez por Lorenz Hiltner (Hiltner, 1904) y se conoce como un ambiente complejo que contiene miles de especies microbianas. Por ejemplo, se ha propuesto que por cada gramo de suelo se pueden encontrar entre 4,000 y 10,000 especies bacterianas (Torsvik et al. 1990). Sin embargo, aunque el grupo de las bacterias pueden ser la más diversa en la rizósfera, la biomasa de hongos puede comprender hasta el 70% del total en la rizósfera.

La comunidad microbiana de la rizósfera es reclutada por el suelo circundante, mientras que las plantas determinan qué miembros de este reservorio prosperaran (Lennon, 2011).

El “Efecto rizósfera" es un término acuñado por Starkey (1938), se define por procesos colectivos que se producen entre el suelo y la raíz de una planta e incluye la exudación de la raíz, la actividad microbiana, el intercambio genético, la transformación de nutrientes y la difusión de gradiente.

Las sustancias liberadas por las raíces de las plantas se denominan colectivamente "rizo deposiciones”. Estos compuestos incluyen Iones solubles en agua y compuestos de bajo peso molecular tales como monosacáridos, aminoácidos y ácidos orgánicos que se pierden pasivamente a lo largo de un gradiente de concentración. Otros compuestos son aquellos de masa molecular alta, tales como carbohidratos, proteínas y lípidos que son transportados activamente a lo largo de un gradiente electroquímico. El mucílago insoluble compuesto de polisacáridos y ácido poligalacturónico, una matriz de metabolitos secundarios, tales como compuestos antimicrobianos, nematicidas y flavonoides asi como los restos de la raíz y células muertas. (Bowen y Rovira, 1991; Hale et al., 1978; Marschner 1995; Whips, 1990).

Los componentes principales de la rizósfera son: la exorizósfera, el rizoplano y la endorizósfera. El rizoplano es la zona de superficie de la raíz donde los microorganismos se adhieren utilizando estructuras superficiales tales como flagelos y fimbrias (Mwajita et al., 2013). El límite entre rizoplano y rizosfera es muy delgada y por lo tanto este hábitat se considera en gran parte como continuo. (Johri et al., 2003). La exorizósfera es la superficie externa de la raíz mientras que la región interna se conoce como endorizósfera (El-Morsy, 2000; Babalola, 2010).

La interacción planta-microbio en la rizósfera puede ser: negativas, positivas o neutras. Las interacciones negativas incluyen la asociación con las plantas parasitarias, bacterias patógenas, hongos oomicetos , nematodos y herbívoros invertebrados mientras que las interacciones positivas incluyen interacciones simbióticas y asociativas con microbios beneficiosos, tales como hongos endo y ecto-micorrizas, bacterias fijadoras de nitrógeno y rizobacterias promotoras del crecimiento vegetal (PGPR).

1.2 Bacterias Promotoras del Crecimiento Vegetal.

Las rizobacterias promotoras del crecimiento vegetal Plant Growth-Promoting Rhizobacteria (PGPR) por sus siglas en inglés, han sido ampliamente estudiadas desde hace varias décadas. Kloepper y Schroth (1978) introdujeron el término "rizobacterias" a la comunidad bacteriana del suelo que competitivamente coloniza las raíces de las plantas y estimula el crecimiento y reduce así la incidencia de enfermedades de las plantas. Posteriormente, se propuso que las las PGPR tienen también capacidades para reducir la incidencia de enfermedades de las plantas.

Las PGPR parecen conseguir establecerse con éxito en el ecosistema rizosférico o en el suelo debido a su gran capacidad de adaptación en una amplia variedad de entornos y adaptándose a cambios en el ambiente los cuales son influenciados por factores bióticos y abióticos (Hernández-Pacheco, et al., 2016; Ettema, y Wardle, 2002), igualmente las PGPR tienen una tasa de crecimiento más rápido y capacidad para metabolizar una amplia gama de compuestos. Cook (2002) considera las PGPR como componente significativo en la gestión de las prácticas agrícolas con potencial genético innato. El concepto de PGPR ha sido ahora confinado a las cepas bacterianas que pueden cumplir al menos dos de los siguientes tres criterios: una colonización agresiva, la estimulación del crecimiento de la planta y el control biológico (Weller et al., 2002; Vessey, 2003).

1.3 El género *Pseudomonas.*

Pseudomonas es un género de bacilos rectos o ligeramente curvados, son organismos ubicuos, bacterias gram negativas, aeróbicos, no esporulan y se clasifican dentro de las proteobacterias. Su diámetro es de aproximadamente 1 μm y su longitud es de 1.5 a 5μm. Son microbiota predominante en la rizósfera de plantas aunque también se han encontrado en ambientes acuáticos.

El género *Pseudomonas* ha sido uno de los más destacados dentro de estos estudios ya que tienen un crecimiento rápido, muestran una agresiva colonización en la rizósfera, puede utilizar diversos sustratos como nutrientes y puede sobrevivir en condiciones que serían adversas y estresantes para otras bacterias.

Su capacidad para producir diversos compuestos de tipo antibiótico y sideróforos, también es crucial para el éxito de su colonización y ocupación de espacios. Entre los que destaca el metabolito fenólico 2,4-DAPG el cual es un componente importante en la supresión natural de ciertos suelos agrícolas, otro antibiótico importante sintetizado por el género *Pseudomonas* son las fenazinas, además del HCN, un anti fúngico volátil que también está relacionado con la supresión de ciertas enfermedades (Voisard et al., 1989).

1.4 Mecanismos Directos e Indirectos de promoción del crecimiento vegetal.

Las PGPR exhiben diversos mecanismos para mejorar y promover crecimiento vegetal. En general y aunque existe cierto traslape entre algunos de ellos, se pueden dividir en dos. Primero, los mecanismos directos incluyen la producción y excreción de compuestos fitoestimulantes que son percibidos por la planta. Por otra parte, los mecanismos indirectos son aquellos que inhiben el crecimiento de potenciales fitopatógenos y permiten la protección vegetal o biocontrol. A continuación se detallan dichos mecanismos.

1.5 Papel de los Sideróforos en la adquisición de Hierro.

El hierro es un catalizador redox-activo versátil y un cofactor requerido dentro de una amplia gama de procesos biológicos. Para casi todos los organismos, el hierro es esencial y potencialmente tóxicos, donde las concentraciones homeostáticas deben mantenerse rigurosamente. Dentro de un hospedero restringido de hierro, la supervivencia y la proliferación de microbios invasores

está condicionada a la explotación de la concentración de hierro adquirido. Las bacterias expresan una multitud de medios complejos y a menudo redundantes de adquisición de hierro, incluyendo las vías de hemo-captación asociadas a la superficie, sideróforos de alta afinidad y transportadores de hierro inorgánico libre. (Sheldon, 2015).

Los Sideróforos son pequeñas moléculas orgánicas producidas por microorganismos bajo condiciones de limitación de hierro que mejoran su absorción para los microorganismos. En el entorno, la forma de hierro férrico es insoluble e inaccesible a pH fisiológico (7.35-7.40). Bajo esta condición, los microorganismos sintetizan sideróforos que tienen alta afinidad por el hierro férrico. A continuación, estos complejos de sideróforos-hierro férricos son transportados al citosol. En el citosol, el hierro férrico se reduce a hierro ferroso y se hace accesible al microorganismo. En los últimos tiempos, los sideróforos han llamado mucho la atención debido a sus posibles roles en diferentes campos (Neilands., 1995).

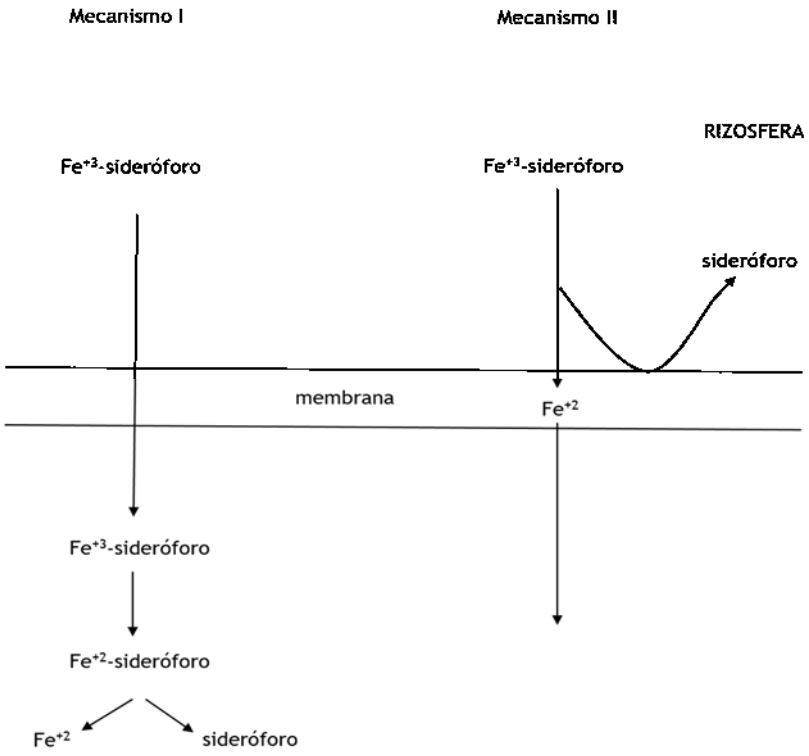

Figura 2. Mecanismos de los sideróforos microbianos para la captura y solubilización del hierro (Modificada de Neilands., 1995). Existen dos mecanismos generales a través de los cuales los microorganismos asimilan el hierro del organismo hospedero. El Mecanismo I, que involucra la adquisición de hierro a través de receptores cognados que usan sideróforos (adquisición indirecta); el Mecanismo II consiste en la adquisición de hierro mediada por receptores a partir de proteínas acarreadoras de hierro (adquisición directa).

Casi todos los sideróforos que se han identificado contienen como grupo ligando al ácido hidroxámico, catecol o al ácido hidroxicarboxílico. De acuerdo con el grupo funcional quelador del hierro, los sideróforos pueden clasificarse en catecolatos (fenolatos), hidroximatos e hidroxicarboxilatos, que forman complejos octaédricos hexadentados con el metal. Algunos sideróforos son más eficaces que otros para quelar el hierro y la gran diversidad de moléculas producidas por estos microorganismos se relaciona con una amplia variedad de sustratos que pueden usar (Marahiel, 1997; Crosa y Walsh, 2002).

Aunque algunas bacterias producen sólo una clase de sideróforos, otras secretan diversos tipos que las hace más eficientes para colonizar diferentes ambientes. Algunas especies del género *Pseudomonas* producen sideróforos del tipo

hidroximato, entre los que se encuentran la ferribactina y pseudobactina, pero otras más producen moléculas denominadas pioverdinas del tipo catecol.

En bacterias Gram negativas y bacterias Gram positivas ricas en AT (adenina y timina), este proceso es usualmente regulado por el represor Fur (ferric uptake regulator o regulador de la captura de hierro férrico), una metaloproteína de zinc (Althaus *et al*., 1999), mientras que el regulador DtxR realiza esta función en bacterias Gram-positivas ricas en GC (guanina y citosina; Miethke y Marahiel, 2007). Los sideróforos son entonces secretados al ambiente extracelular donde secuestran y solubilizan el hierro. A continuación, los sideróforos son reconocidos por receptores específicos ubicados en la membrana externa de la célula (Neilands, 1995). En este punto, el complejo Fe3+-sideróforo puede ser utilizado por las bacterias a través de dos mecanismos (Figura 2).

En muchos casos, todo el complejo Fe3+-sideróforo es transportado activamente a través de la membrana celular, mientras que en hongos y otros eucariotes el complejo Fe3+-sideróforo puede ser reducido extracelularmente a Fe2+ (Fig. 2).

Los sideróforos tienen aplicación en la ecología microbiana para mejorar el crecimiento de varios microorganismos no cultivables y pueden alterar las comunidades microbianas. En el campo de la agricultura, los diferentes tipos de sideróforos promueven el crecimiento de varias especies de plantas y aumentan su rendimiento mediante la mejora de la absorción de Fe a las plantas. Los sideróforos actúa como un potencial agente de biocontrol contra nocivos fito-patógenos y tiene la capacidad de sustituir plaguicidas peligrosos. (Saha., 2015).

Las bacterias promotoras del crecimiento vegetal que poseen la capacidad de producir sideróforos secuestran el hierro al formar un complejo Fe^{+3}-sideróforo, mediante un receptor específico localizado en la membrana bacteriana, lo cual ocasiona que este metal no se encuentre disponible para otros microorganismos que carezcan del sistema de asimilación específico para reconocer dicho complejo. De esta manera, al utilizar todo o la mayoría del hierro disponible en el suelo suprime o inhibe el crecimiento de otros microorganismos patógenos (o

benéficos) presentes en la rizósfera (Compant et al., 2005; Schroth y Hancock, 1982).

1.6 Papel de los sideróforos en la promoción del crecimiento vegetal.

En los años 80´s se publicó un trabajo pionero donde se le atribuía un nuevo mecanismo de promoción del crecimiento vegetal a los sideróforos producidos por rizobacterias del género *Pseudomonas.* En dicho trabajo se propuso que de forma indirecta, los sideróforos excretados al medio por las PGPR captaban el hierro del medio y lo hacían menos disponible para los patógenos, restringiendo así su crecimiento (Kloepper et al., 1980). Posteriormente, a los sideróforos se les atribuyó un papel importante para inhibir el crecimiento de fitopatógenos también en suelos supresores de enfermedades (Kloepper et al., 1980). Actualmente, se ha ampliado el estudio de los sideróforos, sus receptores membranales, su regulación y su papel como mecanismo promotor del crecimiento vegetal en bacterias rizosféricas y aquellas que habitan la endorizósfera (Compant et al., 2005; Santoyo et al., 2012; Santoyo et al., 2016)

De igual manera se ha demostrado que los sideróforos pueden, por sí mismos, actuar como activadores eficientes de los sistemas de resistencia sistémica inducida en las plantas (Ran et al., 2005; Meziane et al., 2005; Bakker et al., 2007). La capacidad de los sideróforos para actuar como supresores de patógenos depende de la planta, del fitopatógeno a controlar, la composición del suelo, la bacteria y la afinidad del sideróforo por el hierro (Glick, 1995; Dellagi et al., 2009). Las plantas no se ven afectadas por el secuestro de hierro por parte de las rizobacterias, ya que la mayoría de ellas son capaces de crecer en medios con concentraciones de Fe^{+3} mucho menores que los microorganismos (O`Sullivan y O'Gara, 1992), además de que algunas son capaces de utilizar los complejos Fe^{+3}-sideróforo bacterianos.

En situaciones de altas concentraciones de hierro en el medio, las proteínas microbianas represoras dependientes de Fe^{+2} se unen al ADN ubicado antes de los genes involucrados en la producción de sideróforos, lo que impide su síntesis. En condiciones de bajas concentraciones de hierro, el Fe^{+2} se disocia de las proteínas represoras lo que activa la transcripción de los genes involucrados en la síntesis de los sideróforos microbianos (Aguado-Santacruz et al., 2012).

1.7 Solubilización de fosfatos.

El fósforo es uno de los elementos indispensables para la vida de todos los organismos, ya que forma parte del ácido desoxirribonuclíco o ADN, así como el ácido ribonucléico o ARN. También es parte esencial del ATP y de otras moléculas que contienen el ion fosfato (PO43-), además de ser parte de los fosfolípidos que integran a las membranas celulares (Fernández y Rodríguez, 2005).

A pesar de que la cantidad de fósforo en el suelo es generalmente bastante alta (a menudo entre 400 y 1,200mg/kg de suelo) la mayor parte de este fósforo es insoluble y por lo tanto no disponible para el crecimiento de la planta. El fósforo insoluble está presente ya sea como un mineral inorgánico, como apatita o como una de varias formas orgánicos incluyendo inositol fosfato (fitato suelo), fosfomonoesteres, y fosfotriésteres (Khan et al, 2007). Además, gran parte del fósforo inorgánico soluble que se utiliza como fertilizante químico se inmoviliza pronto después de ser aplicado de manera que no está disponible para las plantas y por lo tanto se desperdicia. Por lo que, la biodisponibilidad limitada de fósforo del suelo combinado con el hecho de que este elemento es esencial para el crecimiento vegetal significa que la incapacidad de obtener fósforo suficiente limita el crecimiento de las plantas (Feng et al, 2004).

Por todo esto, la solubilización y la mineralización del fósforo por bacterias solubilizante en fosfato es un rasgo importante en las PGPR (Fig. 3). Típicamente, la solubilización del fósforo inorgánico se produce como consecuencia de la

acción de ácidos orgánicos de bajo peso molecular tales como ácido glucónico y ácido cítrico, los cuales son sintetizados por diversas bacterias del suelo (Rodríguez y Fraga, 1999; Bar-Yosef, 1991; Rodríguez et al., 2004). Por otro lado, la mineralización de fósforo orgánico se produce a través de la síntesis de diferentes fosfatasas que catalizan la hidrólisis de los ésteres fosfóricos (Rodríguez y Fraga, 1999).

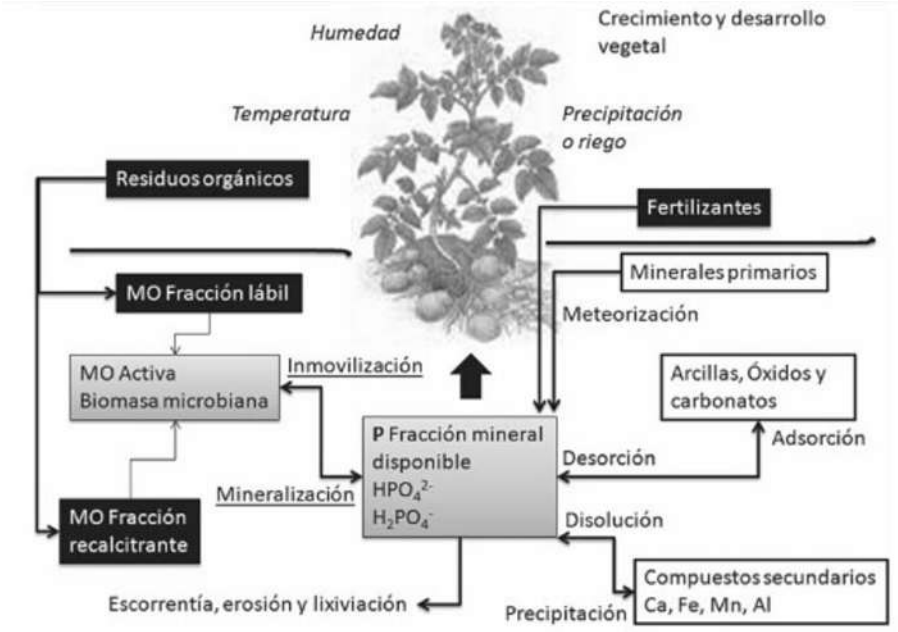

Figura 3. Relaciones entre el ciclo del fósforo y los compartimientos orgánicos y minerales, los cuadros negros son las entradas al sistema, los grises las fracciones disponibles, sin color las fracciones minerales; sin recuadro factores y procesos que tienen influencia en la disponibilidad. MO: materia orgánica (Rincón y Gutiérrez, 2012).

Desafortunadamente, debido a resultados variables, la aplicación comercial de PGPR solubilizadoras de fosfato ha sido bastante limitada. De hecho, los efectos positivos más consistentes se ven cuando estas bacterias son coinoculadas con bacterias con otras capacidades fisiológicas o con hongos micorricicos o no micorricicos (Rojas et al., 2001). Así mismo, se sabe que aproximadamente el 40% de las poblaciones microbianas de la rizósfera pueden tener la capacidad de

solubilizar fosfatos y que su actividad está asociada con la promoción del crecimiento vegetal (Kucey, 1983)

1.8 Producción de antibióticos.

La síntesis de antibióticos por parte de PGPR es un mecanismo importante para controlar patógenos que pueden causar enfermedades en plantas. En especial si son plantas de interés agrícola donde puede ser afectada su producción debido al efecto de patógenos. Los antibióticos producidos por PGPR, además de tener actividad antipatogénica, pueden tener la capacidad de encender el sistema de defensa de la planta (Fernando et al., 2006). Sin embargo, este tema se tratará más adelante.

Existen diversos géneros bacterianos que son ampliamente estudiados como PGPR, incluyendo *Bacillus*, *Rhizobium*, *Serratia*, *Azospirillum* y *Pseudomonas*, por mencionar algunos. El género *Pseudomonas* se encuentra entre los grupos más importantes de bacterias de la rizósfera que contribuyen a la defensa natural de las plantas, además de ser responsables de suprimir enfermedades en suelos (Mendes et al., 2011). *Pseudomonas* es productor de una amplia gama de antibióticos de amplio espectro, incluyendo derivados de fenazinas (fenazina-1-ácido carboxílico), 2,4-diacetilfloroglucinol (2,4-DAPG), pirrolnitrina y pyoluteorina, que actualmente son un foco importante de la investigación de biocontrol, y de los genes implicados en la regulación y la síntesis de estos compuestos son ahora conocidos.

El género *Pseudomonas* se encuentra entre los grupos más importantes de bacterias de la rizósfera que contribuyen a la defensa natural de las plantas. *Pseudomonas* es productor de una amplia gama de antibióticos de amplio espectro, incluyendo derivados de fenazina, 2,4-diacetilfloroglucinol (2,4DAPG), pirrolnitrina y pyoluteorina, que actualmente son un foco importante de la

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1.8.1 2,4-Diacetilfluoroglucinol.

El metabolito secundario producido por el género *Pseudomonas*, 2,4-diacetilfloroglucinol (DAPG) es uno de los compuestos más intrigantes sintetizados por PGPR y que ha sido ampliamente estudiado por sus capacidades antipatogénicas. Este compuesto fenólico se estudió inicialmente por sus propiedades antimicrobianas, posteriormente se han analizado sus propiedades como antifúngico, antibacteriano, antiviral, antihelmíntico, y fitotóxico cuando se utiliza a una alta concentración (Haas y Keel, 2003; Weller, 2007).

Sin embargo, a concentraciones más bajas, DAPG actúa como una molécula señal capaz de influir en la expresión de genes relacionados con biocontrol en *Pseudomonas* y hongos (Baehler et al., 2005; Brodhagen et al., 2004; Lutz et al., 2004). DAPG también puede ser una molécula señal para la inducción de resistencia sistémica de plantas (Iavicoli et al., 2003; Bakker et al., 2007), la estimulación de la exudación de la raíz (Phillips et al., 2004), y la mejora del sistema radicular a través de la vía de señalización dependiente de auxinas (Brazelton et al., 2008).

El locus biosintético incluye el operón de cinco genes *phlACBDE* (Bangera y Thomashow, 1999). Particularmente es digno de mencionar *phlD*, que se requiere para la síntesis de floroglucinol, un precursor de monoacetilfloroglucinol (MAPG) y 2,4-DAPG (Achkar et al. 2005). *PhlD* tiene notable homología con los miembros de la familia de las enzimas de plantas, chalcona sintasa / estilbeno sintasa (CHS / STS) (Bangera y Thomashow, 1999; Ramette et al., 2001). La alineación de la secuencia de aminoácidos indican que la región del probable sitio activo de *PhlD*,

se corresponde bien con la de miembros de la familia CHS / STS, lo que llevó a Bangera y Thomashow (1999) a sugerir que el gen podría haber sido trasladado desde las plantas a la bacterias colonizadoras de estas.

Los marcos de lectura de *phlG, phlA, phlC, phlB, phlD* y *phlE* tienen una orientación transcripcional común, mientras que *phlH* y *phlF* tienen una orientación opuesta (Fig. 4).

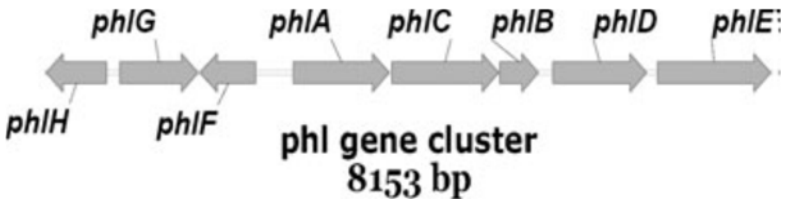


Figura 4. Mapa físico de los genes *phl* en *Pseudomonas fluorescens* F113 involucrados en la biosíntesis de DAPG. (Yang, 2012).

phlA es el gen próximo al promotor en el operón *phlACB*, el producto de *phlA* muestra una alta similitud con una Hidroximetilglutaril-CoA sintetasa (HMG-CoA) de *Archaeoglobus profundus* y una β-cetoacil sintetasa tipo III (FaGH) de *Escherichia coli* (Tsay et al., 1992). *phlB* es una proteína pequeña cuya secuencia tiene homología con varias proteínas de unión a ácidos nucleicos en la base de datos Genbank. El gen *phlC* posee un sitio de unión a acetil-CoA que contiene un residuo de cisteína activo y una región C-terminal rica en glicina que le confieren características estructurales típicas para la condensación de enzimas.

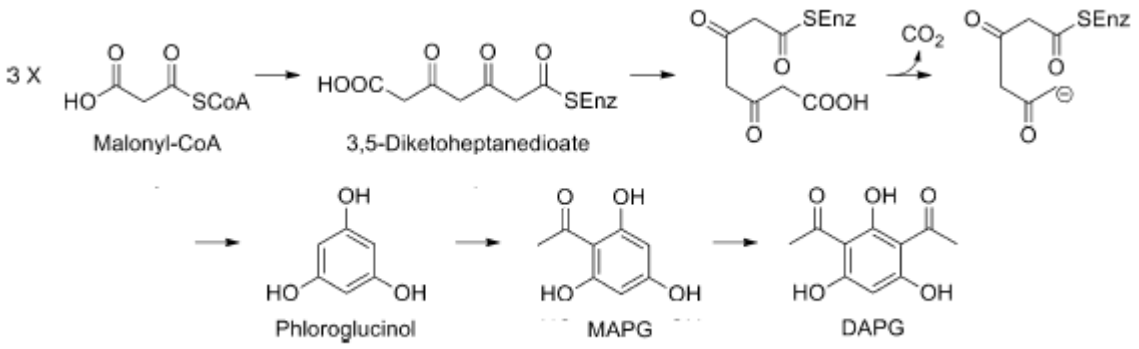


Figura 5. Ruta propuesta para la biosíntesis del DAPG donde tres moléculas de malonil-CoA se condensan para formar un triquétido, 3,5-diketo heptanodiato. La ciclación del triquétido se inicia luego por una descarboxilación del grupo carboxilo, dando lugar a una reacción de condensación de Claisen y resultando floroglucinol, el floroglucinol puede ser acetilado en la posición C2 para formar los MAPG y luego una vez más acetilado en la posición C4 para formar DAPG (Yang, 2012).

phlE se encuentra corriente debajo de los genes *phlACBD,* el alineamiento con las bases de datos sugiere que codifica una permeasa que sirve como proteína exportadora, por lo que se supone que su papel es exportar fuera de la célula intermediarios tóxicos de la degradación del DAPG.

El gen *phlF* está localizado corriente arriba del operón *phlACB* (Fig. 4) Delany et al. (2000) demostró que PhlF se une a la región intergénica phlA-phlF y que la sobreexpresión de *phlF* en *P. fluorescens* resulta en la represión de la producción de DAPG. Mayores estudios demostraron que la represión por PhlF es debida a la interacción con una secuencia repetida invertida denominada phlO, localizada corriente arriba del sitio de inicio de la trascripción de *phlA*. PhlF se une a phlO y bloquea a la ARNpolimerasa, evitando la transcripción de *phlACB*.

Bottiglieri y Keel (2006) designaron a *phlG* como una hidrolasa que cataliza la degradación de DAPG en MAPG y acetato, mediante la escisión de un enlace C-C que une el grupo acetilo al anillo de benceno. El gen *phlH* está localizado corriente arriba de *phlG*, su producto muestra similitud con las proteínas reguladoras IfeR de *Agrobacterium tumefaciens* y AcrR de *E. coli*, sin embargo su actividad precisa no está completamente clara.

1.8.2 Ácido cianhídrico (HCN).

El ácido cianhídrico (HCN) es un compuesto volátil antimicrobiano de amplio espectro que ha sido estudiado en bacterias del género *Pseudomonas*, el cual se le ha asociado en el control biológico de enfermedades de las raíces de diversas especies vegetales. La producción de HCN por *Pseudomonas fluorescens* está implicada en la supresión de enfermedades causadas por hongos fitopatógenos, tales como *Thielaviopsis basicola* (Laville et al 1998; Voisard et al., 1989), *Septoria tritici* y *Puccinia recondita* (Flaishman et al. 1996).

El mecanismo de inhibición del crecimiento en diversos microorganismos por parte del HCN ha sido descrito. Se ha propuesto que tiene la capacidad de inhibir la citocromo c oxidasa en la cadena respiratoria (Knowles 1976), además de unirse a metaloenzimas (Blumer y Haas 2000). Sin embargo, además de su papel en la protección de las plantas, también se ha reportado un efecto deletéreo de HCN microbiano en varias especies de plantas (Alström y Burns 1989; Bakker y Schippers 1987). De hecho, la producción de HCN parece representar un factor de virulencia en *P. aeruginosa* (Gallagher y Manoil 2001). Cepas productoras de HCN incluso se han estudiado para el control biológico de malezas (Kremer y Souissi 2001).

1.8.3 Fenazinas

Las fenazinas incluyen una gran familia de compuestos heterocíclicos que contienen nitrógeno, sintetizados a través de la ruta del ácido shikímico. Las Fenazinas de origen natural son producidas por miembros de varios géneros bacterianos incluyendo *Pseudomonas* (Chin-A-Woeng, et al, 2003; Smirnov y Kiprianova, 1990; Mavrodi et al, 2006; Turner y Messenger, 1986).

La mayoría de las fenazinas son ampliamente activas contra hongos como *Fusarium oxysporum y Gaeumannomyces graminis var. tritici* (Chin-A-Woeng, et al, 2003) así como bacterias Gram-positivas y Gram-negativas (Smirnov y

Kiprianova, 1990) debido a su capacidad para someterse a transformaciones de óxido-reducción (Laursen y Nielsen, 2004;. Price-Whelan et al, 2006).

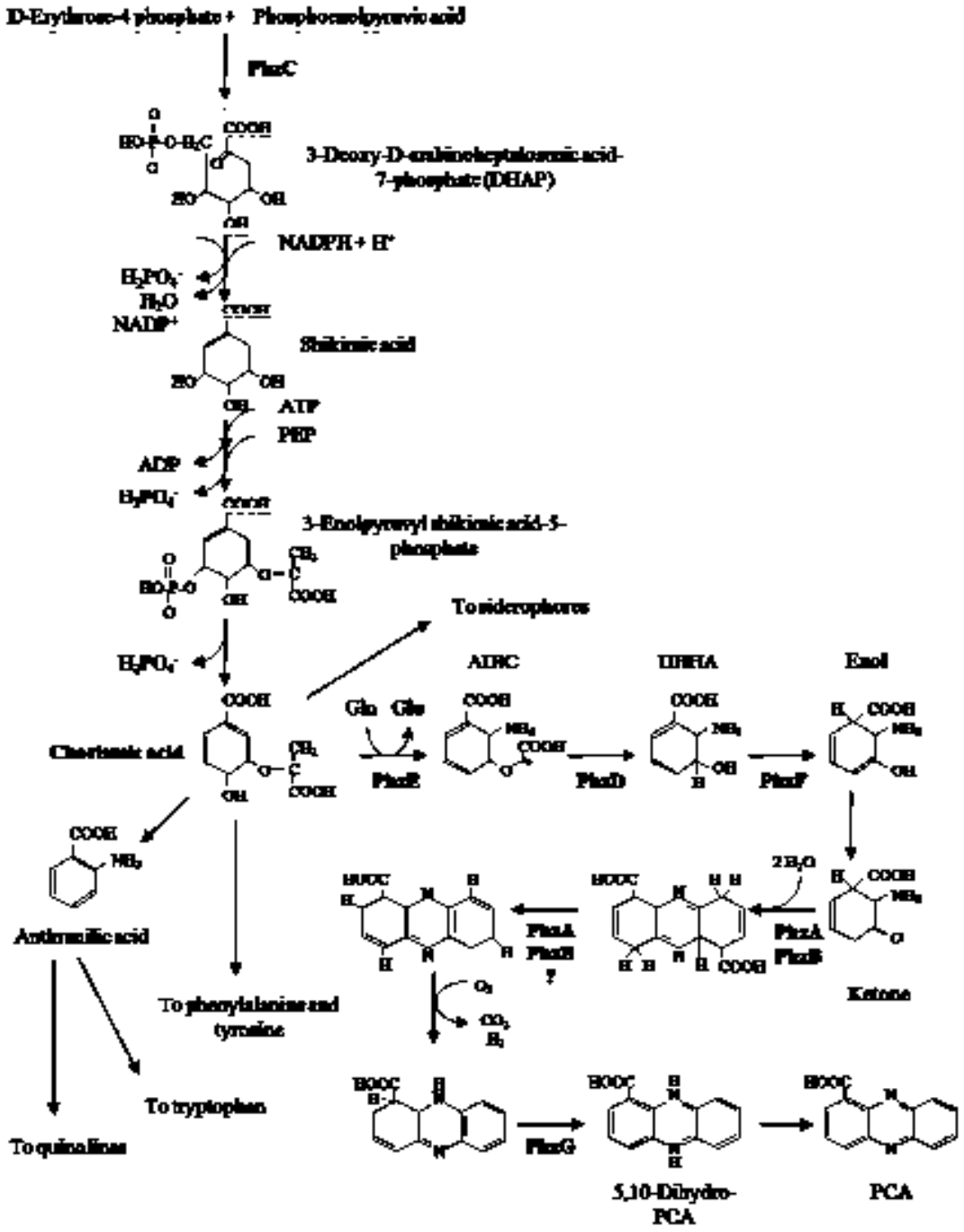


Figura 6. Biosíntesis de las Fenazinas. Ver detalles en el texto. (Pierson y Pierson, 2010).

La biosíntesis de fenazinas es derivada de la vía de síntesis del ácido shikímico (Fig.6), este es convertido a ácido corísmico como parte de la vía común de síntesis de los aminoácidos aromáticos.

El grupo amino de la glutamina es enzimáticamente transferido por PhzE (enzima específica de la síntesis de fenazina) dando lugar al ácido 2-amino-2-deoxiisocorísmico (ADIC), otra enzima específica PhzD cataliza la hidrólisis que convierte a ADIC en ácido trans-2,3-dihidro-3-hidroxiantranílico (DHHA).

Aunque la segunda parte de la vía no es tan clara como la primera, se cree que 2 moléculas de DHHA se condensan para formar ácido 1,6-dicarboxílico, lo cual se observa en cepas de *Streptomyces, Pseudomonas aeruginosa y P. fluorescens*. Sin embargo en otras cepas de Pseudomonas se observa una descarboxilación durante la dimerización formando ácido 1-carboxílico como producto de condensación.

Además de su papel en la supresión de hongos patógenos de plantas también ha habido cierta discusión de un papel para fenazinas en ayudar a la competencia ecológica de *Pseudomona*s en la rizósfera (Mazzola, et al, 1992; Pierson y Pierson, 1996), presumiblemente debido a su capacidad para generar especies reactivas de oxígeno que matan a otros organismos (Hassan y Fridovich, 1980). Así como también, el de activar la resistencia sistémica inducida (ISR) en las plantas (Pierson y Pierson, 1996).

1.9 Producción y regulación de hormonas.

Las fitohormonas u hormonas vegetales son sustancias que actúan como señales químicas producidas por las plantas que coordinadamente controlan el crecimiento y desarrollo de estas incluso en concentraciones extremadamente bajas (Muller y Munné-Bosch, 2011; Muday et al, 2012).

Algunas fitohormonas son producidas no sólo por la planta, sino también por bacterias asociadas a plantas, incluyendo las PGPR. Cabe destacar que las

hormonas excretadas por las PGPR actúan y llegan a activar vías de señalización biosintética en diferentes tejidos vegetales (Roy et al., 2010). Las auxinas son de las principales fitohormonas que regulan el crecimiento de células vegetales. Dado que el nivel de las auxinas presentes en el entorno de la planta afecta críticamente su desarrollo, se especula que la producción de las auxinas microbianas puede alterar su nivel y afectar a todos los procesos fisiológicos regulados por estas, promoviendo así la altura de la planta, la biomasa y el rendimiento del grano o la producción en plantas de interés agrícola (Molina-Favero et al, 2008; Khan y Doty, 2009).

Las auxinas, representadas principalmente por el ácido indol-3-acético (AIA), están implicadas en la regulación del crecimiento y desarrollo vegetal, como habíamos comentado anteriormente. Las auxinas secretadas por las bacterias pueden actuar como moléculas de señalización en la comunicación entre las bacterias para coordinar sus actividades (Ouzari et al., 2008). Una de las características más destacadas de las plantas inoculadas con bacterias promotoras del crecimiento vegetal productoras de auxinas es la modificación de la morfología y el desarrollo de la raíz. Por lo tanto, las bacterias promotoras del crecimiento vegetal promueven el crecimiento de raíces, aumentando el área de superficie de la raíz que a su vez promueve la absorción de nutrientes estimulando indirectamente el crecimiento de las plantas (Egorshina et al., 2012).

Otra fitohormona importante en plantas es el Etileno. La hormona etileno, que se encuentra en todas las plantas superiores, es un modulador importante del crecimiento normal y el desarrollo en plantas, así como una característica clave en la respuesta de estas a una amplia gama de tensiones (Abeles et al. 1992).

Muchos aspectos del crecimiento de los tejidos vegetales, tales como raíces, tallos, hojas, flores y frutos, así como todas las etapas de desarrollo de la planta se ven afectados por el etileno. Una variedad de otros procesos de la planta implica etileno incluyendo nodulación de las leguminosas (Goodlass y Smith 1979), enraizamiento de los esquejes (Li et al., 2005, Mayak et al., 1999 y

Montero-Calasanz et al., 2013), así como la interacción de una planta con micorrizas benéficas (Beyrle, 1995 y Gamalero et al., 2008).

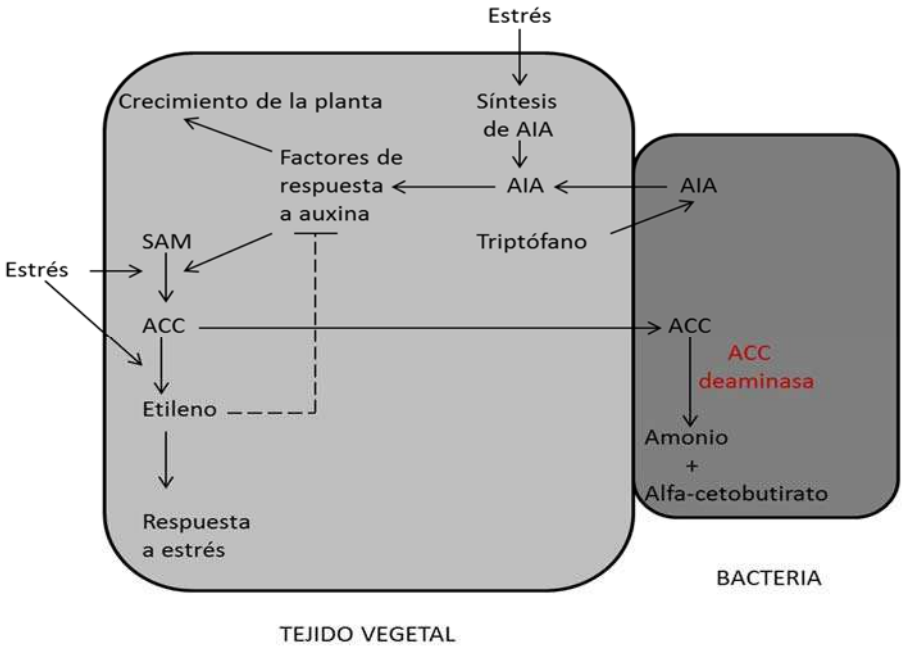


Figura.7. Modelo esquemático de como las bacterias promotoras del crecimiento vegetal que producen ACC deaminasa y AIA puede facilitar el crecimiento de las plantas. Abreviaturas: ACC, 1-aminociclopropano-1-carboxilato; AIA, ácido indol -3- acético; SAM, *S*- adenosil metionina. (Modificado de Glick., 2014).

Desde hace décadas se propuso que las bacterias rizosféricas y endófitas pueden modular los niveles de etileno en plantas, lo cual ha estado asociado a la promoción de su crecimiento y desarrollo (Glick, 2014; Santoyo et al., 2016). Las PGPR (Plant Growth-Promoting Rhizobacteria) y PGPBE (Plant Growth-Promoting Bacterial Endophytes) o PGPB (Plant Growth-Promoting Bacteria) en general, que contienen la enzima desaminasa del ácido 1-aminociclopropano-1-carboxílico (ACC) o ACC desaminasa tienen la ventaja importante de modular los niveles de etileno en plantas.

El mecanismo de acción de la ACC desaminasa es el siguiente: Primero, el ácido 1-aminociclopropano-1-carboxílico (ACC) es exudado por las raíces de las plantas, el cual a su vez es adquirido por la rizobacteria (o bacteria endófita). Segundo, el ACC es convertido en α-cetobutirato (α-CB) y amonio por la ACC desaminasa. De esta manera, las PGPB está actuando como un ¨recipiente¨ para el ACC que proviene de la planta, lo que reduce los niveles de ACC, disminuyendo la cantidad de ACC dentro de la planta que se puede convertir en etileno (Glick, 2014; Santoyo et al., 2016). Adicionalmente, la actividad de ACC desaminasa es un mecanismo que utilizan algunas PGPR para disminuir las concentraciones de etileno y al mismo tiempo, incrementar la disponibilidad de amonio en un ambiente rizosférico.

Así mismo, la actividad ACC desaminasa puede ayudar a mejorar la nutrición vegetal y la resistencia a factores de estrés y actualmente está ayudando a que el uso de PGPB que contienen ACC desaminasa permita mejorar sistemas agrícolas de ambientes áridos, salinos o con problemas de contaminación (Esquivel-Cote et al., 2013).

1.10 Compuestos orgánicos volátiles

Algunas bacterias pueden promover el crecimiento vegetal e inducir la respuesta sistémica (ISR) de las plantas en ausencia de contacto físico, mediante compuestos orgánicos volátiles (VOCs), estos compuestos también tienen la capacidad de inducir tolerancia sistémica (IST) al estrés causado por factores abióticos como sequía y metales pesados (Farag et al., 2013). Los sistemas ISR e IST se describen a detalle más adelante.

Los VOCs son definidos como todos aquellos compuestos que presentan una presión de vapor de 0.01 kPa o más a 20 °C (Insam y Seewald, 2010), por lo que son volátiles a temperatura ambiente. (Valencia-Cantero et al., 2012)
Algunos ejemplos de VOCs son los alcoholes, aldehídos, cetonas y CO2.

Compuestos producidos por PGPR, como la dimetilhexadecilamina, la cual es emitida por *A. agilis* UMCV2 produce un efecto benéfico en el crecimiento vegetal de plantas como *M. sativa* (Orozco-Mosqueda et al., 2013)

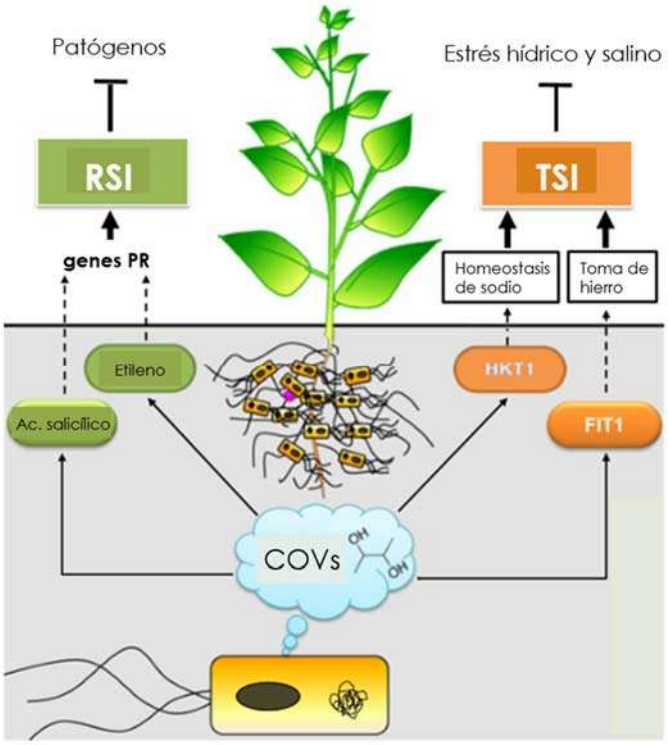


Figura.8. Modelo de la resistencia sistémica inducida (RSI) y la tolerancia sistémica inducida (TSI) provocados por compuestos orgánicos volátiles (COVs) emitidos por rizobacterias promotoras del crecimiento de la planta. Las flechas continuas indican compuestos de la planta afectados por COVs bacterianos, las flechas discontinuas muestran la respuesta de la planta a través de sus componentes reguladores. (Modificado de Farag et al., 2013).

Algunas cepas de PGPR, producen compuestos orgánicos volátiles tales como 2,3-butanodiol, lo que resulta en la sobreexpresión de los genes PR relacionados con la patogénesis a través de las vías de señalización de ácido salicílico y etileno que confieren ISR contra fitopatógenos y herbívoros.

Los VOCs bacterianos reprimen la expresión de HKT1 (Fig. 8), un transportador de Na+ esencial en el mecanismo de tolerancia a salinidad (Almeida et al., 2013), en las raíces pero la estimulan en los tejidos de los brotes. También regulan la

sobreexpresión de FIT1 (Fig. 8), un factor transcripcional que regula las respuestas de captación de hierro (Colangelo y Guerinot, 2004), en toda la planta bajo altas condiciones de sal, toxicidad de metales, y condiciones de sequía (Farag et al., 2013).

1.11 Producción de enzimas líticas.

Los microorganismos son una de las principales fuentes de enzimas del suelo, en el cual las actividades enzimáticas desempeñan un papel importante como degradadoras de la pared celular de organismos patógenos, contribuyendo así con las capacidades de biocontrol de las PGPR. (Hernández-Salmerón, 2014). Las quitinasas, glucanasas, celulasas y proteasas son enzimas que causan lisis y degradación de la pared celular de hongos (Fig.9).

Proteasas extracelulares producidas por *Stenotrophomonas maltophilia W81* están involucradas en el biocontrol de *Pythium ultimum* en la rizósfera de remolacha (*Beta vulgaris*) (Dunnes et al. 1997).

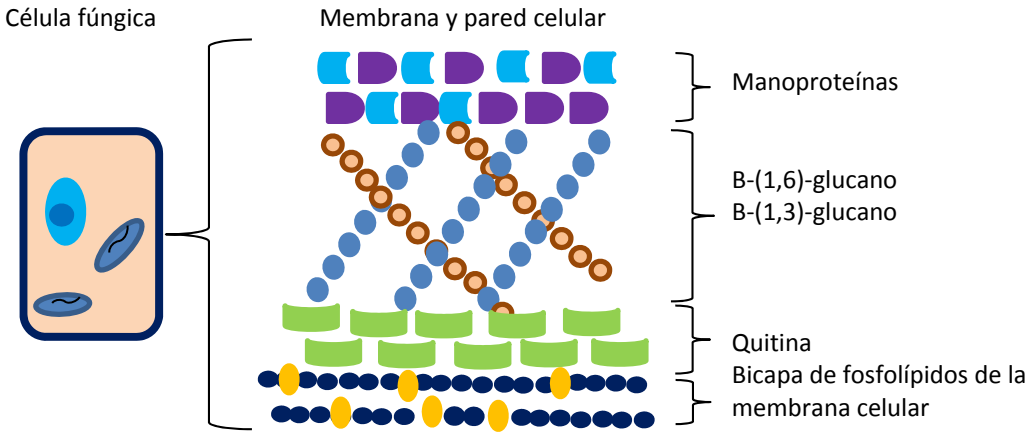


Figura 9. Estructura general de la pared celular de un hongo que muestra los principales blancos de las enzimas líticas.

Otro ejemplo de esto son los aislados de *Bacillus subtilis, Erwinia herbicola, Serratia plymuthica* y un *actinomiceto* que mostraron actividad antifúngica contra *Eutypia lata* a través de la producción de quitinasas, proteasas y celulasas (Schmidt et al. 2001).

La capacidad de producir este tipo de compuestos confiere a las PGPR una gran ventaja para competir en un medio tan activo como la rizósfera, estas capacidades permiten como ya se había mencionado la posibilidad de degradar la pared celular de diversos microorganismos y en algunos casos incluso poder utilizar sus nutrientes.

1.12 Inducción del sistema inmune de la planta.

Las plantas poseen resistencia sistémica adquirida (SAR), que proporciona una defensa a largo plazo frente a un amplio espectro de patógenos. La Inmunidad sistémica adquirida mediada por ácido salicílico provoca la respuesta de defensa en todo el sistema de la planta durante la infección por patógenos en un sitio particular (Muthamilarasan y Prasad, 2013).

Por años la respuesta sistémica inducida (ISR) activada por bacterias promotoras del crecimiento y la respuesta sistémica adquirida (SAR) dependiente de ácido salicílico y activada por organismos patógenos se estudiaron de manera independiente. En los últimos años, se ha demostrado que la respuesta SAR y la vía ISR son compatibles y que no hay significativa discordancia entre ellas. En *Arabidopsis thaliana*, SAR e ISR son eficaces contra un amplio espectro de patógenos, incluyendo el patógeno foliar *Pseudomonas syringae*. La activación simultánea de SAR e ISR da lugar a un efecto aditivo sobre el nivel de protección inducida contra en patógeno. Además, tanto SAR como ISR requieren de la proteína reguladora clave NPR1 (Van Wees et al., 2000).

Actualmente se han realizado varios trabajos que indican que las plantas también poseen, en esencia, dos vías del sistema inmune. Una utiliza un patrón de receptores de reconocimiento transmembrana (PRRS) que responden a patrones moleculares asociados a microbios/patógenos (MAMPs / PAMPs), tales como la flagelina. La segunda actúa en gran medida dentro de la célula, utilizando productos de proteína polimórficos NB-LRR, los cuales llevan el nombre de su característica unión a nucleótidos (NB) y sus dominios repetidos invertidos ricos en leucina (LRR). Efectores de patógenos de diversos reinos son reconocidos por proteínas NB-LRR, y activan las respuestas de defensa (Jones y Dangl, 2006).

Nuestra visión actual del sistema inmunológico planta puede ser representado como un modelo de cuatro fases "zigzag" (Fig.10).

En la fase 1, PAMP (o MAMPs) son reconocidos por PRRs, lo que resulta en una activación de la inmunidad disparada por PAMP (PTI) que puede detener la colonización.

En la fase 2, los patógenos exitosos despliegan efectores de patógenos que contribuyen a la virulencia. Los efectores pueden interferir con PTI. Esto se traduce en la susceptibilidad activada por efectores. (ETS).

En la fase 3, un efector dado se "reconoció específicamente" por una de las proteínas NB-LRR, lo que resulta en la inmunidad activada por efector (ETI). El reconocimiento es bien indirecto o por medio de reconocimiento directo NB-LRR-efector. ETI es una respuesta PTI acelerada y amplificada, lo que resulta en resistencia a la enfermedad y, por lo general, una respuesta de muerte celular hipersensible (HR) en el sitio de la infección.

En la fase 4, la selección natural impulsa a algunos patógenos para evitar la ETI, ya sea por la diversificación del el gen efector reconocido, o mediante la adquisición de efectores adicionales que suprimen la ETI.

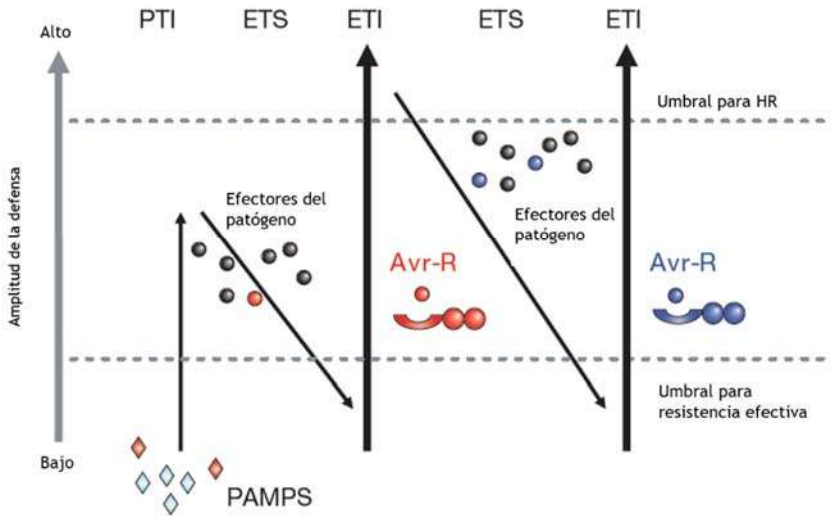


Figura 10. Modelo de zigzag que ilustra la activación del sistema inmune de la planta. En este esquema, la amplitud máxima de la resistencia o la susceptibilidad a la enfermedad es proporcional a [PTI – ETS + ETI]. (Jones y Dangl, 2006).

Sin embargo, este conocimiento sólo se puede apreciar en su totalidad una vez que sepamos hasta qué punto se producen estos mecanismos bajo condiciones realistas en la rizósfera de diversas plantas.

Una mejor comprensión de los factores que modulan las interacciones de *P. fluorescens* con las plantas mejorará la eficacia de la introducción de *P. fluorescens* para mejorar la producción y la defensa vegetal.

Por lo que es importante aislar y caracterizar cepas nativas de *Pseudomonas fluorescens* para determinar si pueden ser consideradas como potenciales bioinoculantes, y así, constituir parte del llamado Control Integrado de Plagas.

2. Antecedentes

Bacterias del género *Pseudomonas* ha sido ampliamente estudiado por sus capacidades relacionadas con el control biológico de fitopatógenos, biofertilización de cultivos y biorremediación (Cabanás et al., 2014; Agaras et al., 2015; Khalid et al., 2016) Cuando se utilizan como bioinoculantes para promover el crecimiento y salúd vegetal, estas *Pseudomonas* son enfrentadas a las condiciones adversas en el ecosistema llamado suelo. La competencia por espacios es complicada, ya que todos los micoorganismos desean llegar a la zona de la rizósfera donde tengan acceso a nutrientes. Sin duda que para poder proliferar en la rizósfera se requiere de un arsenal de mecanismos que les permitan a las bacterias colonizar los mejores nichos.

Se conocen reportes desde 1946 (Krasilnikov y Raznitzyna 1946), de aislados del genero *Pseudomonas* con capacidad de reducir los síntomas de daping-off en *Pinus silvestris.* A partir de este tipo de observaciones, numerosos estudios (Tabla 2) se han enfocado a tratar de explicar los mecanismos por los cuales este género protege diferentes plantas de hongos y bacterias fitopatógenos promoviendo así su crecimiento.

Tabla 2. Trabajos pioneros y relevantes donde se incluyen Pseudomonas y sus capacidades directas e indirectas de promoción del crecimiento vegetal.

Año	Descubrimiento	Referencia
1946	Aislados del género ***Pseudomonas*** reducen síntomas de enfermedad causada por *Fusarium.*	Krasilnikov y Raznitzyna.
1970	Propiedades antibióticas de **DAPG** de *P. fluorescens.*	Reddy y Borovkov et al.
1974	Efecto antibiótico de *Pseudomnas* en **hongos fitopatógenos**.	Kvasnikov et al.
1980	PGPR Productoras de **sideróforos** ejercen su	Kloepper et

	actividad promotora del crecimiento vegetal al privar a la microflora nativa de hierro.	al.
1986	Actividad anti fúngica de un dímero de **fenazina acido carboxílico** aislado de *P. fluorescens*.	Gurusiddaiah et al
1994	La producción de pioluteorina está involucrada en la supresión de damping-off por la cepa **CHA0**.	Maurhofer et al.
2000	Regulación de la producción de DAPG en P. fluorescens **F113.**	Delany et al.
2012	El antibiótico 2,4-DAPG es un determinante importante de la **ISR** en *P. fluorescens.*	Weller et al.
2014	Inhibición de *Colletotrichum musae* por *Pseudomonas fluorescens* FP7	Peeran et al.
2014	*P. fluorescens* PICF7 activa los sistemas de defensa de raíz, tallo y hoja en plantas de aceituna.	Cabanás et al.
2015	Inhibición del crecimiento de hongos fitopatógenos por diferentes especies de *Pseudomonas.*	Agaras et al.
2015	P. fluorescens Pf9A-14 y 80-45 suprimen enfermedades causadas por *Pythium, Phytophthora cansici* y *Rhizoctonia solani*.	Khabbaz et al.
2015	Promoción del crecimiento vegetal por *P. fluorescens* SS101 a través de **compuestos orgánicos volátiles**.	Park et al.

En 1980, Kloepper y col. Presentan evidencia de una cepa de *Pseudomonas fluorescens* capaz de incrementar el rendimiento de papa, remolacha y rábano hasta en un 144% a través de la producción de sideróforos extracelulares que de manera eficiente forman complejos con el hierro del medio ambiente privando de este a la microflora nativa.

Años más tarde, se caracteriza un antibiótico aislado de la cepa de *Pseudomonas fluorescens* 2-79 que mostró una excelente actividad contra varias especies de hongos, incluyendo el patógenos de trigo *Gaeumannomyces graminis*.

Rhizoctonia solani y *Pythium aristosporum*, sugiriéndose que podía tener un papel en la supresión de enfermedades causadas en trigo (Gurusiddaiah et al, 1986). Se propuso entonces que el antibiótico fenazina era el principal producto y que retenía la mayor parte de las características biológicas de la molécula parental.

Los mecanismos de supresión de enfermedades se han estudiado a fondo en los últimos años, *Pseudomonas fluorescens* CHA0 ha sido una de las cepas más estudiadas a nivel molecular. Se encontró que la producción del poliquétido antimicrobiano 2,4-diacetilfloroglucinol (DAPG) es crucial para la supresión de enfermedades causadas principalmente por hongos fitopatógenos (Keel et al., 1992).Sin embargo, el metabolito bacteriano, ácido cianhídrico (HCN) y el antibiótico pioluteorina (Plt) también contribuyen a la capacidad supresora de enfermedades de la cepa CHAO (Voisard et al., 1989; Maurhofer et al, 1994).

En los últimos años, los estudios han sido enfocados hacia la producción de compuestos orgánicos volátiles (VOCs) y la inducción de las respuestas sistémicas de la plata. Estos estudios indican que el antibiótico 2,4-DAPG de *P. fluorescens* es un determinante importante de la ISR (Weller et al., 2012). Y que los VOCs son algunos de los principales metabolitos reguladores de la mejora del crecimiento y desarrollo de las plantas. Además estos compuestos bacterianos contribuyen a las interacciones dadas entre organismos de diferentes reinos tales como plantas, hongos y nematodos y se estudian sus posibles aplicaciones clínicas e industriales (Audrain et al., 2015).

Pseudomonas fluorescens SS101, una rizobacteria no patógena que se aisló de la rizósfera de trigo (*Triticum aestivum*) y ha sido estudiada para determinar la influencia de VOCs en plantas de tabaco (*Nicotiana tabacum*). Se identificaron claramente 11 compuestos químicos diferentes presentes en las muestras inoculadas con Pf.SS101. Por otra parte, las plantas de tabaco expuestas a diversas concentraciones de 13-Tetradecadien-1-ol, 2- butanona y 2-metil-N-1-trideceno, los principales VOCs encontrados, mostraron clara mejoría en la promoción del crecimiento de las plantas (Park et al., 2015). Los VOCs emitidos por diferentes Pseudomonas también han mostrado tener actividad inhibitoria

contra hongos y oomicetos (Zhou et al., 2014; Hunziker et al., 2015). *P. fluorescens* ALEB 7B inhibe significativamente el crecimiento de *Athelia rolfsii*, cuando este es expuesto a los compuestos orgánicos volátiles producidos por la bacteria, de los ocho compuestos identificados el mayor papel se le atribuye al dimetildisulfuro (Zhou et al., 2014).

Otra cepa analizada recientemente es *Pseudomonas fluorescens* N21.4, capaz de desencadenar la biosíntesis de flavonoides en plantas de mora (*Rubus sp*.) como parte de la respuesta sistémica inducida (ISR), para hacer que los niveles de flavonoides aumenten. Se han identificado genes estructurales que codifican enzimas de las vías de fenilpropanoides y de biosíntesis de flavonoides que catalizan la conversión de fenilalanina a los productos finales incluyendo flavonoles, antocianinas y catequinas (García-Seco et al., 2015) mejorando con esto la calidad de los frutos.

Las técnicas moleculares y los estudios de secuenciación masiva, han ayudado a mejorar nuestro conocimiento acerca de este género. A la fecha, los genomas de cuatro cepas de *P. fluorescens* se han secuenciado y se encuentran públicamente disponibles, incluyendo SBW25, WH6, Pf0-1 y Pf-5 (Paulsen et al., 2005; Kimblrel et al., 2010; Silby et al. 2009). Los genomas publicados han revelado que *P. fluorescens* tiene un pan-genoma abierto de aproximadamente 6 a 7 Mb. Además del núcleo de genes, cada cepa posee de 1000 a 1500 genes únicos, sin embargo las funciones de la mayoría de estos genes se desconocen.

A pesar de todos estos avances, en la mayoría de los casos no hemos logado establecer con claridad las condiciones necesarias para explotar todo el potencial que nos brindan estos agentes naturales.

La popularidad de los bioproductos se ha incrementado sustancialmente en los últimos años, incrementándose también los estudios relacionados con las técnicas de producción, almacenaje, transporte y aplicación pero aún se encuentran es desventaja con respecto a los productos químicos utilizados por años.

Por lo que es importante continuar con el estudio de nuevas cepas y las condiciones adaptativas que nos ayuden a conocer las necesidades de estos organismos para el mayor aprovechamiento de sus capacidades.

3. Justificación.

Los cultivos agrícolas continuamente se ven expuestos a potenciales patógenos que afecten la producción, por lo que el uso de químicos es la primera opción para lograr combatirlos y eliminarlos. Sin embargo, múltiples estudios muestran y fundamentan los aspectos negativos de su uso en el ambiente y salud humana. Por lo tanto, la aplicación de agentes biológicos para combatir potenciales fitopatógenos, así como mejorar la promoción del crecimiento, se considera una excelente opción que conduzca hacia una agricultura sustentable. Para lograrlo, se requiere aislar, conocer y caracterizar nuevas cepas bacterianas que exhiban capacidades relevantes y con potencial para ser el componente activo de bioinoculantes. Sin duda que determinar su acción y mecanismo específico contra ciertos fitopatógenos nos conducirá a proponer alternativas de protección y promoción del crecimiento vegetal más eficientes.

4. Hipótesis.

Aislados bacterianos de *Pseudomonas fluorescens* provenientes de la rizósfera de Alfalfa (*Medicago sativa* L.) producen compuestos difusibles y volátiles con actividades específicas de promoción del crecimiento vegetal y de biocontrol.

5. Objetivos

5.1 Objetivo general

Evaluar la actividad de biocontrol y los mecanismos directos de promoción de crecimiento vegetal en cepas de *Pseudomonas fluorescens.*

5.2 Objetivos particulares

1. Aislar y caracterizar molecularmente nuevas cepas del género *Pseudomonas* de la rizósfera de alfalfa (*M. sativa* L.).

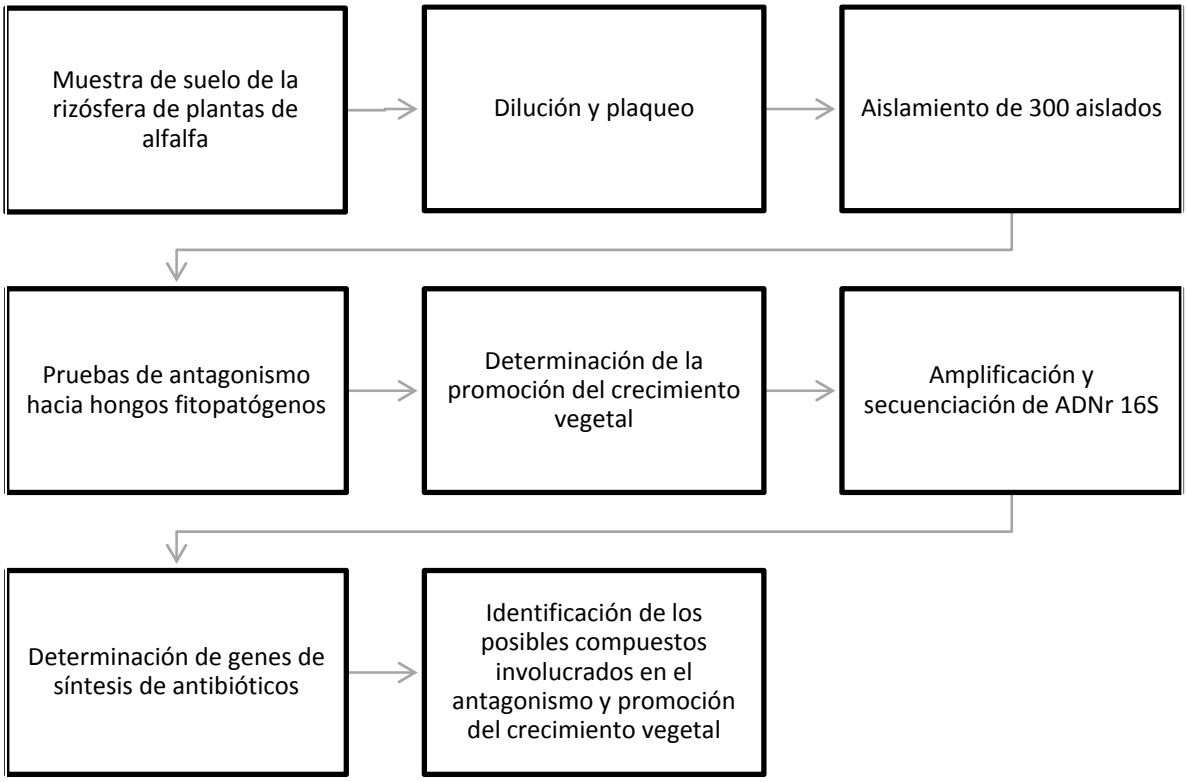
2. Evaluar las actividades de biocontrol y promoción del crecimiento vegetal de compuestos difusibles y volátiles en las cepas seleccionadas.

3. Determinar la presencia y expresión de genes que codifiquen para compuestos involucrados en actividades antagónicas.

4. Identificar los posibles compuestos difusibles y volátiles responsables del antagonismo y promoción del crecimiento vegetal.

5. Evaluar las capacidades de biocontrol del fitopatógeno *Botrytis cinerea* en las cepas de *Pseudomonas*.

6. Metodología.



7. Resultados

Capítulo I

"Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by *Pseudomonas fluorescens* strains"

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Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by *Pseudomonas fluorescens* strains

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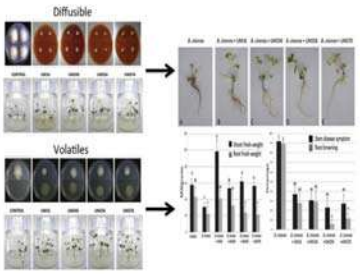
HIGHLIGHTS

- Four *P. fluorescens* antagonists were isolated and characterized.
- All strains showed biocontrol action against *B. cinerea*.
- All strains significantly increased *M. truncatula* biomass and chlorophyll content.
- Multiple antifungal and PGP compounds, including S-containing VOCs were identified.

GRAPHICAL ABSTRACT



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ABSTRACT

We analyzed the antifungal and plant growth-promoting (PGP) effects of diffusible and volatile organic compounds (VOCs) produced by new rhizospheric isolates. The bacterial strains were identified as *Pseudomonas fluorescens* UM16, UM240, UM256, and UM270, based on their complete 16S ribosomal gene sequencing. These pseudomonads showed a high degree of antagonism against the phytopathogen *Botrytis cinerea* during confrontation assays. In addition, all strains significantly increased *Medicago truncatula* biomass and chlorophyll content. Interestingly, these activities were exerted by the emission of either diffusible organic compounds or VOCs. During biocontrol experiments, the four *P. fluorescens* strains were able to protect *M. truncatula* plants from *B. cinerea* infection by reducing stem disease symptoms and root browning. The potential presence of phenazines, cyanogens, and ACC (1-aminocyclopropane-1-carboxylate) deaminase, as well as the production of biofilm, siderophores, proteases, and indole-3-acetic acid were identified in most of the strains. The VOCs emitted by all four *Pseudomonas* strains were similar, and sulfur-containing compounds were among the most abundant, including dimethyl disulfide. Interestingly, the strain UM270 was the only one that produced dimethylhexadecylamine, a compound with antifungal and PGP activities. In conclusion, the *Pseudomonas* strains analyzed here exert multiple antagonistic and PGP mechanisms, and represent an excellent option to be used as either biocontrol or biopromoting agents in crops.

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1. Introduction

Botrytis cinerea is the causal agent of gray mould disease, causing tremendous damage in more than 200 plant species around the world (Nambeesan et al., 2012). In many countries, and especially in developing regions, the first choice to control gray mould disease in diverse crops is the use of agrochemicals, although their toxic effects on the environment have been widely documented (Adesemoye and Kloepper, 2009). Therefore, the biological control of fungal diseases by microbial agents appears to be an excellent option, as secondary damaging effects on the environment can be null or minimal, in addition to the advantage of being able to export products abroad without restriction (versus the use of chemicals). Fortunately, efforts to isolate and characterize endogenous biocontrol agents are ongoing in many research labs worldwide (Selin et al., 2010; Yanes et al., 2012; Guiñazú et al., 2013; Lagzian et al., 2013; Kakar et al., 2014). In some cases, biocontrol agents may present broad antagonism toward plant pathogens, whereas in others, inhibitory effects could be pathogen-specific, or even specific to environmental or regional physico-chemical soil conditions. All these variables remain to be investigated with basic and/or applied research in order to obtain the best results in the field (Singh et al., 2013).

Various studies have reported the capacity of diverse bacterial genera to control fungal diseases; however, the genera *Bacillus* and *Pseudomonas* appear frequently in the literature (Martínez-Absalón et al., 2014; Santoyo et al., 2012). In particular, diverse *Pseudomonas* spp., which are common and abundant inhabitants of the rhizosphere, have the capacity to inhibit or suppress plant diseases (Stutz et al., 1985; Weller et al., 2002; Weller, 2007; Singh et al., 2012, 2013). Similarly, when fluorescent pseudomonads are directly applied to seed or soil inoculation treatments, excellent biocontrol activities have been reported (De la Fuente et al., 2006; Lagzian et al., 2013).

The inhibition of plant pathogens by rhizospheric bacterial strains is considered an indirect mechanism of plant growth promotion (Glick, 2014; Santoyo et al., 2012). *Pseudomonas* spp. produce an antimicrobial arsenal, including hydrogen cyanide (HCN), pyoluteorin, phenazines, pyrrolnitrin, siderophores, cyclic lipopeptides, and 2,4-diacetylphloroglucinol (DAPG), as well as excrete hydrolytic enzymes, such as, proteases, cellulase, chitinase and β-glucanase (Thomashow and Weller, 1995; Weller, 2007). This powerful diversity of antimicrobial compounds is considered part of an indirect strategy to promote plant growth, as well as the ability to induce systemic resistance in plants (Raaijmakers et al., 2009; Glick, 2014). In addition, *Pseudomonas* spp. exhibit not only indirect mechanisms to improve plant health but may directly promote growth by producing and excreting phytohormones (e.g., indole-3-acetic acid, IAA) (Keel et al., 1992). Emission of volatile organic compounds (VOCs) has also been shown to be responsible for growth-promoting activities in plants such as *Arabidopsis thaliana* and *Medicago truncatula* (Farag et al., 2006; Orozco-Mosqueda et al., 2013a). We previously identified the volatile dimethylhexadecylamine (DMHDA), emitted by the plant growth-promoting (PGP) bacterium *Arthrobacter agilis*, as responsible for improving biomass and elevating chlorophyll and iron content in *M. truncatula* plants (Orozco-Mosqueda et al., 2013a). Importantly, DMHDA exhibited *in vitro* inhibitory activities toward fungal pathogens *B. cinerea* and the oomycete *Phytophthora cinnamomi* (Velázquez-Becerra et al., 2013). Other recent studies have shown that *Bacillus* species produce volatile S-containing compounds such as dimethyl disulfide (DMDS), which has been associated with suppression of plant fungal diseases (Huang et al., 2012), as well as growth-promoting effects (Meldau et al., 2013).

We screened and characterized *Pseudomonas fluorescens* isolates from *Medicago* spp. rhizospheres, with the aim of identifying strains with robust direct and indirect mechanisms to promote plant growth. In addition, we identified in most of the strains the potential presence of phenazines, cyanogens, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, as well as the production of biofilm, siderophores, proteases and IAA. Importantly, all strains exhibited PGP and biocontrol activities through VOC emission.

2. Materials and methods

2.1. Biological materials

Bacterial isolates with antifungal capacities were surveyed from *Medicago* spp. plant rhizospheres in an agricultural field in Morelia, Mexico (19° 46′ 6″ N, 101° 11′ 22″ W). Among 1000 bacterial colonies screened, only four strains were found that showed consistent antifungal and PGP effects (*P. fluorescens* UM16, UM240, UM256 and UM270). These strains were grown at 30 °C for 24 h on nutrient agar (NA) and/or King's B medium (KBM), and routinely maintained at 4 °C. The phytopathogenic fungus *B. cinerea* was isolated and grow on potato dextrose agar (PDA) at 30 °C for 3–5 days in total darkness and thereafter stored at 4 °C.

2.2. Molecular characterization of strains

Genomic DNA was isolated from the four *P. fluorescens* strains, and the 16S ribosomal subunit DNA (rDNA) gene was amplified using PCR as previously reported (Martínez-Absalón et al., 2014). Briefly, the universal bacterial primers fD1, 5′-CAGAGTTTGA TCCTGGCTCAG-3′, and rD1, 5′-AAGGAGGTGATCCAGCC-3′, were used. PCR conditions were as follows: an initial denaturation at 95 °C for 3 min, 30 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 53 °C, extension for 2 min at 72 °C, and a final extension step at 72 °C for 5 min. PCR amplifications were performed in GoTaq Master Mix tubes (Promega, Madison, WI, USA) using a TC-412 Techne thermal cycler (Keison Products, Chelmsford, UK). The PCR products were purified and sequenced at the LANGEBIO (Irapuato, Mexico). Nearly complete rDNA sequences were obtained, and homology blast searches were performed against databases, and deposited in GenBank (Accession Numbers: KJ801565–KJ801568).

2.3. Phylogenetic analysis

Phylogenetic relationships of four isolates were analyzed as previously reported (Martínez-Absalón et al., 2014). Multiple sequence alignments were generated with ClustalW (www.ebi.ac.uk/Tools/clustalw2), and the phylogenetic analysis of the 16S rRNA gene sequences was carried out with the MEGA 4.0 program (Tamura et al., 2007). To obtain a confidence value for the aligned sequence dataset, a bootstrap analysis of 1000 replications was performed. A phylogenetic tree was constructed by using the maximum parsimony algorithm.

2.4. In vitro evaluation of fungal antagonism

The evaluation of fungal antagonism was performed as previously reported on petri dish bioassays (Santoyo et al., 2010). Briefly, the bacterial isolates were simultaneously deposited with the pathogenic fungi on either PDA or NA agar plates, with very similar results in both media. Bacteria were streaked onto plates in a cross shape, and a mycelial plug of 4 mm was deposited in the center of each of the quadrants formed. The plates were

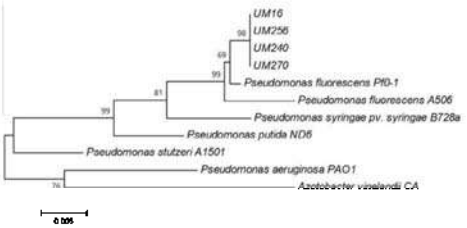


Fig. 1. Phylogenetic tree based on 16S ribosomal gene sequences of *Pseudomonas fluorescens* strains UM16, UM240, UM256, and UM270, showing the relationship with other *Pseudomonas* species (nucleotide sequences can be accessed in GenBank: KJ801565–KJ801568). The phylogenetic tree was constructed using the maximum-parsimony algorithm. Bootstrap analysis of 1000 replications was performed and is expressed as a percentage, and the Pseudomonadaceae strain *Azotobacter vinelandii* CA was used as an outgroup.

incubated in the dark at 30 °C (BOD incubator), and the mycelial growth diameter was measured at day 6. Antifungal effects of volatile compounds emitted by the pseudomonads were evaluated in divided petri plates as follows. A bacterial inoculum of each strain (1×10^6 CFU) was simultaneously deposited on one side of the petri plate and in the other section a mycelial plug of *B. cinerea* (4 mm) was inoculated. As above, the plates were incubated in the dark at 30 °C, and mycelial growth diameter was measured at day 6. Both experiments were independently performed a minimum of three times.

2.5. Evaluation of plant growth-promotion

To evaluate whether the four isolates exhibit direct PGP activities, we employed leaflets of *M. truncatula* (ecotype Jemalong A17) plants and followed a protocol previously reported by Orozco-Mosqueda et al. (2013a). Briefly, *M. truncatula* seeds were subjected to chemical scarification and incubated in plastic petri dishes with Murashige and Skoog (MS) medium at 4 °C for 48 h and then placed in a growth chamber with a photoperiod of 16 h light/8 h dark and a light intensity of 200 mol m^2 s^{-1} a 22 °C. As negative controls, sterile seeds were germinated in petri dishes with MS medium, and then transferred to a growth chamber under the same conditions and photoperiod as described for the scarified seeds. The germinated sprouts were placed in glass flasks with 200 mL of Hoagland nutrient medium plus 6 g of agar (Phytotechnology, Shawnee Mission KS, US) per liter. After 5 d of growth, an inoculum (approximately 1×10^6) of each bacterial strain was placed on media, close to the roots of the plant. Control plants were not inoculated with bacteria. The experiment was maintained until the plants reached 20 d of age (15 d of interaction), after which the plants were weighed ($n = 12$ in triplicate experiments) and the concentration of chlorophyll was determined by a spectrophotometric method (Orozco-Mosqueda et al., 2013a). Briefly, chlorophyll content was quantified using a CCM-200 chlorophyll meter

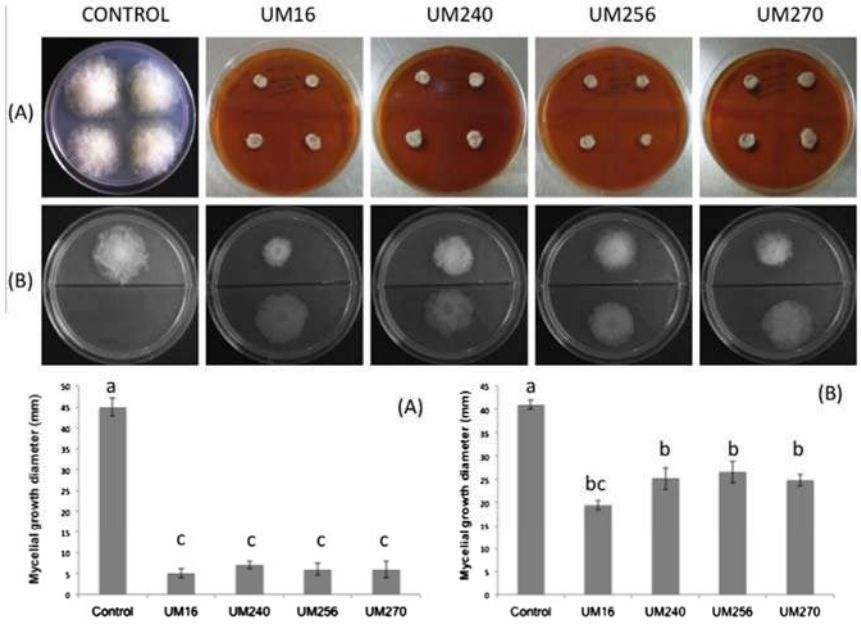


Fig. 2. Antifungal effect of co-inoculation of *Pseudomonas fluorescens* strains UM16, UM240, UM256, and UM270 with the phytopathogen *Botrytis cinerea*. Top panel (A) shows the direct effect of diffusible compounds produced by the pseudomonads on mycelial growth of *B. cinerea*. The bacterial isolates were streaked onto plates in a cross shape, and mycelial plugs of 4 mm in diameter were deposited in the center of each of the quadrants formed. Top panel (B) shows the effect of volatile compounds emitted by the pseudomonads in divided petri plates. Here, an inoculum of each strain (1×10^6 CFU) was simultaneously deposited in one side of the petri plate and in the other section was inoculated a mycelial plug of *B. cinerea* (4 mm). Both experiments were independently performed a minimum of three times. The plates were incubated and mycelial growth was measured at day 6. The percentage of growth inhibition was measured as follows: % of growth inhibition = $[(Ac - Ab)/Ac] \times 100$, where Ac is the control mycelial area and Ab is the mycelial area with treatment. The respective graphs of each experiment are shown in bottom panels (A) and (B), where bars represent the mean ± SE values ($n = 12$). Letters indicate that the means differ significantly by Duncan's multiple range test ($p < 0.05$).

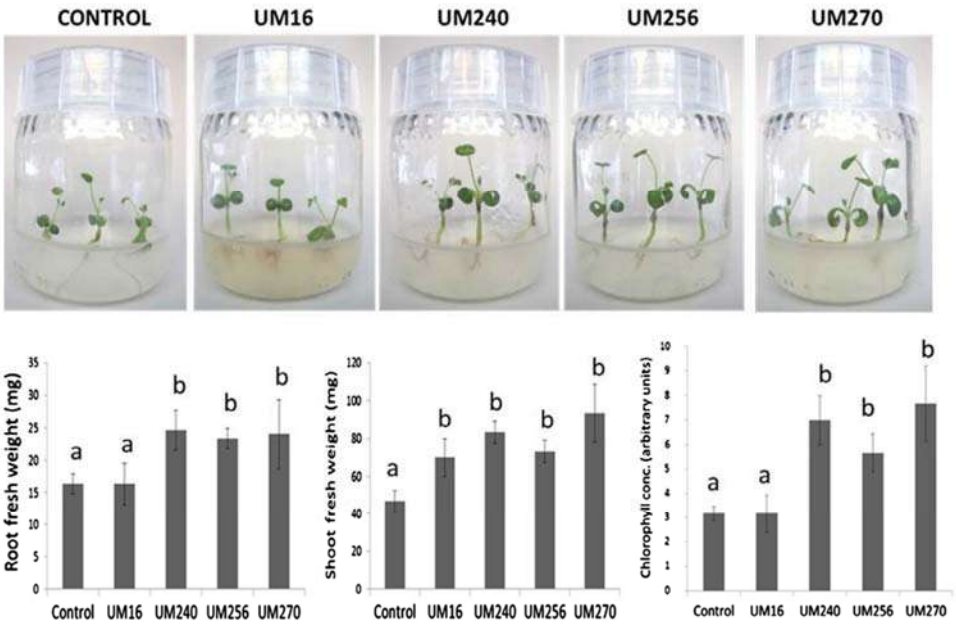


Fig. 3. Growth-promoting effects of direct co-inoculation of *Pseudomonas fluorescens* strains UM16, UM240, UM256, and UM270 with *Medicago truncatula*. Direct co-inoculation allows exchange of diffusible compounds between the bacteria and the plant. Germinated seedlings of *M. truncatula* were placed in glass flasks containing MS medium. After 5 d, pseudomonads strains (1×10^6 CFU) were directly inoculated into the medium. Upper panel shows the general view of the plants after 15 d of interaction. Lower panel shows the graphs corresponding to the measurements of shoot and root fresh weight (FW) and chlorophyll content. Bars represent the mean ± SE values ($n = 12$). Letters indicate that means differ significantly by Duncan's multiple range test ($p < 0.05$).

(Opti-Sciences, Inc., Hudson, NH, USA), which measures chlorophyll concentrations (in arbitrary units) based on the rates of transmitted radiation (940 and 660 nm) through a leaf.

2.6. Evaluation of plant growth-promotion by VOCs emission

We evaluated the plant growth-promotion by VOC emission from *Pseudomonas* strains as previously reported (Orozco-Mosqueda et al., 2013a,b) and as mentioned above, except that bacteria (approximately 1×10^6) were inoculated in 4 mL vials with NA media. Each vial was placed within the flasks containing three *M. truncatula* plants, avoiding any direct bacteria-plant interactions, except for the VOC emission. Control experiments did not contain bacterial inoculum. The experiment was maintained until the plants reached 20 d of age, after which the plants were weighed ($n = 12$ in triplicate experiments) and the concentration of chlorophyll was determined by the spectrophotometric method described previously (Orozco-Mosqueda et al., 2013a).

2.7. Protection assay using the M. truncatula–B. cinerea pathosystem

We used the previously reported *M. truncatula–B. cinerea* pathosystem (Martínez-Absalón et al., 2014). Briefly, the isolates studied were assessed for their efficiency in suppressing *B. cinerea* infection of *M. truncatula* plants. *M. truncatula* (ecotype Jemalong A17) seeds were subjected to chemical scarification and germinated in petri dishes with MS medium and then transferred to a growth chamber with the following photoperiod: 16 h light/8 h dark with a light intensity of 200 mol m^2 s^{-1} at 22 °C. It is important to mention that antagonism in Petri plates was also evaluated at 22 °C with identical results. Recently scarified seeds were placed in petri dishes with MS medium at 4 °C for 48 h and then placed in a growth chamber with the same photoperiod. The germinated sprouts were placed in glass flasks with 100 mL of Hoagland nutrient medium and 6 g of agar (Phytotechnology) per liter. After 11 d of growth, a bacterial inoculum of 1×10^6 CFU suspended in 100 µl or sterile water (100 µl for control plants), was placed 1 cm from the roots of the plant. Another set of plants was inoculated with the same bacterial inoculum plus 1×10^6 spores suspended in 100 µl of *B. cinerea* or spores without bacteria (control plants). The experiment continued until the plants reached 21 d of age. At this time, plants were measured, weighed, and evaluated for *B. cinerea* infection, including stem and leaf disease symptoms, such as yellow chlorosis, presence of gray mould, and root browning and necrosis (RBN; $n = 12$ in triplicate experiments). The percentages were measured according to the formulas: SLDS = (diameter of wounded area/total area) × 100 and RBN = (length of browning and necrotic zone/total root length) × 100.

2.8. Detection of antibiotic genes by PCR amplification

Genomic DNA from four strains was used as a template for PCR amplification of the following genes by using the gene-specific primers, PCA2a and PCA3b for phenazine (*phzCD*) (Zhang et al., 2006) and Phl2a and Phl2b for DAPG (*phlD*) (Raaijmakers et al., 1997). For the HCN-synthase gene (*hcnCB*), we used the primers Fhcn, 5′-CTCAAGCTGCACGGCATT-3′ and Rhcn 5′-ATCCGGCAAC GTCGGTTTT-3′. For detection of the ACC deaminase gene, we used the specific primers AzbAc F and AzbAc R previously reported by Farajzadeh et al. (2010) and for *hcnAB* genes the primers HCNP1F

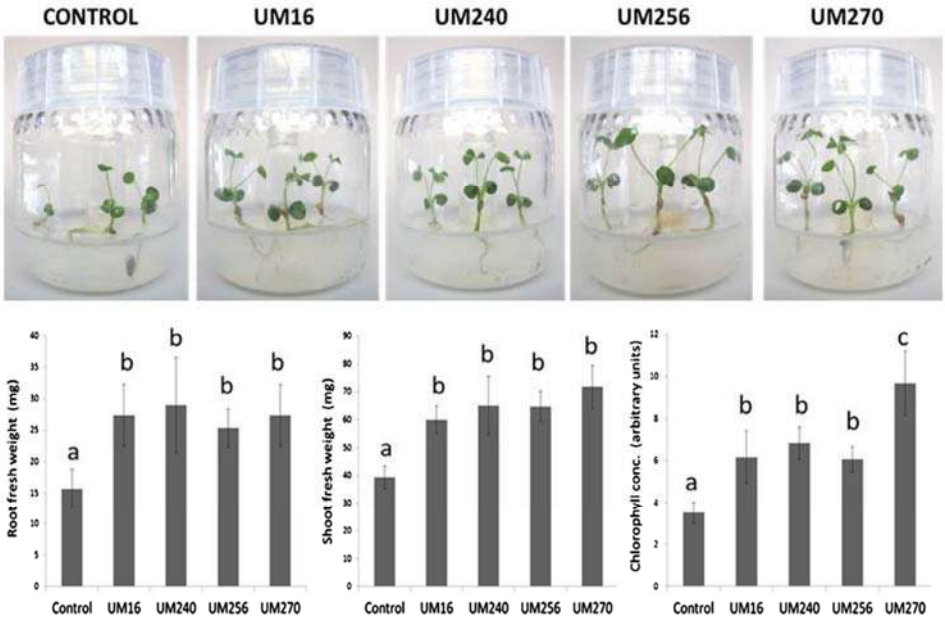


Fig. 4. Growth-promoting effects of volatile organic compounds (VOCs) produced by *Pseudomonas fluorescens* strains UM16, UM240, UM256, and UM270 on *Medicago truncatula*. Germinated seedlings of *M. truncatula* were placed in glass flasks containing MS medium. After 5 d, pseudomonads strains (1×10^6 of CFU) were inoculated in vials containing NA medium. Upper panel shows the general view of the plants after 15 d of interaction. Lower panel shows the graphs corresponding to the measurements of shoot and root fresh weight (FW) and chlorophyll content. Bars represent the mean ± SE values ($n = 12$). Letters indicate that means differ significantly by Duncan's multiple range test ($p < 0.05$).

and HCN2L (5′ CTCAAGCTGCACGGCATT 3′ and 5′ ATCCGGCAACG TCGGTTTT 3′). PCR amplified products were purified and sequenced as above at LANGEBIO (Irapuato, Mexico) to confirm similarity to their respective biosynthesis gene sequences.

2.9. Identification of biocontrol and plant-growth promotion determinants

Strains UM16, UM240, UM256, and UM270 were analyzed for diverse biocontrol and/or plant-growth promotion traits. Siderophore production was determined by the chrome azurol S (CAS) assay (Schwyn and Neilands, 1987), while Skim Milk agar (SM) plates were used to detect protease production (Kumar et al., 2005). Other lytic activities, such as cellulase, chitinase, and glucanase were analyzed, using *Bacillus thuringiensis* UM96 as a positive control (Martínez-Absalón et al., 2014); however, none of these activities was found in any of the *P. fluorescens* strains. Biofilm formation capacity in bacteria was analyzed following the protocol by Wei and Zhang (2006). IAA production was analyzed in filtered-culture supernatants from the four strains by GC–MS (Gas Chromatograph 6850 Series II – Mass Spectrometry detector 5973; Agilent, Foster City, CA, USA) analysis, as previously reported with some modifications for specific estimation in bacteria (Contreras-Cornejo et al., 2009). NA cultures were supplemented with tryptophan and incubated with agitation (250 rpm) at 30 °C for 48 h. The identity of IAA was confirmed by comparison of the retention time in the bacterial extracts with samples of the pure IAA standard (Sigma–Aldrich, St. Louis, MO, USA). IAA amounts produced by strains were estimated using calibration curves for each strain. All experiments described in this section were performed in triplicate.

VOCs produced by strains UM16, UM240, UM256, and UM270 were analyzed by solid phase microextraction–gas chromatography–mass spectrometry (SPME–GC–MS) on PDMS/DVB fibers (Supelco, Inc., Bellafonte, PA, USA) as previously reported (Gutiérrez-Luna et al., 2010), except that the GC–MS was equipped with a DB-23 capillary column (30 m × 0.32 mm × 0.25 μm). Operating conditions consisted of helium as the carrier gas (1 mL/min) and a detector temperature of 250 °C. The column was held for 1 min at 40 °C, and then programmed to increase at a rate of 3 °C per minute to a final temperature of 180 °C, which was maintained for 1 min. Source pressure was 7 Pa, filament voltage was 70 eV and the scan rate was 1.9 scan s^{-1}. The compounds were identified by comparison with data from the Mass Spectra Library (NIST/EPA/NIH, "Chem Station" Agilent Technologies Rev. D.04.00 2002). Three independent determinations were made for each *Pseudomonas* strain.

3. Results

3.1. Selection and identification of bacterial antagonists

Approximately 1000 bacterial colonies were isolated from the rhizospheres of wild *Medicago* spp. plants. During screening analysis for antagonistic activity against diverse fungal pathogens, 49 showed consistent and notable antagonistic activity toward the pathogenic fungus *B. cinerea* (data not shown). As the goal of this work was to analyze not only those strains with antifungal activity

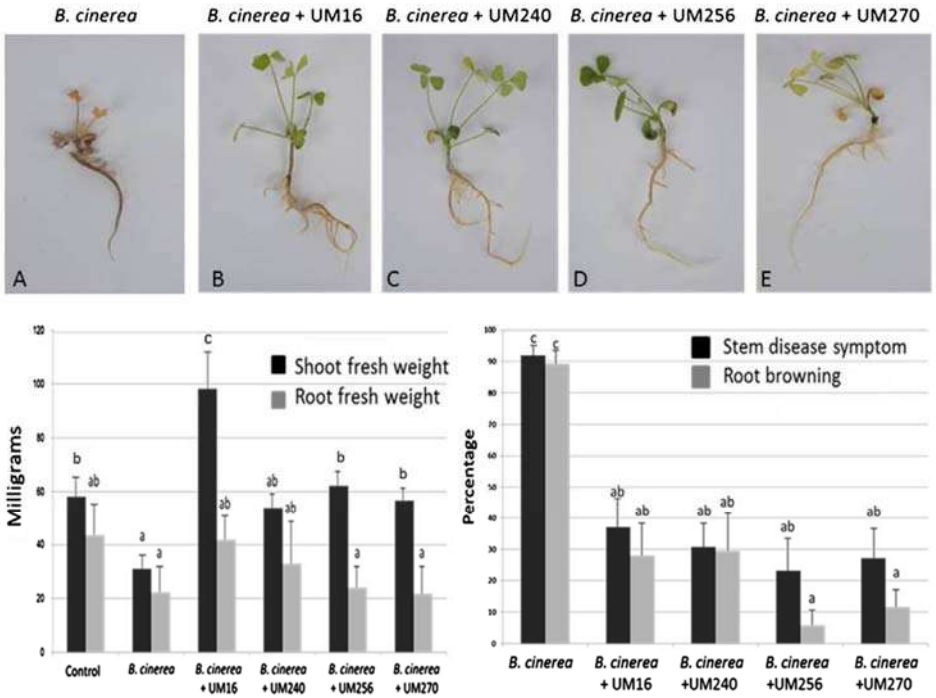


Fig. 5. Biocontrol capacity of the *Pseudomonas fluorescens* strains UM16, UM240, UM256, and UM270 against *Botrytis cinerea* in *Medicago truncatula* seedlings. Representative plants ($n = 12$) are shown in different panels (A–E). (A) Control plant inoculated with *B. cinerea* fungus. (B) Plants coinoculated with *B. cinerea* and UM16 (B), UM240 (C), UM256 (D) and UM270 (E). Left graphic shows the shoot (black bars) and root fresh weights (gray bars). Right graph shows stem disease symptoms (black bars) and root browning (gray bars). Letters indicate that means differ significantly by Duncan's multiple range test ($p < 0.05$).

but also those with direct PGP activity, we further analyzed those with antifungal activity and found that only four strains exhibited both activities. The selected strains were named UM16, UM240, UM256, and UM270. Molecular characterization by full-length16S ribosomal gene sequencing placed the four strains in a cluster with *P. fluorescens* species, including a 99–100% identity with the biocontrol strain *P. fluorescens* Pf0-1 in Blast analysis (Fig. 1). Since four strains showed high identity to *P. fluorescens* species, we performed a 16S gene sequence alignment of the four strains, and we detected only four single-nucleotide polymorphisms between their ribosomal sequences (NCBI accession numbers: KJ801565–KJ801568). The previous result and further analysis showed different behavior during assessment of the antagonism and PGP activities (see Supplemental Fig. 1); we therefore maintained the four isolates as separate strains.

3.2. Antagonism against B. cinerea

Antagonism toward the phytopathogen *B. cinerea* was tested either by action of diffusible (direct co-inoculation) or VOCs (inoculation on divided petri dish plates) produced by the *Pseudomonas* strains (Fig. 2). These four *P. fluorescens* strains showed a more robust inhibitory effect on the *B. cinerea* mycelial growth diameter during co-inoculation bioassays compared to the effect of VOC emission, although both mechanisms were significantly inhibitory ($p < 0.05$). In terms of percentage, diffusible compounds reduced the mycelial growth diameter by up to 86%, while VOC emission inhibited the fungal growth by up to 53%. Notably, *Pseudomonas* strains also showed different grades of antagonism against other important fungal phytopathogens, such as *Rhizoctonia solani*, *Diaporthe phaseolorum*, and *Colletotrichum lindemuthianum* (see Supplemental Fig. 1).

3.3. Plant growth promotion by Pseudomonas strains

The *P. fluorecens* strains UM16, UM240, UM256, and UM270 were isolated from a rhizospheric zone and selected for their antagonistic activities against diverse phytopathogens, with a particular emphasis on antagonism toward *B. cinerea*. However, a desirable trait in any biocontrol agent is the capacity for directly inducing the growth of plants. Hence, we tested this activity in bacterial strains in *M. truncatula* leaflet-inoculation experiments *in vitro*, and observed the effects of diffusible compounds and VOC emission. Fig. 3 shows the effect of direct inoculation of each of *Pseudomonas* strains on the *M. truncatula* plants, where strains UM240, UM256 and UM270 induced significant increases in shoot and root weights, as well as elevated chlorophyll concentrations. Strain UM16 only promoted an increase in shoot weight, while no differences were observed in root fresh weight or chlorophyll content. With regard to the effect of the VOCs, all four strains increased the *M. truncatula* plant biomass and induced higher chlorophyll concentrations (Fig. 4). These results suggest that

Pseudomonas strains UM16, UM240, UM256, and UM270 produce diffusible and VOCs as a mechanism to promote plant growth.

3.4. Biocontrol efficacy of Pseudomonas strains

We have developed a *M. truncatula–B. cinerea* pathosystem (Martínez-Absalón et al., 2014), which was useful for ascertaining the biocontrol efficacy of *Pseudomonas* strains. Control plants were inoculated with 1×10^6 spores/mL of *B. cinerea*, where diverse disease symptoms were evident after 21 d post-pathogen inoculation. Disease symptoms included growth of mycelia on the plant (gray mould), yellow chlorosis in leaves, root browning, and necrosis in different plant tissues (Fig. 5). Visually, the shoots and roots in plants inoculated only with the fungal spores were affected, though no significant difference was observed in root fresh weight compared with control (Fig. 5). When spores were co-inoculated with each *Pseudomonas* strain (1×10^6 CFU/mL), we observed that disease symptoms were significantly reduced and plants appeared healthier (Fig. 5). No significant differences were observed between control plants and those co-inoculated with fungal spores and bacteria, except for strain UM16, which was able to increase its shoot weight, even in the presence of the phytopathogen. Importantly, the general stem disease symptoms, root browning and necrosis, were significantly reduced in plants co-inoculated with all strains by up to 60% (Fig. 5). These results demonstrate the plant-protective effects of strains UM16, UM240, UM256, and UM270 against infection by the gray mould phytopathogen *B. cinerea*.

3.5. Identification of biocontrol and PGP determinants

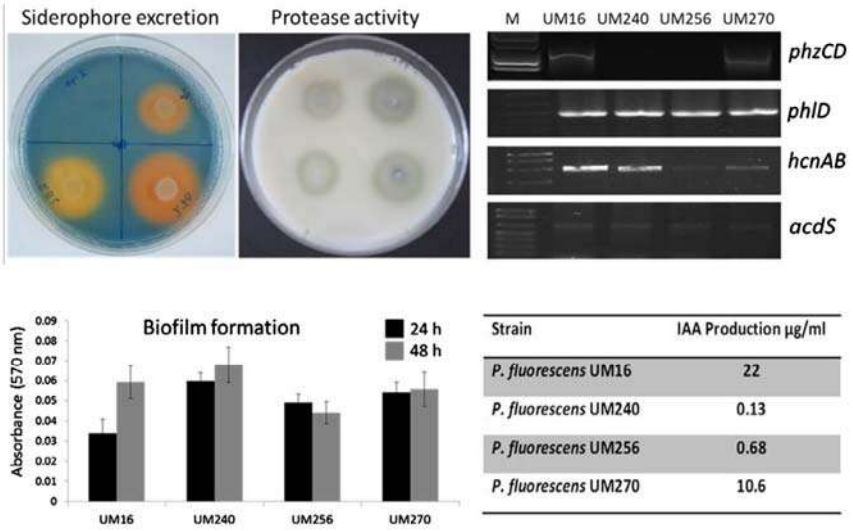
Fluorescent pseudomonads produce an arsenal of antimicrobial compounds. Here, we detected potential biocontrol determinants, such as cyanogens, DAPG synthesis, ACC deaminase, biofilm formation and proteases in four *P. fluorescens* strains (Fig. 6). The potential presence of phenazines was detected in strains UM16 and UM270. The siderophore production was observed by an orange halo formation in three strains, except for the UM240 strain. The production of IAA was also different in the four strains as determined by GC–MS analysis, where UM240 and UM256 showed the lowest levels (0.13 and 0.68 µg/mL, respectively), while strains UM16 and UM270 showed robust production of the phytohormone auxin (22 and 10.6 µg/mL, respectively). These results demonstrate the metabolic plasticity of the *P. fluorescens* strains UM16, UM240, UM256, and UM270, as well as the putative synergistic action of diverse compounds against *B. cinerea*.

3.6. Analysis of VOCs

VOCs emitted by all four *Pseudomonas* strains were analyzed by SPME–GC–MS. The volatile blends of all four pseudomonads strains were very similar and 19 volatile compounds were identified (Table 1). Those volatiles that were detected in NA media without bacterial strain inoculation were eliminated. VOCs produced by the bacterial strains included aldehydes, ketones, alcohols and sulfur-containing compounds. Interestingly, the most abundant and commonly emitted by all four strains were the sulfur-containing volatiles, including methanethiol, dimethyl sulfide, DMDS and dimethyl trisulfide. Another interesting volatile, DMHDA, was produced solely by the strain UM270. This result suggests that *Pseudomonas* strains UM16, UM240, UM256, and UM270 produce a similar cocktail of VOCs, among which the most abundant are sulfur-containing compounds as putative candidates for the biocontrol and PGP activities.

4. Discussion

The rhizospheric microbiome is composed of diverse beneficial microbial species; however, genera, such as, *Pseudomonas*, are predominately isolated and cultivated in the lab due to their



Strain	IAA Production µg/ml
P. fluorescens UM16	22
P. fluorescens UM240	0.13
P. fluorescens UM256	0.68
P. fluorescens UM270	10.6

Fig. 6. Potential biocontrol and plant growth-promoting trait of *Pseudomonas fluorescens* strains UM16, UM240, UM256, and UM270. Siderophore production was determined by the chrome azurol S (CAS) assay and Skim Milk agar (SM) plates were used to detect protease activity. Production of indole-3-acetic acid (IAA) was determined from filtered-culture supernatants from respective strains by GC/MS analysis. Detection of genes responsible for the production of phenazines (*phzCD*), DAPG (*phlD*), hydrogen cyanide (*hcnAB*), and ACC deaminase (*acdS*) by PCR. See Section 2 for details. All experiments were performed in triplicate with three independent trials.

Table 1
Analysis of volatile organic compounds produced by *Pseudomonas fluorescens* strains and detected by GC/MS analysis.

		UM16	UM240	UM256	UM270
Volatile compound	Rt (min)	%	%	%	%
Methanethiol	0.28	12.34	10.57	12.99	15.13
Dimethyl sulfide	0.36	16.86	17.12	13.26	23.4
2-Butanone	0.96	0.23	n.d.	0.24	n.d.
1-Nonene	1.05	1.09	1.03	1.75	2.02
Methyl thiolacetate	1.46	0.15	1.36	1.35	1.17
Dimethyl disulfide	1.72	30.26	22.89	13.84	5.62
1-Decene	1.95	1.53	1.82	1.13	0.53
1-Undecanol	3.56	35.07	38.77	52.50	50.01
2,4-Dithiapentane	5.32	1.00	0.96	0.52	n.d.
1-Dodecene	6.09	n.d.	0.44	0.48	n.d.
Dimethyl trisulfide	7.28	0.42	1.07	0.70	0.57
S,S-Dimethyl dithiocarbonate	9.15	0.13	0.52	n.d.	n.d.
2-Nonanone	11.65	0.26	0.54	0.18	n.d.
Decyl oxirane	16.83	0.30	1.03	0.46	n.d.
Methyl methylthiomethyl disulfide	17.07	0.12	0.41	n.d.	n.d.
2-Amino-5-methyl benzoic acid	17.69	n.d.	n.d.	n.d.	n.d.
Thiazole	18.66	n.d.	0.58	0.31	0.41
Butylated hydroxytoluene	26.22	0.25	0.86	0.30	0.49
Dimethylhexadecilamine	31.4	n.d.	n.d.	n.d.	0.64

Green color indicates sulfur-containing volatiles.

metabolic plasticity when grown in different media and conditions (Mendes et al., 2011). *P. fluorescens* has been attracting particular attention for several decades due to its capacity to protect plants from phytopathogenic attack (Santoyo et al., 2012). Current efforts to isolate, characterize and select the best bacterial antagonists to control phytopathogens worldwide are continuously reported in the literature (Yanes et al., 2012; Guiñazú et al., 2013; Mavrodi et al., 2012; Rashid et al., 2012), though very few studies have demonstrated the presence of direct or indirect mechanisms in the reported antagonists. Therefore, in this study, four novel rhizospheric strains belonging to the *P. fluorescens* species were isolated and characterized for their significant antagonism toward the gray mould phytopathogen *B. cinerea*. Further analysis showed that, in addition to the biocontrol activities, the four new *Pseudomonas* strains are able to promote *M. truncatula* growth, and protect them from *B. cinerea* attack. The modes of action of these pseudomonads can be explained by direct and indirect mechanisms. Some other studies have also found in diverse bacterial genera a synergistic action of multiple compounds against plant pathogens. For instance, *Serratia plymuthica* showed multiple mechanisms in the biocontrol of diseases by *B. cinerea* and *Sclerotinia sclerotiorum* (Kamensky et al., 2003). Similarly, the *Pseudomonas brassicacearum* strain J12 produced diverse antifungal metabolites against *Ralstonia solanacearum* (Zhou et al., 2012), and *Pseudomonas chlororaphis* subsp. *aurantiaca* strain Pa40, which exhibited multiple mechanisms toward the suppression of sharp eyespot of wheat, caused by *Rhizoctonia cerealis* (Jiao et al., 2013). Therefore, it is not surprising to find that our strains contain multiple mechanisms against phytopathogens such as *B. cinerea*. However, functional genomics, random and direct gene knockouts and analytical chemistry approaches will be necessary to correlate specific metabolites or enzymatic activities with biocontrol activities, while random transposon mutagenesis will allow us to detect novel antagonistic determinants in our *P. fluorescens* strains.

Pseudomonas spp. possess a large arsenal of antifungal metabolites, and assigning a specific role for each metabolite is usually difficult (Haas and Défago, 2005). The *P. fluorescens* UM16, UM240, UM256, and UM270 strains exhibited several antifungal traits (potential presence of phenazines, DAPG, HCN, ACC deaminase, production of biofilm, IAA, siderophores, and proteases), which could be acting during the antagonism toward *B. cinerea*. Synthesis of siderophores has been associated with the iron sequestration to restrict pathogen growth (Santoyo et al., 2010; Weller, 2007). The biocontrol strain *P. fluorescens* CHAO produces extracellular proteases and the volatile HCN involved in the protection of tobacco against diverse plant pathogens (Voisard et al., 1989). The *Pseudomonas* strain 2-79 produces phenazine with concomitant biocontrol of *Gaeumannomyces graminis* (Thomashow and Weller, 1988). The production of biofilm and IAA has not been directly associated with biocontrol activities, though it has been observed that bacteria containing these two activities have increased capacities for colonizing, adapting, persisting, and surviving in rhizospheric environments (Beyeler et al., 1999; Bianco et al., 2006; Duca et al., 2014).

Interestingly, the four strains analyzed here produced a red pigment that covered the petri dishes during direct confrontation bioassays with *B. cinerea*. Similar red pigments have been associated with the synthesis of the antibiotic DAPG, which is involved in fungal antagonism (Keel et al., 1996). As this red pigment production has been associated with DAPG production, we analyzed the expression pattern of the *phlD* gene in the presence or absence of *B. cinerea*. Four operon genes, *phlACBD*, are required for the synthesis of DAPG (Bangera and Thomashow, 1999), where *phlD* encodes for a type III polyketide synthase, indispensable for the biosynthesis of monoacetylphloroglucinol (MAPG). PhlA, PhlC, and PhlB are necessary for the transacetylation of MAPG to produce DAPG (Wu et al., 2012). Interestingly, *B. cinerea* induced higher expression levels of the *phlD* gene, compared to those in which the bacteria were grown without the fungus (Supplemental Fig. 2). Paulin et al. (2009) obtained similar results, in which the *phlD* gene from *Pseudomonas* sp. LBUM300 was upregulated when confronted with *Verticillium dahliae* on PDA.

In addition, the *Pseudomonas* strains described here also produced VOCs with antifungal activity. We analyzed by SPME–GC–MS the abundant production of sulfur-containing volatiles, including DMDS, which has been previously associated with antifungal and plant protective activities (Huang et al., 2012). One proposed mode of action of DMDS is that it acts as an eliciting factor during the induced systemic resistance in plants (Huang et al., 2012). The precise mechanism by which sulfur-containing volatiles inhibit *B. cinerea* mycelia is currently unknown and awaits further investigation. Interestingly, strain UM270 was the only one that produced DMHDA, which has been associated with antifungal and PGP activities in previous studies in our lab (Orozco-Mosqueda et al., 2013a; Velázquez-Becerra et al., 2013). However, no significant difference was found when compared with the antifungal or PGP activities of the other three strains. Therefore, such production and excretion of diffusible and VOC emission may be acting as antifungal determinants during antagonism and protection of *M. truncatula* plants.

P. fluorescens strains UM16, UM240, UM256, and UM270 were also selected by their direct activities to promote plant growth through the action of diffusible (direct coinoculation) or volatile compounds (separate compartments). PGP rhizobacteria may promote plant growth through a wide variety of mechanisms (Santoyo

et al., 2012). Here, the *acdS* gene was detected, and therefore these *P. fluorecens* strains have the potential to express the ACC deaminase protein. ACC deaminase cleaves ACC to α-ketobutyrate and ammonia, and as a consequence, the ethylene levels in plants are decreased (Glick, 2014).The synthesis of IAA by bacteria is another obvious trait for growth promotion, because this plant hormone controls virtually every aspect of plant growth and development. In our results, we did not detect a specific correlation between IAA production by our strains and the promotion of plant growth. Strains UM16 and UM270 produced the highest levels of IAA, while UM240 and UM256 were low IAA producers. It has been shown that the range in which IAA induces beneficial effects is very narrow and that high concentrations could cause plant-deleterious effects (Kawaguchi and Syono, 1996; Suzuki et al., 2003). Interestingly, only strain UM16, which produced the highest level of IAA, was able to induce shoot growth in *M. truncatula* plants, even in the presence of the phytopathogen. As a prelude to what volatiles are responsible for the improvement of plant health, we analyzed the VOCs emitted by all four strains. As previously mentioned, the most abundant VOCs produced by the strains were the sulfur-containing compounds. DMDS has previously been identified as a VOC produced by diverse rhizobacteria (Farag et al., 2006; Huang et al., 2012; Meldau et al., 2013). In a recent study by Meldau et al. (2013), the authors describe a mechanism by which DMDS induces growth of *Nicotiana attenuata* plants. They demonstrated that DMDS released by *Bacillus* B55 contributes to *N. attenuata* sulfur nutrition and revealed interesting connections between ethylene and sulfur metabolism. Taken together with our data, these results suggest that such a mechanism might not be species-specific, since our strains belong to a distinct bacterial division, but might be specific to plant-associated rhizobacteria. Another study by Huang et al. (2012) reported that the application of DMDS, also produced by a *B. cereus* strain, is able to suppress plant fungal diseases under greenhouse conditions. Here, another interesting compound, DMHDA, was produced by the UM270 strain. DMHDA was recently identified in an investigation into the VOCs produced by the PGP *A. agilis*, and was assigned a role in biomass improvement and elevated chlorophyll and iron content in *M. truncatula* plants (Orozco-Mosqueda et al., 2013a). In addition, the application of pure DMHDA exhibited strong inhibitory activities toward fungal pathogens (Velázquez-Becerra et al., 2013). It would be interesting to test the application of both compounds, DMDS and DMHDA, in greenhouse experiments to observe possible synergistic effects on plant protection and growth promotion.

In conclusion, this work describes the characterization of four new *P. fluorescens* strains, which exhibit greater and more diverse capabilities of antagonizing phytopathogens compared to other known PGP bacteria. These four strains also exhibited robust protection and plant growth promotion, opening a new door for their promising use as biocontrol and/or biopromoting agents in crops.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2014.11.011.

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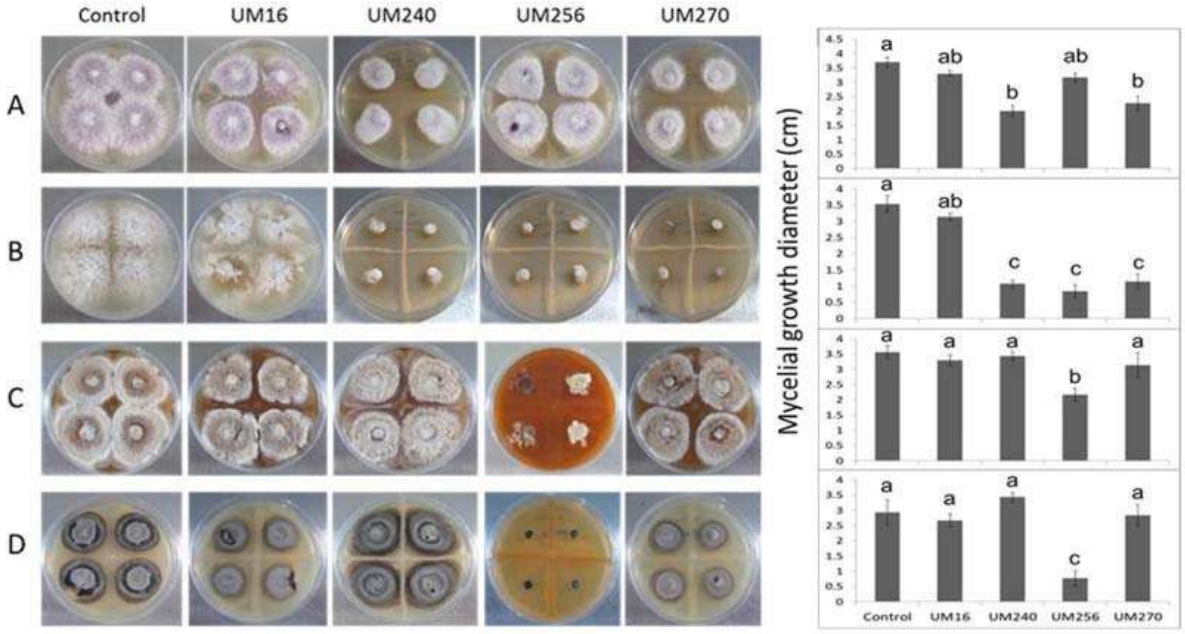
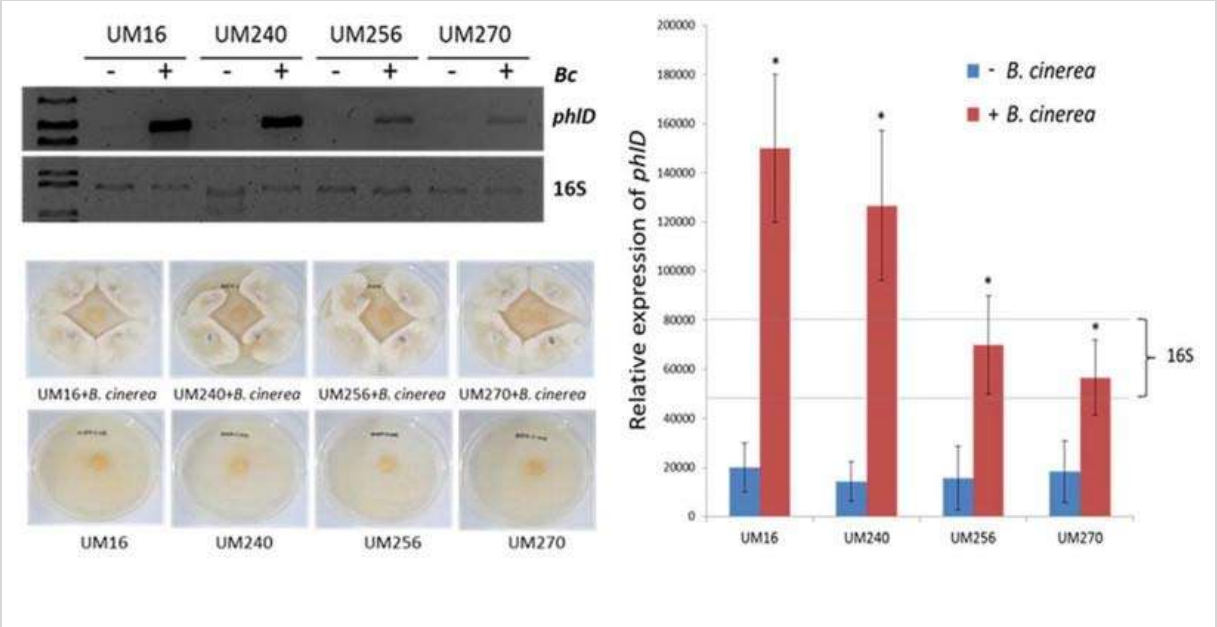
Supplementary data



Supplementary Fig. 1.

Antifungal effect of co-inoculation of *Pseudomonas fluorescens* strains UM16, UM240, UM256 and UM270 with the phytopathogens (A) *Fusarium oxysporum*, (B) *Rhizoctonia solani*, (C) *Diaporthe phaseolorum* and (D) *Colletotrichum lindemuthianum*. The bacterial isolates were streaked onto plates in a cross shape, and a mycelial plug of 4 mm in diameter was deposited in the center of each of the quadrants. Experiments were independently performed a minimum of three times. The plates were incubated and mycelial growth was measured at day 6 or 7. The percentage of growth inhibition was measured as follows: % of growth inhibition = [(Ac − Ab)/Ac] × 100, where Ac is the control mycelial area and Ab is the mycelial area with treatment. The respective graphs of each experiment are shown to the right, where bars represent the mean ± SE values (n = 12). Identical letters above the bars indicate that the means differ significantly by Duncan's multiple range test ($p < 0.05$).



Supplementary Fig. 2.

Semiquantitative PCR analysis of *phlD* gene expression. Four mycelial plugs (4 mm) of the phytopathogen *Botrytis cinerea* were inoculated on PDA agar plates and incubated in the dark at 30 °C for 48 h. After this time, 50 µL from a bacterial overnight culture (O.D. = 1.0) were inoculated at the center of the petri dish during 24 h of co-inoculation. Control experiments consisted of the inoculation of the same amount of bacterial cells (50 µL) on separate petri dishes without fungal mycelium. After this time, RNA was isolated from the bacterial cell pellets using an SV Total RNA Isolation System (Promega). The RNA was treated with DNase I to remove residual genomic DNA, and control experiments were performed to confirm this. The RNA samples were run in a 1.2% agarose gel and stained with ethidium bromide to confirm the quality of the RNA. Finally, cDNA was synthesized with the GoScript Reverse Transcription Kit (Promega). cDNA was quantified, and 0.1 µg of template was added to each amplification tube plus 50 pM of each primer, Master mix (Promega), and sterile DNAse-free water. For semiquantitative RT-PCR analysis, we used following primers: Phl2a and Phl2b for *phlD* amplification (Raaijmakers et al., 1997), and for the 16S ribosomal gene we used: F16S (5′-CCTGGGATTTCACCCCTGAC-3′) and R16S (5′-CGGTATCCGGAGAAGAAGCC-3′). The expression level of the 16S ribosomal gene was used as the internal control. Relative levels of mRNA in each sample were calculated by using the Quantity One Software imaging analyzer (Bio-Rad) and plotted as bar graphs on the right. Asterisks indicate significant differences ($p \leqslant 0.05$) between the treatments by using the Student's *t*-test

8. Discusión

El microbioma rizosférico esta compuesto por una gran diversidad de especies microbianas, las cuales interactúan estrechamente con las raíces de la planta. Sin embargo, para que las bacterias puedan ser competitivas y lograr acceder a los beneficios que les brinda la rizósfera, deben poseer una serie de características o mecanismos que les permitan colonizar espacios y ser altamente competitivas. Además de esto, deben tener una amplia platicidad metabólica que les permita enfrentar los diversos factores bióticos y abióticos (Santoyo et al., 2012).

Desde hace algunas décadas, las bacterias que brindan algún beneficio a la planta con la que interactúan se les ha denominado bacterias promotoras del crecimiento vegetal o PGPR, por sus siglas en inglés. En muchos casos, las PGPR poseen ciertos mecanismos de promoción de crecimiento vegetal o de biocontrol (el cual se considera un mecanismo indirecto de promoción), pocas son los reportes de cepas que cuentan con las dos características a la vez (Cabanás et al., 2014; Agarás et al., 2015; Cawoy et al., 2015). El género *Pseudomonas*, es uno de los más estudiados debido a su capacidad para metabolizar diversos compuestos cuando crece en diferentes medios (Mendes et al., 2011). A pesar de contener diferentes especies de interés (Zhou et al., 2014; Jiao et al., 2013), *P. fluorescens* ha sido la más estudiada en los últimos años por sus amplias capacidades antifúngicas. En este trabajo, se analizaron cuatro nuevas cepas de *P. fluorescens* que poseen la capacidad promover el crecimiento vegetal de *M. truncatula*, estas fueron denominadas UM16, UM240, UM256 y UM270, algunos de los mecanismos que fueron analizados y por los cuales se proponer ocurra esta promoción incluyen, la producción de ácido indol acético, ACC demianasa, biofilm y sideróforos. Estudios recientes muestran que las cuatro cepas de *Pseudmonas* muetsran una alta capacidad para colonizar las raíces de planats de maíz, así como ser altamente competitivas.

Estudios similares han mostrado que algunas PGPR, tales como *Serratia plymuthica*, mostraron múltiples mecanismos en el biocontrol de enfermedades causadas por fitopatógenos como *Botrytis cinerea* y *Sclerotinia sclerotiorum* diseases (Kamensky *et al*., 2003). De igual manera la cepa *Pseudomonas brassicacearum* J12 exhibió la producción de diversos metabolitos con actividad antifúngica contra *Ralstonia solanacearum* (Zhou et al., 2012). Algunas otras cepas como *Pseudomonas chlororaphis* subsp. *aurantiaca* strain Pa40 también han mostrado no sólo un mecanismo en particular para inhibir el crecimiento de fitopatógenos como *Rhizoctonia cerealis*, causante de diversas enfermedades vegetales, sino que son diversos los mecanismos que permiten a la bacteria suprimir al fitopatógeno (Jiao et al., 2013). Por lo tanto, no fue realmente sorprendente encontrar ambos mecanismos en nuestras cepas. Pero el objetivo de nuestro trabajo no es solamente describir funciones ya conocidas en otras cepas, sino describir nuevas funciones en genes desconocidos. Asi que, si se desea descubrir nuevos mecanismos de bicontrol o promoción del crecimiento vegetal en las cepas de *Pseudomonas* que aislamos, proponemos que el uso de transposones para generar múltiples mutantes en el geneoma, las cuales bajo una búsqueda de pérdida de funciones benéficas, se podrían encontrar nuevos elementos genéticos que permitan asignar funciones benéficas en la interacción con plantas.

En el caso particular de la cepa UM16, cuando es inoculada en plantas de *M. truncatula* en condiciones “in vitro”, se puede observar un acortamiento de la longitud de la raíz, sin embargo, se sabe que los efectos de las auxinas en el crecimiento de la raíz son dependiente de la concentración (Muday et al, 2012). Las auxinas promueven el crecimiento de la raíz a un nivel óptimo. Las concentraciones más altas causan un retraso en el desarrollo de la raíz (Facella et al, 2012). Esta explicación es congruente con el hecho de que la cepa UM16 es, de las cuatro estudiadas, la que produce la mayor concentración de AIA (22 μg/ml). Los efectos inhibidores de crecimiento de altas concentraciones de auxinas en raíces, son consecuencia del aumento de la síntesis de etileno estimulado por los niveles de auxina través de una mayor biosíntesis de ACC (ácido 1-

aminociclopropano-1-carboxílico), precursor inmediato de etileno. (Glick., 1995; Shah et al., 1997; Shah et al, 1998; Glick 2005). En este trabajo, también se logró amplificar el gen *acdS*, que codifica para una 1-aminocyclopropano-1-carboxilato desaminasa, en cada una de las cepas estudiadas. Por lo tanto, se requiere de mayores estudios de expresión del gen *acdS* y actividad ACC desaminasa en nuestras cepas de *Pseudomonas* para determinar si efectivamente dicha enzima participa como uno de los principales mecanismos de promoción del crecimiento en plantas de *M. truncatula*.

La producción de sideróforos es otro mecanismo reportado por medio del cual las PGPRs pueden inhibir el crecimiento de fitopatógenos (Kloepper, 1980) y promover el crecimiento de las plantas. Las cuatro cepas estudiadas son capaces de producir estos compuestos. Aunque no logramos determinar el tipo de sideróforos producidos, sería interesante determinarlo en estudios posteriores. Además de promover el crecimiento de *M. truncatula*, las cepas mencionadas poseen la capacidad de inhibir el crecimiento de hongos fitopatógenos como*, Fusarium oxysporum, Rhizoctonia solani, Diaporthe phaseolorum* y *Colletotrichum lindemuthianum* y de proteger a *M. truncatula* de la infección de *B. cinerea* disminuyendo los síntomas de la infección causada por este. A este respecto se encontraron genes que están involucrados en la síntesis de 2,4-DAPG, fenazinas y HCN que podrían ser los causantes de la capacidad de biocontrol de estas cepas, sin embargo es difícil asignar un papel específico para cada metabolito (Haas y Défago, 2005).

El gen *phlD*, que fue identificado en las cuatro cepas, es uno de los genes indispensables para la síntesis de 2,4-DAPG, la sobre expresión de este gen en presencia de *B. cinérea* fue analizada, el mecanismo por el cual el hongo es capaz de percibir a las bacterias se desconoce, aunque existen trabajos que sugieren que algunos hongos pueden poseer un sistema receptor que detecte a los microorganismos (Paoletti y Saupe., 2009). Paulin y colaboradores en el 2009, analizaron la expresión de genes relacionados con la síntesis de 2,4-

diacetilfluoroglucinol y HCN a diferentes tiempos de interacción con los hongos *Phytophthora cactorum* y *Verticillium dahliae,* concluyendo que existe modulación en la expresión genética, sin embargo mayores estudios son necesarios para determinar cuál es el mecanismo por el cual las baterías pueden percibir al hongo y viceversa.

Los compuestos orgánicos volátiles producidos por las PGPR son uno de los mecanismos más novedosos que han sido propuestos para estar involucrados en la estimulación del desarrollo vegetal. Los VOCs desempeñan un papel clave en la modulación de crecimiento de las plantas y la resistencia sistémica inducida (ISR) a patógenos. A pesar de su importancia, las funciones fisiológicas de los compuestos orgánicos volátiles específicos producidos por *Pseudomonas fluorescens* no se han dilucidado con precisión (Park et al., 2015). A través de un análisis de cromatografía de gases acoplada a espectrometría de masas (GC-MS) se analizaron los compuestos orgánicos volátiles producidos por UM16, UM240, UM256 y UM270. Este trabajo abre una puerta hacia este tipo de estudios, en especial de compuestos orgánicos volátiles azufrados, ya que se han sugerido como un mecanismo de promoción de crecimiento de las plantas que mejora la disponibilidad de azufre, lo que es particularmente beneficioso para las plantas que crecen en suelos deficientes en este elemento (Meldau et al., 2013). El DMDS es uno de los compuestos volátiles que contiene azufre y ha sido relacionados con la inhibición de hongos fitopatógenos (Huang et al., 2012) se ha propuesto su capacidad para inducir resistencia sistémica en plantas (Huang et al., 2012). Uno de los compuestos que han sido ampliamente estudiados en nuestro grupo de trabajo es la dimetilhexadecilamina, para la cual se han reportado las capacidades de promoción de crecimiento vegetal y biocontrol (Becerra et al., 2013; Orozco-Mosqueda et al., 2013). Únicamente la cepa UM270 es capaz de producir este compuesto. El análisis genético también es una herramienta que nos brinda gran información de las bacterias estudiadas, como parte de un nuevo proyecto, el genoma de la cepa UM270 ha sido secuenciado (Hernández-Salmerón et al.,

2016) para analizar su potencial genético y así conocer de una manera más amplia las capacidades que esta posee.

Ciertamente es importante determinar y desarrollar el gran potencial que existe para la selección de microorganismos biocontroladores y la fabricación de bioproductos a partir de recursos microbiológicos locales. Además el aporte científico de cuatro cepas que poseen múltiples características que pueden estar confiriendo sus capacidades de biocontrol y que pueden ser estudiadas como organismos modelo, es parte importante de este estudio.

9. Perspectivas.

- Dilucidar el principal mecanismo que confiere a las cepas UM16, UM240, UM256 y UM270 las características de promoción de crecimiento vegetal mediante la construcción de mutantes.
- Modificar las vías metabólicas que estén involucradas en biocontrol y promoción del crecimiento vegetal.
- Determinar la actividad y el papel de la ACC deaminasa en las cepas de *P. fluorescens*.
- Realizar experimentos de biopromoción y protección vegetal contra hongos fitopatógenos en invernadero y campo.
- Analizar la expresión global del genoma de las cepas en presencia de hongos fitopatógenos.
- Conocer el perfil proteómico de las cepas en presencia de hongos fitopatógenos y en interacción con plantas.

10. Coautorías.

10.1 Potential use and mode of action of the new strain *Bacillus thuringiensis* UM96 for the biological control of the grey mould phytopathogen *Botrytis cinerea*

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RESEARCH ARTICLE

Potential use and mode of action of the new strain Bacillus thuringiensis UM96 for the biological control of the grey mould phytopathogen Botrytis cinerea

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The potential use of Bacillus thuringiensis UM96 as a biocontrol agent for the grey mould phytopathogen Botrytis cinerea was evaluated. In order to dissect the mode of action of this UM96 strain, we also examined the role of lytic activities in the antagonism. First, B. thuringiensis UM96 was characterised based on 16S rRNA and gyrA gene sequencing and phenotypic traits. Petri dish biocontrol assays demonstrated that when strain UM96 was inoculated 24 h previous to B. cinerea, the mycelial growth was inhibited by up to 70%. Test for lytic enzymes activities of cellulase and glucanase was negative. Chitinase was the only positive enzyme activity in two different culture media. PCR detection of the chiB gene was also positive. Chitinolytic supernatants, obtained from rich and minimal media supplemented with colloidal chitin as the sole carbon source, from B. thuringiensis UM96 showed a strong inhibitory effect of B. cinerea that was not observed with heat-treated supernatant. Interestingly, when the supernatant was supplemented with 100 µM allosamidin, a chitinase specific inhibitor, the antagonistic activity was suppressed significantly. A lack of chitinase activity was also observed in allosamidin-treated supernatants. Our pathogenic B. cinerea strain also exhibited susceptibility to pure Streptomyces griseus chitinase. Finally, the chitinolytic strain B. thuringiensis UM96 was able to protect Medicago truncatula plants in vitro from B. cinerea infection and significantly reduced the necrotic zones and root browning of the plants. Together, these results suggest a potential use of B. thuringiensis UM96 for the biological control of B. cinerea and a role for chitinases during the antagonism displayed.

Keywords: Bacillus thuringiensis; chitinases; biological control; Botrytis cinerea

Introduction

Botrytis cinerea is a fungal phytopathogen that affects many plant species of agricultural importance, causing grey mould disease, serious declines in fruit production and significant economic loses. The damaging effects of B. cinerea have

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been reported in at least 200 plant species worldwide (Nambeesan et al., 2012). Because of this, the use of agrochemicals to diminish or reduce the damage caused by this fungal phytopathogen is a common practice although their toxic effects on the environment as well as animal and human health are well documented (Adesemoye & Kloepper, 2009). Rhizobacteria are eco-friendly and, therefore, are possibly interesting biocontrol agents for many plant diseases caused by fungi, including *B. cinerea*. *Bacillus* spp. have been widely reported as biocontrol agents (Ashwini & Srividya, 2014; Santoyo, Orozco-Mosqueda, & Govindappa, 2012). In fact, several *Bacillus*-based fungicides are being marketed (Cawoy, Bettiol, Fickers, & Ongena, 2011). Unfortunately, the use of biocontrol agents in agriculture is still limited in many countries.

Bacillus spp. can inhibit the growth of fungal phytopathogens through the synthesis of many metabolites, proteins and cell wall-attacking enzymes, including β-1,3-glucanases, lipases, cellulases and chitinases (Dahiya, Tewari, & Hoondal, 2006; Rao, Tanksale, Ghatge, & Deshpande, 1998). Chitin is an insoluble linear β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), and chitinases can selectively degrade it by hydrolyzing the β-1,4-glycosidic bonds that link GlcNAc (Dahiya et al., 2006; Swiontek Brzezinska, Jankiewicz, Burkowska, & Walczak, 2014). *Bacillus* spp. have been widely reported to synthesise chitinases with antifungal activity (Liu, Cai, Xie, Liu, & Chen, 2010; Neeraja et al., 2010; Sampson & Gooday 1998; Swiontek Brzezinska et al., 2014). For instance, Kishore and Pande (2007) evaluated *Bacillus cereus* chitinase action against *B. cinerea* by foliar supplementation and showed a reduced severity in grey mould disease symptoms in chickpea plants. Unfortunately, *B. cereus* is a potential human pathogen (Drobniewski, 1993), which restricts its use in potential biocontrol rhizosphere formulations.

Bacillus thuringiensis belongs to the *B. cereus* group and it is widely known for being the most important entomopathogenic bacterium (de Maagd, Bravo, Berry, Crickmore, & Schnepf, 2003; Rasko, Altherr, Han, & Ravel, 2005). This insecticidal activity is very interesting, but only a few works demonstrate its importance as fungal antagonist (Chang, Chen, & Wang, 2010; Liu et al., 2010). For instance, chitinases produced by *B. thuringiensis* var. *israelensis* have shown antagonistic and biocontrol activity against the phytopathogen *Sclerotium rolfsii* in soybean seeds, improving their germination percentages (Reyes-Ramírez, Escudero-Abarca, Aguilar-Uscanga, Hayward-Jones, & Barboza-Corona, 2004). Recently, the production of chitinases from *B. licheniformis* and *B. thuringiensis* strains collected from rhizospheric systems in Egypt was evaluated (Gomaa, 2012). Interestingly, chitinases from *B. thuringiensis* were more effective in degrading cell walls from many fungi than those from *B. licheniformis*. This result demonstrates the utility of chitinolytic strains of *B. thuringiensis* in the inhibition of fungal growth phytopathogens.

The UM96 strain, belonging to the genus *Bacillus*, has been previously isolated and partially characterised. This strain showed excellent antifungal activity against diverse phytopathogens (Martínez-Absalón et al., 2012). Here, by employing molecular and phenotypic methods, the UM96 strain was found to belong to the *B. thuringiensis* species. Additionally, the chitinolytic activity of *B. thuringiensis* UM96 supernatants against *B. cinerea* was investigated. Using a chitinase specific inhibitor, allosamidin (Sakuda, Inoue, & Nagasawa, 2013), chitinases of

B. thuringiensis UM96 were found to play a relevant role in the biocontrol of the grey mould pathogen, *B. cinerea*.

Materials and methods

Biological material

B. thuringiensis UM96 was previously isolated from a sorghum plant rhizosphere (*Sorghum* spp.) in an agricultural field in Morelia, Mexico (Martínez-Absalón et al., 2012). The strain was grown at 30°C for 24 h on nutrient agar (NA) and routinely maintained at 4°C. The *B. cinerea* fungus was inoculated on potato dextrose agar (PDA) at 30°C for 3–5 days in darkness and maintained at 4°C.

Molecular and phenotypical characterisation of strain UM96

Genomic DNA was isolated from *B. thuringiensis* UM96, and the subunit of the 16S ribosomal DNA (rDNA) gene was amplified using PCR (Sambrook & Russel, 2001). The universal bacterial primers fD1, 5′-CAGAGTTTGATCCTGGCTCAG-3′, and rD1, 5′-AAGGAGGTGATCCAGCC-3′, were used. In addition, internal primers were designed (at http://www.yeastgenome.org/cgi-bin/web-primer) to complete the full ribosomal gene sequence (601F, 5′-TGATGTGAAAGCCCACGG-3′; 1201F, 5′-CATCATGCCCCTTATGAC-3′; 27R, 5′-ACACTGGGACTGAGA-CAC-3′; 929R 5′-AAAGGAATTGACGGGGGC-3′). To amplify the *gyrA* gene the following primers were used: (gyrAF, 5′-CAACAAGCACGAATTCGAGA-3′; gyrAR, 5′-CTTCTTCTTCATCTTTCTGTG-3′). PCR conditions were as follows: an initial denaturation at 95°C for 3 min, 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 53°C, and extension for 2 min at 72°C, and a final extension step at 72°C for 5 min. PCR amplifications were performed using a TC-412 Techne Thermal Cycler (Keison Products, Chelmsford, UK). GoTaq® Master Mixes tubes (Promega, Madison, WI, USA) were used. The PCR product was additionally purified, and the rDNA 16S and *gyrA* genes from bacterial isolate UM96 were sequenced at the LANGEBIO (Irapuato, Mexico). The ribosomal sequence was obtained (GenBank Accession No. JF430006) and compared with those deposited in GenBank database using the nucleotide Basic Local Alignment Search Tool (BLAST) program. In order to observe the *B. thuringienesis* characteristic parasporal body, we followed, with some modifications, the protocol reported by Rampersad and Ammons (2005). Briefly, 5 µl was taken from a 3-ml sporulated culture of *B. thuringiensis* UM96 to be slide fixed and stained with Coomassie Brilliant Blue and viewed under Brightfield Microscopy using a 100X immersion oil objective. Amplification of the cry1A genes in *B. thuringiensis* UM96 was carried out with specific universal primers for cry1A gene group (Juarez-Perez, Ferrandis, & Frutos, 1997). Gene sequencing confirmed high identity with the cry1A gene group of other *B. thuringiensis* strains.

Phylogenetic analysis

Multiple sequence alignment was generated with ClustalW (www.ebi.ac.uk/Tools/clustalw2), and the phylogenetic analysis of the 16S rRNA and *gyrA* gene sequences was carried out with the MEGA 4.0 program (MEGA, Paris, France; Tamura, Dudley, Nei, & Kumar, 2007). To obtain a confidence value for the aligned sequence

data-set, a bootstrap analysis of 1000 replications was performed. A phylogenetic tree was constructed by using the maximum parsimony algorithm.

In vitro *evaluation of fungal antagonism*

The evaluation of fungal antagonism was carried out as previously reported on Petri dish assays (Santoyo, Valencia-Cantero, Orozco-Mosqueda, Peña-Cabriales, & Farías-Rodríguez, 2010). Briefly, the UM96 isolate was inoculated with the pathogenic fungi on PDA or NA agar plates either simultaneously, 24 h before or 24 h after, showing identical results on both media. The bacterial UM96 isolate was streaked onto plates in a cross shape, and a mycelial plug of 4 mm was deposited in the centre of each of the quadrants formed. The plates were incubated in darkness at 30°C, and mycelial growth was measured from day 1 to day 6. The experiments were performed in at least triplicate independent replicates. The percentage of growth inhibition was measured using the following formula: % of growth inhibition = $[(A_c - A_b)/A_c] \times 100$, where A_c is the control mycelial area and A_b is the mycelial area with treatment (Ben Slimene et al., 2012).

PCR amplification of the* chiB *gene

Genomic DNA was isolated from *B. thuringiensis* UM96, which was used as the template, to amplify a partial segment of the *chi* gene using the following primers: Chi-151/F, 5′-AATCACCCCCAGCTCCCTTG-3′ and Chi-1201/R, 5′-TTACGTC-TAGTTTTCGCTAA-3′. The PCR conditions were as follows: initial denaturation at 95°C for 3 min, 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 53°C, extension for 2 min at 72°C and a final extension step at 72°C for 5 min. The PCR amplifications were performed with a TC-412 Techne Thermal Cycler with the Promega reagents listed above.

Determination of the lytic activities and antifungal action of* B. thuringiensis *UM96 supernatants

Cellulase, chitinase and glucanase activities were measured as previously reported (de los Santos-Villalobos, Barrera-Galicia, Miranda-Salcedo, & Peña-Cabriales, 2012). Briefly, 20 mL supernatant aliquots were obtained in nutrient broth medium (NBM) to quantify these lytic activities in *B. thuringiensis* UM96. One unit of activity was defined as the amount of enzyme that releases one micromole equivalent of GlcNAc or glucose per minute under specific assay conditions. The enzyme yield was expressed as 1 mol/(g substrate/min). For chitinase and glucanase/cellulose activities, the released reducing sugar was measured at 565 nm by 3,5-dinitrosalicylic acid using GlcNAc and glucose as standards, respectively.

Chitinase activity of the UM96 strain was induced by growing the strain on minimal media with chitin (MMCH). Antifungal activities of supernatant samples from both media were tested in plate bioassays containing PDA. Supernatants from *B. thuringiensis* cultures were filter-sterilised with 0.2-μm membranes and treated for 1 h at 50°C, 60°C, or 70°C. One mL of each liquid media, having approximately 0.2 and 0.5 units of chitinase activity in NBM and MMCH media, respectively, was inoculated onto the plates and left alone until dry. Then, a 4-mm mycelial plug was

placed in the centre of each PDA plate and incubated in darkness at 30°C. The mycelial growth diameter was measured every 24 h for 7 days.

The specific chitinase inhibitor allosamidin (Sakuda et al., 2013) was added at a final concentration of 100 μM to supernatants to inhibit the chitinase activity of *B. thuringiensis* UM96 supernatants from NBM and MMCH media. Similar concentrations of allosamidin have been previously reported by Sampson and Gooday (1998) to completely suppress chitinolytic activity. Supernatants supplemented with allosamidin were also filter-sterilised and similarly tested on PDA Petri dishes as above to evaluate antagonism *towards B. cinerea*. Increasing concentrations of commercial *Streptomyces griseus* chitinase (Sigma) were added to PDA plates containing a 4-mm mycelial plug placed in the centre. Mycelial growth diameter was measured as previously.

UM96 biocontrol of the Medicago truncatula *and* B. cinerea *system*

The UM96 strain was assessed for its efficiency to suppress *B. cinerea* infection of *M. truncatula* plants. *M. truncatula* (ecotype Jemalong A17) seeds were subjected to chemical scarification and immersed in a vial containing 1 mL of concentrated anhydrous sulphuric acid in continuous agitation until black spots were observed on the integument (10–15 min). The excess acid was removed, and the seeds were rinsed with 5–10 washes of sterile deionised water. For sterilisation, seeds were soaked in a solution of sodium hypochlorite (12%) for three minutes and rinsed with 5–10 washes of sterile deionised water. Seeds were germinated in Petri dishes with MS medium and then transferred to a growth chamber with the following photoperiod: 16 h light/8 h dark with a light intensity of 200 mol·m^2/s at 22°C (Orozco-Mosqueda et al., 2013).

Recently scarified seeds were placed in plastic Petri dishes with MS medium at 4°C for 48 h and then placed in a growth chamber with previously described photoperiod. The germinated sprouts were placed in glass flasks, which measured 12 cm height and 9 cm in diameter, with 100 mL of Hoagland nutrient medium and 6 g of agar (Phytotechnology, Shawnee Mission, KS, USA) per litre (L). After 11 days of growth, a bacterial inoculum of 1×10^6 Colony Forming Units (CFU)/mL of the UM96 strain or sterile water, for control plants, was placed by the roots of the plant (1 cm from the plant). Another set of plants was inoculated with the same bacterial inoculum plus 1×10^6 spores/mL of *B. cinerea* or spores without bacteria for control plants. The experiment went on until the plants were 21 days of age. At this time, plants were measured, weighed and evaluated for *B. cinerea* infection, including stem and leaf disease symptoms (SLDS), such as yellow chlorosis, presence of grey mould and root browning and necrosis (RBN; $n = 12$ by triplicate experiments). The percentages were measured according to the formulas SLDS = (diameter of wounded area/total area) ×100 and RBN = (length of browning and necrotic zone/total root length) ×100. The relative significance of experiments was analysed using the Student's *t*-test ($P < 0.05$) calculated at the following website: www.physics.csbsju.edu/stats/t-test.html. The biocontrol experiment was statistically analysed by a Duncan's multiple range test ($P < 0.05$).

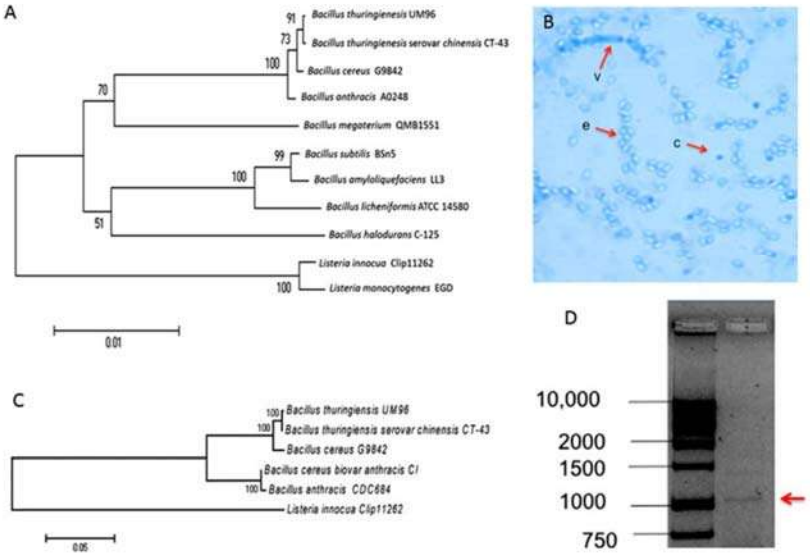

Figure 1. (Colour online) Molecular and phenotypic characterisation of strain UM96. Panel A shows phylogenetic analysis based on complete 16S ribosomal (Panel A) and *gyrA* (Panel C) gene sequences showing the relationship between *B. thuringiensis* UM96 and the *B. thuringiensis* species. The phylogenetic tree was constructed based on the neighbour-joining method. Other algorithms show similar tree topologies. Bootstrap analysis of 1000 replications was performed and expressed as percentages. Two or one species of *Listeria* were used as an outgroup. Panel B shows parasporal bodies (c), vegetative cells (v) and endospores (e). Bacterial cells of the *B. thuringiensis* UM96 strain were stained with Coomassie Brilliant Blue and viewed under Brightfield Microscopy using a 100X objective. Panel D shows the PCR fragment amplification of the gene *cry1A*.

Results

The UM96 strain belongs to the B. thuringiensis *species*

Bacterial strain UM96 was previously isolated from sorghum rhizosphere plants and selected because of its strong antifungal activity. Partial molecular sequencing of the 16S rDNA gene showed that the UM96 strain belonged to the *Bacillus* genus (Martínez-Absalón et al., 2012). Here, we completed the sequence of the 16S rDNA and *gyrA* genes, which showed a high identity match with *B. thuringiensis* species in the NCBI database. This was confirmed with phylogenetic analysis, in which the UM96 isolate clustered with other *B. thuringiensis* species (Figure 1A and C). Microscopic phenotypic observation of the parasporal body and PCR fragment amplification of *cry1A* genes coding for Cry proteins, characteristics of the *B. thuringiensis* species were also confirmed in the UM96 strain (Figure 1B and D).

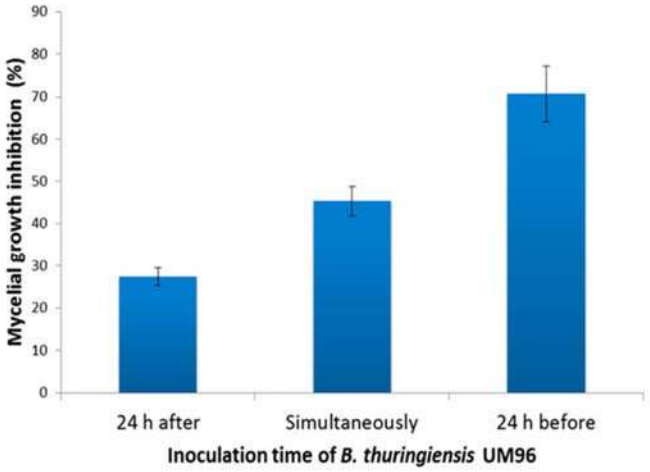

Figure 2. (Colour online) Inhibitory effect of the *B. thuringiensis* UM96 strain against *B. cinerea* growth inhibition. Fungal growth diameter is presented as the mean of at least three independent replicates measured from day 1 to day 6, compared to the control experiment (without bacterial inoculation). Statistically significant growth inhibition was observed between treatment and control experiments (marked by asterisks; Student's *t*-test $P < 0.05$).

B. cinerea *inhibition bioassays*

The inhibitory effect of *B. thuringiensis* UM96 against the *B. cinerea* was evaluated *in vitro*. As shown in Figure 2, the inoculation time of *B. thuringiensis* UM96 was important to observe a high degree of inhibition of the phytopathogen mycelia. When the bacterium was inoculated 24 h after or simultaneously with the fungus, it was observed a percentage of inhibition of mycelium diameter of 28%. However, when the bacteria had time to grow 24 h before *B. cinerea*, inhibition percentages were by up to 70%. This suggests that the establishment of the bacteria, as well as possibly the synthesis of antifungal metabolites in the medium, prior to the presence of phytopatogen, is significant to establish a high degree of antagonism.

Detection of the* chiB *gene and chitinase activity in supernatants and allosamidin inhibition

The presence of *chiB*, a gene encoding for an endochitinase, in the genome of the UM96 strain was detected by PCR with the expected size band. After detection, we evaluated supernatant lytic activities, including cellulase, glucanase and chitinase, which may have been involved in antagonism towards *B. cinerea*. Analysis of *B. thuringiensis* UM96 supernatant showed no cellulase or glucanase activity, but the supernatant did show good chitinase activity. When *B. thuringiensis* was grown in NBM, the observed chitinase activity in the supernatant was 0.2 ± 0.03 U (µmol/g substrate/min) and was increased at nearly 2.5 fold (0.49 ± 0.03 U) in supernatants from minimal medium containing chitin (MMCH) as the sole source carbon (Figure 3). Allosamidin, a specific family 18 chitinase inhibitor, was added to the

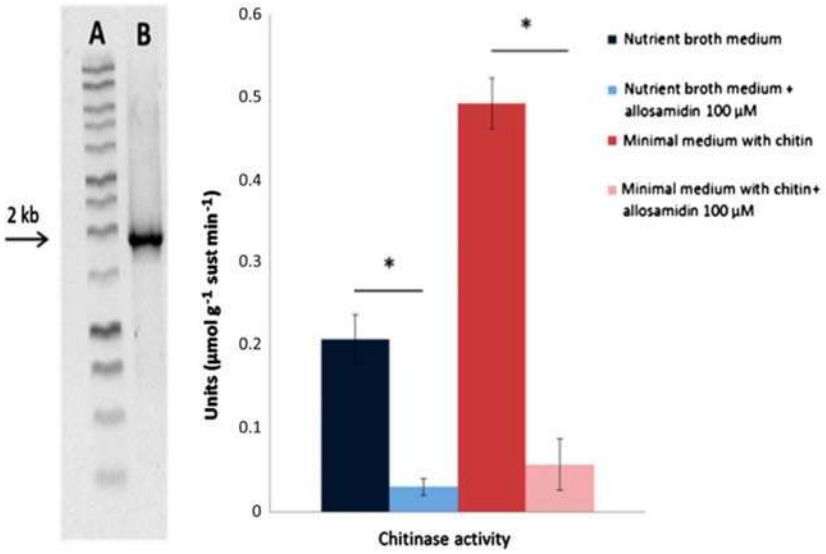

Figure 3. (Colour online) PCR detection of the *chiB* gene and chitinase activity in *B. thuringiensis* UM96 supernatants. Left, PCR amplification of the *chiB* gene at the expected size (A, molecular marker; B, expected size band of the *chiB* gene). Right, Chitinolytic activity in supernatants obtained from NBM and Minimal Medium with Chitin (MMCH) with or without 100 µM allosamidin. Statistical differences (asterisks) were observed between supernatants treated with allosamidin, in which chitinolytic activity was diminished (Student's *t*-test $P < 0.05$).

supernatants to inhibit the chitinase activity of *B. thuringiensis* UM96 supernatants from NBM and MMCH. As shown in Figure 3, 100 µM allosamidin inhibited the chitinase activity of both supernatants, which only showed the basal activity of less than 0.1 U.

Antifungal activity of chitinolytic supernatants

The response *B. cinerea* growth to the addition of chitinolytic *B. thuringiensis* supernatants obtained from rich media (NBM) and MMCH was analysed. Both supernatants were heat treated to eliminate protein activity and treated with allosamidin to inhibit chitinase activity. We also evaluated *B. cinerea* sensitivity to commercial *S. griseus* chitinase. The mycelial growth diameter of *B. cinerea* was measured for seven days in all treatments. As Figure 4 shows, both chitinolytic supernatants (NBM and MMCH) significantly ($P < 0.05$) inhibited mycelial growth from days 4 to 7, compared to the control treatment. Heated supernatants lost their antagonistic activity, suggesting a role for a protein activity. Importantly, supernatants treated with 100 µM allosamidin did not restrict the growth of *B. cinerea*, confirming the specific role of protein chitinases in growth inhibition. *B. cinerea* also showed to be sensitive to *S. griseus* chitinase. As previously observed (Figure 3), allosamidin inhibited the chitinolytic activity in supernatants from cells grown in

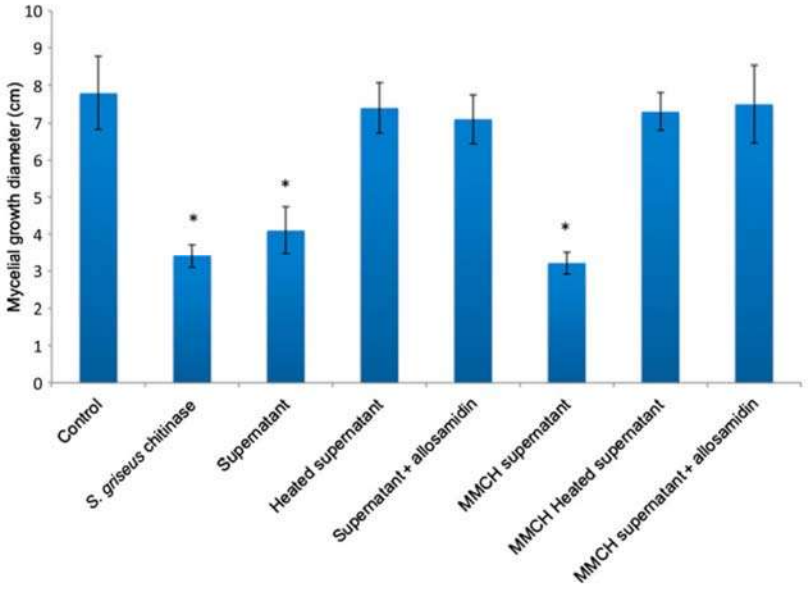

Figure 4. (Colour online) The effect of differentially treated *B. thuringiensis* UM96 supernatants and *S. griseus* chitinase on the *B. cinerea* growth inhibition. Fungal growth is presented as the mean in cm of at least three independent replicates of mycelium diameter measured at day 7. Asterisks show statistical differences compared to control experiment (Student's *t*-test $P < 0.05$).

minimal media supplemented with chitin, where higher chitinase activity was observed.

Plant protection and biocontrol evaluation

For biocontrol assays, we utilised the *M. truncatula* and *B. cinerea* system. First, we conducted analysis to demonstrate the ability of *B. cinerea* spores to infect and pathogenise *M. truncatula* plants in vitro. As shown in Figure 5, inoculation of 1×10^6 spores/mL caused severe stem and root disease symptoms in *M. truncatula* plants, including the visual growth of mycelium on the plant tissue (grey mould), yellow chlorosis, and root browning and necrosis, and shoot and root fresh weights were also affected. When spores were inoculated along with the bacterial inoculum (1×10^6 CFU/mL), disease symptoms were significantly reduced, and plants were visually healthier, similar to plants inoculated only with the bacteria or control plants (Figure 5B–D). Importantly, a protective effect in plants inoculated with the UM96 strains was observed. Shoot fresh weights recovered, and stem and leaf disease symptoms were also significantly reduced by up to 70% (Figure 5E and F).

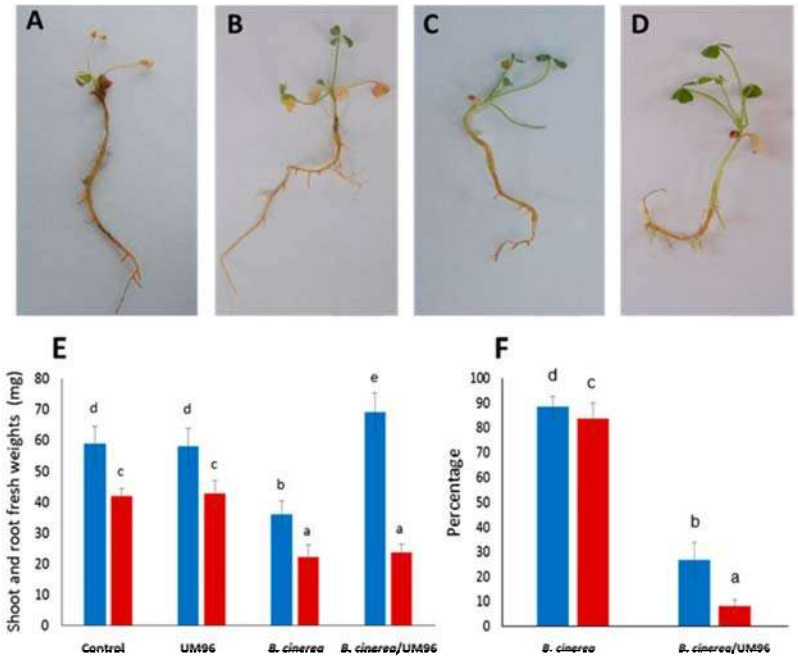

Figure 5. (Colour online) Biocontrol capacity of the chitinolytic *B.thuringiensis* UM96 strain against *B. cinerea* in *M. truncatula* seedlings. Representative plants (n = 12) are shown in different panels (A–D). (A) Plant inoculated with *B. cinerea* fungi. (B) Plant coinoculated with *B. cinerea* and *B. thuringiensis* UM96. (C) Plant inoculated with *B. thuringiensis* UM96 (D) Control plant. Panel E shows the shoot fresh weights (red bars) and root fresh weights (blue bars). Panel F shows stem and leaf disease symptoms (red bars) and root browning and necrosis (blue bars). Lower case letters indicate significant differences between treatments ($P < 0.05$; duncan's multiple range test). Figure S1 shows the ANOVA analysis.

Discussion

B. cinerea causes grey mould disease around the world, severely damaging fruit production and causing enormous economic losses in agriculture. In fact, grey mould has been reported to affect more than 200 plant species (Nambeesan et al., 2012). In this work, we characterised the antagonistic and biocontrol activity of *B. thuringiensis* UM96 chitinases against the phytopathogen *B. cinerea*. The genus *Bacillus* has been widely reported as an antagonist of fungal organisms (Ashwini & Srividya, 2014; Santoyo et al., 2012). In the case of *B. thuringiensis*, its entomopathogenic capacity is an interesting research topic (de Maagd et al., 2003). *B. thuringiensis* are a close relative of potential human pathogens, such as *B. cereus*, making it difficult to *utilise B. cereus* in future research as biocontrol or biopromoting inoculants even though these bacteria exhibit good biocontrol traits (Kishore & Pande, 2007). In fact, phylogenetic analysis of the *B. thuringiensis* 16S

ribosomal gene sequence groups them into one clade with human *Bacillus* pathogens with no clear differences at this level of comparison. In this work, we detected the parasporal body and *cry* coding genes, which are characteristic of *B. thuringiensis*. In fact, the Cry protein crystals in the spore produced by *B. thuringiensis* can be an advantage in the soil environment upon sporulation (Jensen, Hansen, Eilenberg, & Mahillon, 2003; Rasko et al., 2005). According to Rasko et al. (2005), as well as other authors (Bravo et al., 1998) *B. thuringiensis* is phenotypically distinguished from *B. cereus* only by the formation of intracellular protein crystals during sporulation, meanwhile Kolstø, Lereclus, and. Mock (2002) considered that 'by definition, *B. cereus* is acrystalliferous, but a *B. cereus* strain carrying a functional cry gene is considered as a *B. thuringiensis* strain'. Here, the molecular and phenotypic characterisation allowed us to conclude that the UM96 strain belongs to the *B. thuringiensis* species since we detected functional cry genes.

When *B. thuringiensis* UM96 was co-inoculated with the fungus, the UM96 strain inhibited mycelial growth by up to 70%, compared to control experiments. Similar results have been found with other biocontrol bacteria, including *Bacillus* species and members of other genera, such as *Pseudomonas fluorescens* (Santoyo et al., 2010, 2012). The rationale is that bacteria need some time to produce metabolites or enzymes in the rhizosphere. Secondary metabolism is inducible under certain conditions, including the presence of inducers and abiotic conditions. Chitinase enzyme production and excretion are regulated by repressor and inducer systems and can be induced by chitin in the media. On the other hand, chitinases are repressed by glucose or other sources of carbon (Sahai & Manocha, 1993; Swiontek Brzezinska et al., 2014). We have observed in confrontation assays that *B. cinerea* induces *phlD* gene expression, which codes for a type III polyketide synthase involved in the biosynthesis of 2,4-diacetylphloroglucinol, in *P. fluorescens* strains (Hernández-León, unpublished results). Currently, we are interested in determining what *B. cinerea* factors induce chitinase production in *Bacillus* species with different carbon sources in the media.

Bacillus rhizobacteria produce several lytic enzymes as an antagonistic mechanism to compete for spaces and nutrients in the rhizosphere, either displacing or inhibiting the growth of other microorganisms (Glick, 2012). β-1,3-Glucanases, cellulases, lipases and chitinases are examples of widely lytic enzyme activities in bacterial antagonists (Dahiya et al., 2006; de los santos-villalobos et al., 2012; Rao et al. 1998). Here, *B. thuringiensis* UM96 did not have glucanase and cellulose activities, but for the UM96 strain did exhibit chitinase activity in supernatants extracts. Here, it was evident that allosamidin-treated supernatants significantly lost the capacity to inhibit the growth of *B. cinerea*. This suggested a specific role of chitinases in the antifungal activity. Chitinolytic enzymes produced by many rhizosphere microorganisms have been postulated to play an important role in the antagonism and biocontrol of fungal organisms (Swiontek Brzezinska et al., 2014). However, few studies have clearly associated chitinase production with antifungal activity because many bacteria produce other metabolites or enzymes (i.e., lipopeptides and antibiotics) that could also be involved in antagonism. Thus, the use of specific inhibitors, like allosamidin, can elucidate the association between chitinase production and fungal antagonism (Sampson & Gooday, 1998).

This work demonstrates a role for *B. thuringiensis* UM96 chitinases in bioassays and the important biocontrol of *B. cinerea* in the infection of *M. truncatula* plants.

M. truncatula plants inoculated with the UM96 strain had significantly reduced disease symptoms in leaf and stem tissues, and necrosis and browning in roots were decreased. Interestingly, plants inoculated with bacteria had similar shoot weights to control plants without pathogen. The antifungal and protective effects of *Bacillus* chitinases have been investigated by a few other studies. For example, purified chitinases from *Bacillus subtilis* NPU 001 showed inhibition of hyphal growth of the fungus *Fusarium oxysporum* (Chang et al., 2010). In another study, addition of chitinases from *B. thuringiensis* var. *israelensis* to soybean seeds infected with *S. rolfsii* resulted in a protective effect and increased germination percentages (Reyes-Ramírez et al., 2004). Similarly, Liu et al. (2010) found that purified chitinase A (chiA) from *B. thuringiensis* subsp. *colmeri* 15A3 inhibited spore germination in many species of fungi, including *Rhizoctonia solani*, *B. cinerea*, *Penicillium chrysogenum*, *Physalospora piricola*, *Penicillium glaucum* and *Sclerotinia fuckelian*. Interestingly, ChiA protein also showed larvicidal activity against *Spodoptera exigua* and *Helicoverpa armigera*. This latter result suggests that *Bacillus* chitinases should be evaluated in other biotechnological applications. It will also be useful to test the entomopathogenic potential of the UM96 strain because the chitinase synthesis in other *B. thuringiensis* strains has been associated with a higher insecticidal capacity (Sampson & Gooday, 1998). In conclusion, our work demonstrates that chitinases produced by *B. thuringiensis* UM96 play a role in the biocontrol of the grey mould phytopathogen *B. cinerea*, and they could potentially be used to neutralise the phytopathogen.

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Supplemental data

Supplemental data for this article can be accessed here: http://dx.doi.org/10.1080/09583157.2014.940846.

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10.2 Draft Genome Sequence of the Biocontrol and Plant Growth-Promoting Rhizobacterium ***Pseudomonas fluorescens*** Strain UM270

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SHORT GENOME REPORT Open Access

Draft Genome Sequence of the Biocontrol and Plant Growth-Promoting Rhizobacterium ***Pseudomonas fluorescens*** strain UM270

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Abstract

The *Pseudomonas fluorescens* strain UM270 was isolated form the rhizosphere of wild *Medicago* spp. A previous work has shown that this pseudomonad isolate was able to produce diverse diffusible and volatile compounds involved in plant protection and growth promotion. Here, we present the draft genome sequence of the rhizobacterium *P. fluorescens* strain UM270. The sequence covers 6,047,974 bp of a single chromosome, with 62.66 % G + C content and no plasmids. Genome annotations predicted 5,509 genes, 5,396 coding genes, 59 RNA genes and 110 pseudogenes. Genome sequence analysis revealed the presence of genes involved in biological control and plant-growth promoting activities. We anticipate that the *P. fluorescens* strain UM270 genome will contribute insights about bacterial plant protection and beneficial properties through genomic comparisons among fluorescent pseudomonads.

Keywords: *Pseudomonas fluorescens*, Biocontrol, PGPR

Introduction

Plant pathogens cause diverse crop plant diseases resulting in drastic economic losses around the world. An alternative to the use of chemicals to control plant pathogens is the employment of eco-friendly bacterial agents [1, 2]. An ideal bacterial biocontrol agent would be one with the additional capacity to directly stimulate plant growth [3]. Here, we report the draft genome sequence of the novel strain *Pseudomonas fluorescens* strain UM270. This strain was previously isolated and characterized for its excellent capacities for biocontrol of phytopathogens and plant growth promotion [4].

In a previous report, our group showed that the *P. fluorescens* strain UM270, among other three pseudomonad strains, was the best in promoting the growth of *Medicago truncatula* Gaertn. plants by significantly increasing biomass and chlorophyll content. During confrontation assays, strain UM270 inhibited the growth of agro-economically important fungal phytopathogens such as *Botrytis cinerea*, *Rhizoctonia solani*, *Diaporthe phaseolorum*, and *Colletotrichum lindemuthianum* [4]. In biocontrol experiments, the strain UM270 protected *M. truncatula* plants from *B. cinerea* infection, reducing general stem disease symptoms, root browning and necrosis [4].

Importantly, the strain UM270 exerted these activities through the emission of either diffusible compounds (such as phenazines, cyanogens, 1-aminocyclopropane-1-carboxylate deaminase, siderophores, proteases and indole-3-acetic acid) or volatiles (like dimethyl disulfide and dimethylhexadecylamine) [4], revealing that the strain UM270 contains direct and indirect mechanisms to promote plant growth [5].

Organism Information

Classification and features

P. fluorescens strain UM270 is a Gram-negative, non-sporulating, motile, rod-shaped bacterium belonging to the Order *Pseudomonadales* and the Family *Pseudomonadaceae* (Fig. 1). The strain exhibits the general and

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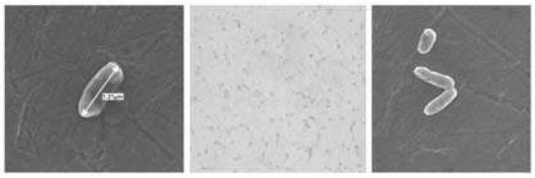

Fig. 1 Images of *P. fluorescens* strain UM270 using scanning electron microscopy (left and right) and phase-contrast (center)

common features of a *Pseudomonas* species phenotype (Table 1) [6].

The UM270 strain was isolated from the rhizosphere of *Medicago* spp. located in an agricultural field in Morelia, Michoacán, México. As mentioned above, this bacterium was further characterized and found to produce several diffusible and volatile compounds involved in biocontrol against several fungal pathogens, particularly effective against the grey mold disease caused by *Botrytis cinerea* [4]. Recent work in our lab

Table 1 Classification and general features of *Pseudomonas fluorescens* strain UM270

MIGS ID	Property	Term	Evidence code[a]
	Current classification	Domain *Bacteria*	TAS [14]
		Phylum *Proteobacteria*	TAS [15]
		Class *Gammaproteobacteria*	TAS [16, 17]
		Order *Pseudomonadales*	TAS [18, 19]
		Family *Pseudomonadaceae*	TAS [18, 20]
		Genus *Pseudomonas*	TAS [18, 21]
		Species *Pseudomonas fluorescens*	TAS [18, 22]
		Strain UM270	TAS [4]
	Gram stain	Negative	TAS [6]
	Cell shape	Rod-shaped	TAS [6]
	Motility	Motile	NAS [6]
	Sporulation	None	NAS
	Temperature range	Mesophilic	IDA
	pH range; Optimum	6-8.5;7-8	IDA
	Optimum temperature	28 °C	IDA
	Carbon source	Heterotroph	IDA, [6]
	Energy source	Chemoorganotroph	NAS
MIGS-6	Habitat	Rhizospheric soil	TAS [4]
MIGS-6.3	Salinity	NaCl 1-4 %	IDA
MIGS-22	Oxygen Requirement	Aerobic	IDA
MIGS-15	Biotic relationship	*Medicago* spp. root associated	TAS [4]
MIGS-14	Pathogenicity	Non-pathogenic	TAS [4]
MIGS-4	Geographic location	Morelia, México	TAS [4]
MIGS-5	Sample collection	March, 2012	NAS
MIGS-4.1	Latitude	19° 46' 6" N	TAS [4]
MIGS-4.2	Longitude	101° 11' 22" W	TAS [4]
MIGS-4.3	Depth	10-20 cm	NAS
MIGS-4.4	Altitude	1800 M.A.S.L.	NAS

[a]Evidence codes - IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project

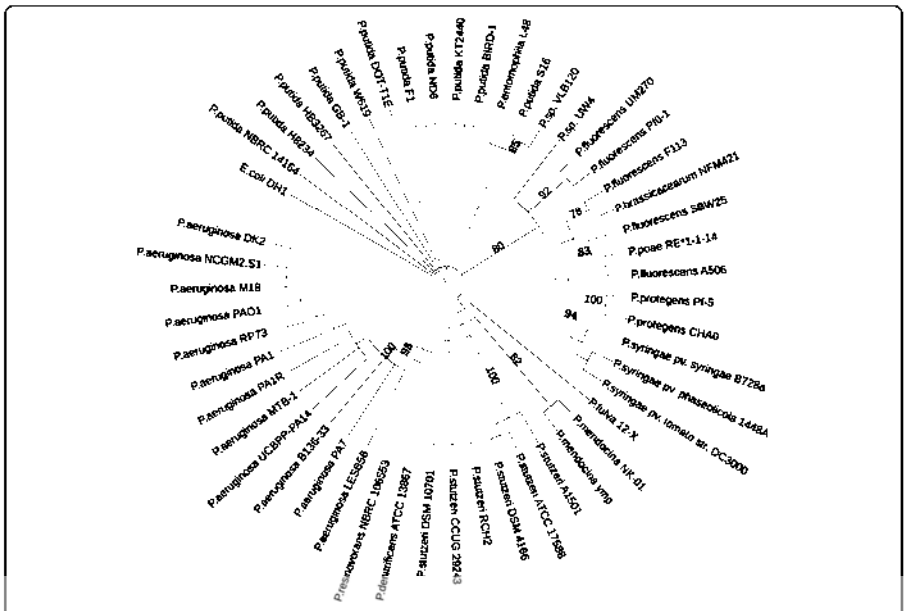

Fig. 2 Phylogenetic tree showing the close relationship of *P. fluorescens* strain UM270 with *P. fluorescens* Pf0-1, as well as with other *Pseudomonas* species based on aligned sequences of the 16S rRNA gene. Phylogenetic analyses were performed using SeaView and edited in iTol. The tree was built using the maximum likelihood method. Bootstrap analysis (1000 replicates) was performed to assess the support of the clusters. *E. coli* was used as an outgroup

Table 2 Project information

MIGS ID	Property	Term
MIGS 31	Finishing quality	High-quality draft (Full genome representation)
MIGS-28	Libraries used	3 libraries of 400–450 bp, 600 bp and 1,000 bp.
MIGS 29	Sequencing platforms	Illumina MiSeq
MIGS 31.2	Fold coverage	45.0 ×
MIGS 30	Assemblers	Newbler v. 2.9
MIGS 32	Gene calling method	NCBI Prokaryotic Genome, Annotation Pipeline
	Locus Tag	RL74
	Genbank ID	JXNZ00000000
	GenBank Date of Release	2014-12-09
	GOLD ID	Gb0118948
	BIOPROJECT	PRJNA269735
MIGS 13	Source Material Identifier	UM270
	Project relevance	Agriculture, Plant-Bacteria Interaction, Biocontrol

Table 3 Genome statistics

Attribute	Value	% of total
Genome size (bp)	6,047,974	100.00
DNA coding (bp)	5,284,158	87.00
DNA G + C (bp)	3,772,331	62.00
DNA scaffolds	524	100.00
Total genes	5,509	100.00
Protein coding genes	5,396	98.00
RNA genes	59	-
Pseudo genes	110	1.90
Genes in internal clusters	NA	-
Genes with function prediction	4,490	82.00
Genes assigned to COGs	3,821	68.00
Genes with Pfam domains	4,297	78.00
Genes with signal peptides	5	0.09
Genes with transmembrane helices	30	0.50
CRISPR repeats	0	-

has demonstrated that this strain is highly competitive and an efficient root and rhizosphere colonizer, as well as an inducer of ISR (Induced systemic resistance) in plants [Rojas-Solis and Santoyo, Unpublished results]. The Minimum Information about the Genome Sequence of *P. fluorescens* strain UM270 is summarized in Table 1. Its phylogenetic position is shown in Fig. 2, where the 16S rRNA gene of *P. fluorescens* strain UM270 is 99 % similar to that of *P. fluorescens* strain Pf0-1 [7–9].

Genome sequencing information

Genome project history

The *P. fluorescens* strain UM270 was selected among other pseudomonads for its higher ability to control fungal pathogens and protect *Medicago truncatula* Gaertn. from *B. cinerea* infection [4], for being highly competitive, an excellent root and rhizosphere colonizer of maize plants and for inducing ISR in plants (Rojas-Solis and Santoyo, Unpublished results). A high-quality draft sequence of the genome has been deposited at DDBJ/EMBL/GenBank. A summary of the project information is shown in Table 2.

Growth conditions and genomic DNA preparation

From a single colony culture the *P. fluorescens* strain UM270 was inoculated on 50 ml of King's B medium [10], grown overnight at 28 °C with in agitation (250 rpm). One milliliter of the culture was serially diluted to be analyzed further. We confirmed the morphology and antibiotic-resistance phenotype of the strain. From the culture, 20 ml were taken to isolate the genomic DNA by using the Wizard® Genomic DNA Purification Kit following manufacture's instructions (Promega). DNA samples were subjected to an additional purification step with the same Wizard®

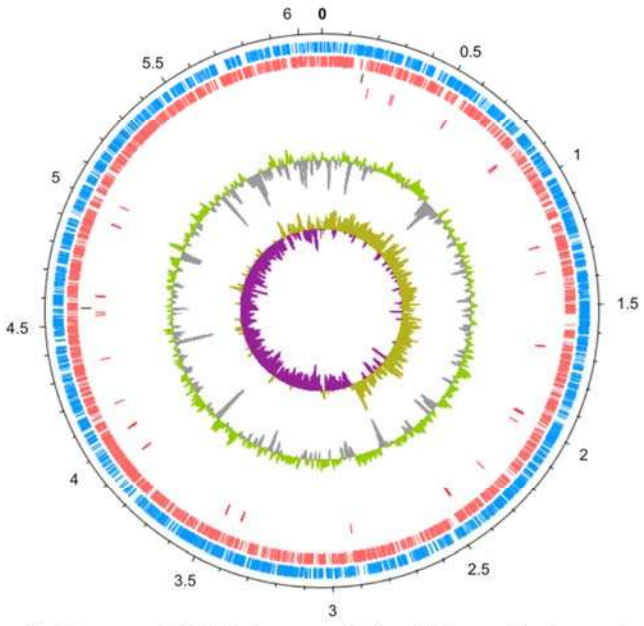

Fig. 3 Graphical map of the *P. fluorescens* strain UM270. Numbers represent Megabases (Mb). From outside to the center: Genes on forward strand (blue), Genes on reverse strand (red), RNA genes (rRNAs black color, tRNAs red color) G + C% (green and gray), G + C skew (purple and yellow). To display the *P. fluorescens* strain UM270 draft genome we ran a blastn comparison using the contigs as query, against the genome sequence of *P. fluorescens* strain Pf0-1 as target reference. We then used these results to order the contigs following the matching coordinates of the reference genome. Contigs not matching the reference genome were ordered from largest to smallest and appended to the contigs matching the genome of reference. The ordered contigs were joined with 50 bp of "N" to draw this figure using the DNA plotter software

Genomic DNA Purification Kit (Promega). The quality and quantity of the final DNA sample were evaluated by agarose gel electrophoresis and by using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Genome sequencing and assembly

Genomic DNA samples of *P. fluorescens* strain UM270 were sent to a sequencing service at the LANGEBIO-Irapuato, México. Genome sequencing was performed using a MiSeq Sequencer (Illumina, Inc.) generating three paired-end libraries (400–450 bp, 600 bp and 1,000 bp, respectively) with a coverage of approximately 45×. The *P. fluorescens* strain UM270 draft genome we ran a blastn comparison using the contigs as query, against the genome sequence of *P. fluorescens* Pf0-1 as target reference. To order the contigs we followed the matching coordinates of the reference genome. Project information is shown in Table 2.

Genome annotation

Genome annotation was carried out with RAST [11] and the Prokaryotic Genome Annotation Pipeline tools [12]. Statistics for the genome assembly were calculated using software Newbler v2.9 (Roche) and are shown in Table 2. This Whole Genome Shotgun sequence project has been deposited at DDBJ/EMBL/GenBank under accession JXNZ00000000. The version described in this paper is version JXNZ00000000.

Genome Properties

The total length of the assembled sequences obtained was 6,047,974 bp belonging to one chromosome, with a G + C content of 62.66 %. The sequenced fragments of the genome are predicted to contain 5,509 genes, consisting of 5,396 coding sequences, 59 RNA genes, 110 pseudogenes and 14 frameshifted genes. Genome statistics are in Table 3 and a graphical map is represented in Fig. 3. The

Table 4 Number of genes associated with the 25 general COG functional categories

Code	Value	% of total[a]	Description
J	159	2.94	Translation, ribosomal structure and biogenesis
A	0	0.00	RNA processing and modification
K	342	6.33	Transcription
L	117	2.16	Replication, recombination and repair
B	3	0.00	Chromatin structure and dynamics
D	32	0.59	Cell cycle control, cell division, chromosome partitioning
Y	0	0.00	Nuclear structure
V	55	1.01	Defense mechanisms
T	216	4.00	Signal transduction mechanisms
M	212	3.92	Cell wall/membrane biogenesis
N	142	2.63	Cell motility
Z	0	0.00	Cytoskeleton
W	0	0.00	Extracellular structures
U	55	1.01	Intracellular trafficking and secretion
O	150	2.77	Posttranslational modification, protein turnover, chaperones
C	244	4.52	Energy production and conversion
G	190	3.52	Carbohydrate transport and metabolism
E	434	8.04	Amino acid transport and metabolism
F	78	1.44	Nucleotide transport and metabolism
H	143	2.65	Coenzyme transport and metabolism
I	185	3.42	Lipid transport and metabolism
P	226	4.18	Inorganic ion transport and metabolism
Q	67	1.24	Secondary metabolites biosynthesis, transport and catabolism
R	364	6.74	General function prediction only
S	372	6.89	Function unknown
-	1,610	29.83	Not in COGs

[a]The total is based on the total number of protein coding genes in the annotated genome

Table 4 presents the number of genes associated with the COG functional categories.

Insights from the genome sequence

The draft genome sequence reported here covers its full genome and at first analysis reveals the presence of multiple genes participating in the synthesis of diffusible metabolites and volatile organic compounds produced by *P. fluorescens* strain UM270. Some of this antimicrobial arsenal includes compounds like phenazine (*phzFABCD*), pyocyanin (*pcnCDE*), pyoverdine (*pvdPD*), 2,4-diacetylphloroglucinol (*phlACBD*) and the volatile hydrogen cyanide (*hcnCB*), important for the biological control of several plant diseases caused by phytopathogenic fungi, oomycetes, and bacteria [2]. Other plant-bacteria communication genes detected in the strain UM270 genome are *acdS* and *iaaMH*, encoding for an ACC deaminase (1-aminocyclopropane-1-carboxylate) protein and IAA (indole-3-acetic acid) biosynthesis. The synergistic interaction of ACC deaminase and both plant and bacterial auxin, IAA, is relevant for the optimal functioning of PGPR to directly promote plant growth and also protect plants against environmental stresses, and bacterial and fungal pathogens [5]. Other genes such as *pcdQ*, which codes for an Acyl-homoserine lactone acylase, important for bacterial communication and biofilm formation, were detected, as well as Secretion Systems Type II to VI and orthologs of the toxin-antitoxin loci *vapBC-1* and *vapXD*. These last determinants are important for survival, competence and colonization of the rhizosphere and root systems [13].

Conclusions

The strain UM270 was selected for genome sequencing due to its biocontrol and plant growth promoting properties [4]. The plant beneficial mechanisms exerted by this rhizobacterium involved direct and indirect mechanisms. Here, the draft genome sequence of the *P. fluorescens* strain UM270 revealed further genetic elements involved in plant-bacterial communication, as well as in rhizosphere competence and colonization. We anticipate that the genome of *P. fluorescens* strain UM270 will contribute to new insights about biocontrol and plant beneficial activities through genomic comparisons among available complete genomes of pseudomonad strains.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JHS, RHL and MCOM performed the experiments. JHS and GM-H performed the annotation and sequence homology searches. GM-H and GS wrote the manuscript. EVC and GS conceived and designed the experiments. All authors commented on the manuscript before submission. All authors read and approved the final manuscript.

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10.3 Recent advances on the role of abiotic factors modulating the plant-microbe-soil interactions

Title: Recent advances on the role of abiotic factors modulating the plant-microbe-soil interactions

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Abstract

Microbial soil communities are active players in biogeochemical cycles, impacting soil fertility and interacting with aboveground organisms. Although soil microbial diversity has been studied in good detail, the factors that modulate its structure are still relatively unclear, especially the environmental factors. Several abiotic elements may play a key role in modulating the diversity of soil microbes, including those inhabiting the rhizosphere (known as the rhizosphere microbiome). This review summarizes relevant and recent studies that have investigated the abiotic factors at different scales, such as pH, temperature, soil type, and geographic and climatic conditions, that modulate the bulk soil and rhizosphere microbiome, as well as their indirect effects on plant health and development. The plant–microbiome interactions and potential benefits of plant growth-promoting rhizobacteria are also discussed. In the last part of this review, we highlight the impact of climate change on soil microorganisms via global temperature changes and increases in ultraviolet radiation and CO_2 production. Finally, we propose the need to understand the function of soil and rhizospheric

ecosystems in greater detail, in order to effectively manipulate or engineer the rhizosphere microbiome to improve plant growth in agricultural production.

Keywords: Abiotic factors, Plant Growth-Promoting Rhizobacteria, Rhizosphere Microbiome, Sustainable Agriculture, Bulk Soil.

INTRODUCTION

An ecosystem is made up of a community of living organisms interacting with each other and their environment, also known as the abiotic environment or environmental factors. However, gaining a detailed understanding of an ecosystem can be quite difficult due to the potential complexity of multiple and often multifactorial ecological interactions (Chapin et al., 2011). However, determining the effects of various components on an ecosystem, including both biotic and abiotic factors, is highly relevant to understanding how an ecosystem works as a whole. Indeed, studies on multi-trophic interactions and multi-communities have shown that the biota of the surface of the soil can affect the biota beneath soil (Wardle et al., 2004).

The part of the soil that is influenced by the roots of plants is known as the rhizosphere (Hiltner 1904). This micro-ecosystem is composed of a biota (mainly microorganisms) that inhabits the soil and can form complex communities that interact in beneficial, harmful, or neutral way with plants. Beneficial plant–microbe interactions are of particular interest, since we can take advantage of these functions in order to improve and promote the growth, development, and health of plants for several applications, including agricultural purposes (Santoyo et al., 2012; Santoyo et al., 2015; Glick et al., 2014.). Therefore, the microbial communities inhabiting the rhizosphere, also known as the rhizosphere microbiome, have been studied for decades and recognized as an important factor influencing the physiology and development of plants; some have even gone so far to define the rhizosphere biome as a second plant genome (Berendsen et al., 2012). Similarly, these microorganisms receive benefits from plants that select for and promote their development in several ways, either by increasing their survival under stress conditions, or by producing hormones that stimulate microbial growth and/or eliminate pathogens in the soil (Berendsen et al., 2012; Santoyo et al.,

2012; Santoyo et al., 2015; Hernández-León et al., 2015); this aspect of the interaction will be discussed in more detail below. Moreover, neither plants nor rhizosphere microorganisms can be considered separately from the abiotic components of the ecosystem.

Several studies have emphasized the importance of differentiating between the influence of abiotic and biotic factors on the generation and maintenance of microbial diversity in the rhizosphere. According to the theory of Bass-Becking who stated that "Everything is everywhere, but the environment selects" (Wit & Bouvier 2006), many researchers have attempted to identify the specific factors that determine microbial biodiversity and their particular influence on an ecosystem, using various theoretical and experimental approaches, both in the laboratory and the field.

From this ecological perspective, Wardle (2006) recently reviewed the influence of several sources of biotic origin on soil microbial diversity, including plant species, interactions between organisms within and beyond the rhizosphere, and animal and human activities. In the present review, we focus on the influence of abiotic factors on the diversity of soil microbes, especially those inhabiting the rhizosphere, that may be relevant to plant development, including type of soil, pH, temperature, and geographical and other environmental characteristics (Figure 1).

Moreover, increasing evidence demonstrates the impacts of climate change on the biota of the planet, and life in the soil is no exception. From this perspective, we propose various scenarios in which the rhizosphere microbiome can be used to improve plant growth, with particular focus on agricultural crops, to alleviate the negative consequences of climate change on agricultural productivity.

PLANT–MICROBIOME BENEFICIAL INTERACTIONS AND BIOTECHNOLOGY POTENTIAL

For more than a century, the rhizosphere has been recognized as a microenvironment where microorganisms inhabit and have a great influence on the development and health of plants. The rhizosphere is influenced by the exudates excreted from the plant roots, which are secondary metabolites that can have various functions in the rhizosphere. For example, exudates such as carbohydrates, organic acids, vitamins, or amino acids may be chemo-attractants to microorganisms, which can metabolize the nutrients and allow for population growth

(Bais et al., 2006; Bertin et al., 2003). Other exudates such as phenolic derivatives, in particular flavonoids, specifically attract rhizobia bacteria, a heterogeneous group of bacteria that includes the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium,* and *Allorizhobium* (Hernández-Salmerón et al., 2013). In general, rhizobia can form symbiotic associations with legumes and fix atmospheric nitrogen to convert it into ammonium, thereby making nitrogen available for the plant (Biate et al., 2015). Moreover, some exudates are part of the plant defence system, such as phenolic compounds and terpenoids, which have efficient antibacterial and antifungal capacity. In addition, some volatile compounds emitted by the root were recently identified as part of the plant defence system (Niederbacher et al., 2015).

The rhizosphere is an ecosystem with particularly high microbial diversity and is thus a great source for discovering new taxa and genetic material with high biotechnological potential, including for agrotechnology (Handelsman 2004; Hernández-León et al., 2010; Escalante-Lozada et al., 2004). The proliferation of beneficial microorganisms, particularly bacteria in the rhizosphere, is of particular interest for generating new bioinoculants or biopromoters that do not have negative impacts on the environment or human and animal health (Santoyo et al., 2012; Owen et al., 2015). It has been proposed that plants can interact with plant growth-promoting bacteria, better known as plant growth-promoting rhizobacteria (PGPR). PGPR-relevant genera include the nitrogen-fixing rhizobia *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Erwinia*, *Serratia*, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Caulobacter*, and *Chromobacterium* (Bhattacharyya & Jha, 2012; Santoyo et al., 2012). Several studies have demonstrated the important contributions of PGPR to plants, implicating both direct and indirect mechanisms (Glick 2012), including conferring resistance to different stresses such as drought (Rolli et al., 2014), temperature (Alexandre & Oliveira, 2013), salinity (Kang et al., 2014), tolerance to heavy metals (Glick 2014), and biocontrol pathogens (Hernandez Leon et al., 2015; Martinez-Absalón et al., 2014). Therefore, the rhizosphere microbiome can significantly influence the development, health, and survival of plants in unfavourable conditions; in this way, the rhizosphere biota can be considered as a type of intrinsic mechanism of plant survival, and has thus coevolved with plants.

The importance of the composition of microorganisms in the rhizosphere is well-established, as well as their influence on the specific types of ecological functions that can be performed to benefit plants. These interactions can either have a moderate influence or be crucial and indispensible for plant survival under

particularly adverse or stressful situations. The rhizosphere microbiome may benefit the plant through various mechanisms, such as the promotion of plant growth (Lugtenberg & Kamilova, 2009), symbiosis (Gage 2004; Koch et al., 2010), provision of nutrients through nitrogen fixation (Van Rhijn & Vanderleyden 1995; Raymond et al., 2004) solubilization of phosphate (Vassilev et al., 2006), remove soil contaminants (Kuiper et al., 2004) and biocontrol of phytopathogens (Compant et al., 2005; Zhuang et al., 2007).

The rhizobial microbiome is also recognized as a potential source for the entry of bacteria into the plant roots (Marquez-Santacruz et al., 2010). Bacteria with capacity to colonize and survive within the internal tissues of a plant are collectively known as bacterial endophytes. According to Kado (1992), endophytic bacteria inhabiting the interior of diverse plant tissues should not trigger any harm to the host plant. The mechanisms by which bacterial endophytes promote plant growth are similar to those of rhizospheric bacteria, and have been classified into direct and indirect mechanisms (see Glick 2012). Some researchers have ranked the importance of bacterial endophytes beyond that of rhizospheric bacteria for plants, since they are in closest contact with the plant and may therefore be better able to exert their beneficial effects on plant health (Rashid et al., 2012). Indeed, endophytic bacteria have been considered as a plant's internal microbiome (Gaiero et al., 2013), and their potential for improving plant growth and other biotechnological applications such as phytoremediation have been widely recognized (Brader et al., 2014; Gaiero et al., 2013; Newman & Reynolds 2005).

However, research into the factors responsible for the selection and structure of the rhizosphere microbiome is still in the early phases, and thus forms the focus of this review to highlight the work carried out to date, potential of these findings, and remaining questions to be tackled. Other excellent reviews are available for the reader that describe the detailed mechanisms or potential of PGPR for reducing environmental stresses or other more general areas such as agriculture (Bhattacharyya & Jha, 2012; Glick 2014; Santoyo et al., 2012; Santoyo et al., 2015).

ABIOTIC FACTORS AFFECTING THE SOIL AND RHIZOSPHERE MICROBIOME

The soil and rhizosphere ecosystems are affected and modulated by several environmental factors. Since an ecosystem is composed of multiple interacting biological and non-biological elements, it is hard to classify them and study them as separate pieces. Indeed, in several cases, the soil or rhizosphere microbiome is affected by two or more abiotic factors, thereby complicating analysis of the specific effects of single factors (Cleveland et al., 2007; Beauregard et al., 2010; Drenovsky et al., 2004; Das et al., 2011; Fierer & Jackson 2006; Liu et al., 2000; Roesch et al., 2007; Stomeo et al., 2012; Andrew et al., 2012; Van Horn et al., 2013; Rousk et al., 2010; Castro et al., 2010). Nevertheless, we have tried to summarize these individual effects as much as possible, and have classified them according to our best criteria in the following sections. Table 1 resumes relevant works of diverse abiotic factors modulating the microbial diversity.

Structure and soil type

The soil is considered to be a complex environment, whose origin is derived from the mixture of minerals, gases, liquids, organic matter, and living organisms that sustain plant growth (Bronick & Lal 2005). The main mineral constituents of the soil are sand, silt, and clay. The specific mineral composition defines the soil type, because the mineral content will influence the porosity and soil moisture; for example, fertile soil requires a porosity of 50%, in which half the soil is occupied by gas and the other half by liquids. These interfaces open up the possibility of forming gradients of nutrients, pH, and gases, leading to innumerable microenvironments that offer distinct ecological niches. The organisms living in the soil, including bacteria, fungi, and viruses (Hättenschwiler et al., 2005), may have important ecological roles because of their participation in the cycle of nutrients through the degradation of organic matter and minerals, which in turn provides essential nutrients for plant growth (Uroz et al., 2009).

It has been argued that the physiological effects of plants should be considered to have equal importance to any other abiotic factor for the soil microbiome, because of the consequent effects on the functions of the organisms living in the soil ecosystem (Verville et al., 1998). The presence of a plant not only promotes the growth of soil microbial communities directly but also influences the abiotic properties that influence their

growth indirectly (Singh et al., 2009). Other studies have suggested that soil characteristics (Girvan et al., 2003) and the geographic factors are the most important factors in shaping the structure of the soil microbial communities; however, soil microorganisms can also have a significant effect on the formation of soil aggregates (Tisdall 1996; Bronick & Lal 2005). In particular, soil moisture content shows the strongest impact on the microbial community structure among other factors, even greater than the effect of nutrients in the soil (Singh et al., 2009). This has been documented in research conducted on soils under extreme environmental conditions such as in the tundra of the Canadian Arctic (Chu et al 2011), Antarctic soils (Yergeau et al 2007), and the Tibetan permafrost soil (Zhang et al., 2013). In an analysis of soil bacteria diversity, Zhang et al., (2013) found that *Proteobacteria* was the dominant group and was significantly associated with the amount of soil moisture. Geyer et al., (2014) investigated the association between the type of soil and bacterial diversity in Polar desert soils, and also found that moisture content was closely related to the abundance of several bacterial genera.

In one particularly interesting study, the ability of microorganisms to move according to the amount of soil moisture was analysed (Bashan et al., 1996). Mobility is important for bacteria to colonize other soil or rhizosphere spaces that are closest to the plant root exudates or nutrients. For example, the genus *Azospirillum* was found to travel a distance of 40–60 mm in 96 hours in a sandy soil with a moisture content of 16%; however, in soils with only 10% moisture, displacement was reduced to 20 mm over the same time period (Bashan et al., 1996). These findings indicated that soil moisture is directly related to an organism's ability to colonize the rhizosphere. Precipitation is closely associated with the formation of the specific type and structure of soils, especially arid or semiarid soils. In this regard, Bachar et al., (2010) studied the effect of rainfall in arid and semi-arid soils, noting that the abundance of bacteria decreased with respect to precipitation; however, bacterial diversity was independent of the precipitation gradient.

Soil aggregates have also been shown to be an important element allowing for the selection or survival of certain microbial groups; for example, the division *Acidobacteria* is often found in soil macroaggregates but not soil microaggregates (Mummey et al., 2006). Moreover, communities can also vary according to the size of the pore dwellings (Ruamps et al., 2011), which influences carbon mineralization (Ruamps et al., 2013). For example, in forest soils, species richness was shown to be modified according to the soil horizon, which

promotes an organic layer for bacteria and a mineral layer that is mostly inhabited by Archaea (Uroz et al., 2013).

Furthermore, the effectiveness of bacterial communities in promoting plant growth has been investigated in different soils. Egamberdiyeva (2007) observed that genera, including *Pseudomonas*, *Bacillus,* and *Mycobacterium*, that are more efficient in stimulating the uptake of N, P, and K in corn plants were more likely to grow in nutrient-deficient soils compared to nutrient-rich soils. Moreover, apparent changes in the bacterial communities in the rhizosphere were observed in an experiment where microbial diversity was compared in three soil types with three types of plants using independent molecular techniques such as 16S ribosomal gene sequencing and denaturing gradient gel electrophoresis (DGGE) (Marschner et al., 2004). Several factors were found to contribute to the species composition in the rhizosphere, but the plant roots, through excretion of exudates, showed a highly selective effect that was comparable to the influence of soil type (Marschner et al., 2004). Therefore, although much has been discovered about the influence of the soil type as an abiotic modulating factor of the soil microbiome, more work is needed to gain a detailed picture of the relative effect of soil type on modulating the rhizosphere microbiome. The development of novel *in vitro* methods could help to unveil these gaps in knowledge, since an open system can be hard to control for single-factor evaluations. This type of research is currently lacking.

Soil pH

The pH indicates the concentration of hydronium ions [H3O]+ present in the soil (or another system), and therefore determines the acidity or alkalinity of the soil; thus, pH is a key factor in many soil science studies. Soil pH is also regarded as one of the main elements defining the structure of microbiome communities (Andrew et al., 2012; Zhalnina et al., 2014; Lauber et al., 2009). Soil pH varies substantially from the regional to the global scale, and therefore can affect microbial communities, as soil microbes show a wide range of optimal pH tolerance. Several studies have focused on the effect of pH at different scales. For example, a continent-wide study clearly showed an association between soil pH and the presence of certain microbial communities (Fierer and Jackson, 2006; Lauber et al., 2009), demonstrating that pH was the main factor responsible for this variation. A pioneering study in biogeography of soil microbial diversity at the continental

scale was carried out by Fierer & Jackson in 2006. The authors collected 98 soil samples from North and South America, and characterized and compared the bacterial community composition using the ribosomal DNA-fingerprinting method. Their results showed that bacterial diversity was unrelated to site temperature, latitude, and other variables, and the community composition was instead largely independent of geographic distance. Interestingly, the diversity and richness of the soil bacterial communities differed by ecosystem type, which the authors mainly attributed to differences in the soil pH. Similarly, other researchers have studied the effect of soil pH at the regional level and found an association of microbial diversity and soil pH and/or pH as a key modulating factor for variation in community composition (Rousk et al., 2010).

Furthermore, pH was recently proposed to be the best predictor of microbial diversity at the phylum level (Geyer et al., 2014), which is consistent with a recent study of the presence of *Acidobacteria* along an elevational gradient (Zhang et al., 2014). *Acidobacteria* is one of the most dominant soil genera, which could reflect its metabolic plasticity. The authors selected four elevation gradients (from 1000 to 2800 m) of a mountain in central China. Interestingly, they observed a significant single-peak distribution pattern between the OTU number and elevation. Their Jaccard and Bray–Curtis index analyses showed that the *Acidobacteria* compositional similarity significantly decreased with an increase in elevation distance. Finally, the authors concluded that soil pH, soil temperature, and plant diversity may be the key factors shaping the soil *Acidobacteria* community.

Soil pH, as a measure of alkalinity or acidity, is directly related to the availability of nutrients for plants by controlling the chemical forms of the soil compounds. This has also been suggested to be an indirect limiting factor for microbial soil communities (Zhalnina et al., 2014). Neutral soils generally harbour a greater microbial or bacterial diversity, while acidic soils tend to show lower diversity indices (Fierer & Jackson 2006; Lauber et al., 2009; Rousk et al., 2010). Note that soil pH would only affect the survival of certain microbial species and is not a general factor for all species.

On the other hand, other studies have found no association between soil pH and the bacterial diversity of the ecosystem. For example, in a biogeographic study of the nitrogen-fixing rhizobacterium *Sinorhizobium meliloti* across several regions of Croatia, various abiotic factors were analysed, including the soil pH; however, only soil type and other geographical factors appeared to be responsible for shaping the genetic

diversity of the 128 isolates analysed (Donnarumma et al., 2014). Likewise, in a separate study conducted over a mountain range in China from tropical rainforests to boreal coniferous forests across various altitudes, climates, and soils, pH was not always identified as the most important factor contributing to the composition and diversity of soil microbial communities, which were associated with multiple factors (e.g., nutrient variability, temperature, altitude) that vary in each region (Singh et al., 2013; Angel et al., 2010). Therefore, based on the evidence collected to date, pH appears to be an important agent that can influence soil microbial diversity; however, this is not a generality. This highlights the importance of analysing the various abiotic factors contributing to each specific ecosystem in order to best understand the possible effects of local abiotic components and their association with soil microbiome diversity.

Soil nutrients

The effect of nutrients in the soil and their impacts on plants, as well as agricultural production has been extensively studied in various regions worldwide (Ryan & Sommer 2012). Therefore, in this section we will focus on certain studies that have become particularly relevant with respect to the impact of major soil nutrients and their direct effects on the rhizosphere microbiome.

In agricultural soils, one of the major constraints to production is an infertile soil, determined by the three main nutrients: nitrogen (N), carbon (C), and phosphorus (P) (Reich & Oleksyn, 2004; Ryan & Sommer, 2012). Other nutritional factors such as iron can also affect the abundance of the rhizosphere microbiome, particularly bacteria (Yang & Crowley, 2000). Nutrient limitation problems are usually readily solved immediately with the application of chemical fertilizers, which have negative effects for the environment and for human and animal health (Geiger et al., 2010). In addition, this is only a short-term solution to infertile soil and is not sustainable. The fertility of a soil is the product of complex biotic and abiotic interactions in which soil microorganisms play a major role in the decomposition of organic matter, generating available nutrients for plants. In turn, the improved plant growth allows for exploration of the roots for nutrient acquisition, thereby allowing the soil microbes to adhere to and inhabit the roots, and thus the rhizosphere. Therefore, soil nutrients and their bioavailability have both direct and indirect (through plants) effects on the diversity and abundance of the rhizosphere microbiome (Berendsen et al., 2012). Furthermore, an imbalance

in the proportions of nutrients in the soil can impact biodiversity in many ways through different processes that change the characteristics of terrestrial environments. Similarly, the function and land use can be affected by cultural practices that in turn affect the soil microbial community (Joergensen & Emmerling 2006).

Nitrogen enrichment is a predominant factor in some soil types that can have substantial effects on both plant productivity and the composition of bacterial communities (Clark et al., 2007; Turner et al., 1997). In some cases, experimental enrichment of nitrogen was found to result in an increase in plant productivity, but at the expense of lower plant and bacterial community species richness and diversity (Suding et al., 2005). Levels of nitrogen enrichment in these experimental studies often exceed the current rates of the atmospheric deposition of N; however, even relatively low levels of chronic nitrogen enrichment are a threat to the conservation of different environments such as grasslands (Clark & Tilman 2008).

Carbon is also one of the main determinants of the structure and function of microbial communities in the soil (Ahmed et al., 2008; Degens et al., 2000; Drenovsky et al., 2004). Degens et al., (2000) used the microbial catabolic evenness as a measure of soil microbial diversity in soils with different organic C pools; for example, total organic C, microbial biomass C, and potentially mineralizable C. Their results showed that land use was significantly associated with microbial catabolic evenness, since certain practices deplete organic C stocks in soils, which may cause declines in the catabolic diversity of soil microbial communities.

Another important soil nutrient is phosphorus, which is also a modulating factor of the rhizosphere microbiome. For example, Beauregard (2010) analysed the effect of P application for eight years on the soil microbial diversity of alfalfa monocultures, using the microbial diversity profile of phospholipid fatty acids and DGGE. Their results showed that application of P modified the structure of the communities of fungi and bacteria, but did not influence species richness. Coolon et al., (2013) reported that anthropogenic activities such as burning of grasslands could increase the availability of nutrients such as N or P, and analysed the effects of N and P enrichment in grasslands in North America on the structure of bacterial populations. To detect changes in diversity, they sequenced the V3 region of the 16S ribosomal gene. Their results showed that the enrichment of soil N, but P, significantly altered the bacterial community diversity, structure, and abundance of individual taxa. This study has important implications for the management of rangeland

ecosystems and further highlighted the modulating effects of N and P as significant nutrient factors in natural ecosystems.

Geographical factors: Altitude, Latitude, and Longitude

There have been a breadth of studies conducted on the influence of geographical factors such as altitude (defined as the height of a point on the Earth's surface above sea level), latitude (the angular distance between the equator and a given point on Earth), and longitude (angular distance between a given point and the Greenwich meridian or prime meridian, which is measured from 0° to 180° East or West), on species distributions and diversity; however, research on their influence on microorganisms is extremely scarce to non-existent. Recently, Van Horn et al., (2013) published a paper on the influence of certain abiotic factors (pH, sulphates, organic matter) that control the biodiversity of soils in Antarctica. The authors found that in sites rich in organic carbon, with a low elevation, members of the phyla *Actinobacteria* and *Acidobacteria* were frequent, whereas *Firmicutes* and *Proteobacteria* were dominant at sites of high elevation and low humidity. Of note, the microbial parameters were significantly related to soil water content and soil characteristics, including soil pH, organic matter, and sulphates. However, the magnitude and even the direction of these relationships varied among basins, and application of models showed evidence of significant contextual effects at the local and regional levels. The authors thus concluded that their study demonstrated the importance of geographical scale sampling to determine the specific geographical elements controlling the characteristics of the soil microbial community.

In this sense, it is widely accepted that microbial diversity is affected by multiple factors, since both abiotic and biotic elements form pieces of the same puzzle (i.e., the ecosystem). For example, some studies have shown a pattern of decline, both with respect to the richness and diversity of bacteria along an elevation gradient; i.e., lower species richness and diversity is observed at higher elevations (Wang et al., 2014; Kerkhoff et al., 2008). However, these studies also suggest that altitude, longitude, and latitude are not acting as unique modulating factors, but rather it is the combination of different abiotic elements such as atmospheric pressure, temperature, solar radiation, and the ultraviolet-B (UV-B) radiation fraction, that is responsible for this biogeographical phenomenon. However, this effect cannot be generalized for

microorganisms. As one example, Fierer & Jackson (2006) provided evidence that bacterial diversity cannot be clearly defined in terms of latitude in analysis throughout the American continent; they concluded that although there was a trend of diversity change along latitude, this was not a significant determining factor of bacterial biodiversity. Other studies showed that the soil bacterial diversity in the Arctic was not fundamentally different from that found in other biomes (Chu et al., 2010). Therefore, more large-scale studies on this topic are clearly needed to clarify these relationships.

GLOBAL CLIMATE CHANGE: EFFECTS OF INCREASED UV RADIATION, CO_2, AND TEMPERATURE

Climate change is defined as a dramatic and constant change in the global climate. This drastic and fast change in climate could result in an increase in temperature, causing frequent droughts and atypical rains in several regions of the planet. Although the causes may be different, there is clear evidence that climate change affects life on earth and its biological processes. Heterotrophic microorganisms are not protected from these consequences, since they are part of biogeochemical processes such as C and N cycles.

Furthermore, climate change has great impacts on plant biology, which can lead to consequent changes in the associated rhizosphere microbiome. The interaction between the root exudates and rhizosphere microbiome is the result of a long co-evolutionary process (Badri & Vivanco 2009). Recently, different chemical compositions of root exudates from *Arabidopsis* were found to be selected by various specific microbial communities (Badri et al., 2013) that respond to environmental changes. Therefore, temporal changes in root exudates appear to show great potential to affect the microbial community in response to climate change.

Bardgett et al., (2013) documented the effect of elevated concentrations of CO_2 generated through root exudates of the plant on modulating the structure and function of the rhizospheric soil microbial community. Likewise, the presence and expression of functional genes in soil microbial communities have been shown to change in response to climate change disturbances. For example, bacteria with spore-forming ability are expected to predominant and withstand adverse conditions such as a drought season better than others; this mechanism allows these bacteria to survive for long periods of stress, but is not generally applicable for all

species such as gram-negative bacteria (Drenovsky et al., 2004), which hypothetically would not be able to survive as well in the face of drastic environmental changes over either the short or long term.

UV radiation (UVR)

Approximately 3.2% of the total solar energy is in the UV range from 290 to 320 nm (Cutchis 1974). Photons of these wavelengths are sufficient to cause direct DNA damage, thereby exerting damage to organisms with high or prolonged UV exposure (Sundin & Jacobs 1999). The main damage includes thymine and citocine dimer formation and single- or double-stranded breaks, leading to mutations or loss of genetic information generated during the DNA repair processes, ultimately affecting cell viability (Santoyo & Romero 2005).

The depletion of the stratospheric ozone layer, in part due to the accumulation of chlorofluorocarbons and accumulation of CO_2, has allowed for an increase in the flow of solar UV-B, in the range of 280–320 nm (Müller et al., 1997). These effects are stronger in the Polar regions (Caldwell et al., 1982) where the ozone layer has thinned, and ecosystems are particularly sensitive to disturbance (Callaghan et al., 1995). Polar plant communities highly depend on the nutrient cycling carried out by soil microorganisms. In this sense, UVR is an abiotic factor that has direct effects on soil microorganisms (Formánek et al., 2014). UV-B radiation has direct effects on soil microorganisms, including a change in pigment content, growth, and induction of carbon assimilation in amino acid synthesis (Sinha et al., 1999).

The rhizosphere is a habitat primarily influenced by the carbon sources and nutrients released by plants; in response to stress, plants can alter metabolism of the roots, which can have clear consequences for the selection of different bacterial communities. This is because the rhizosphere microbial composition is largely influenced by plants, and therefore the composition of root exudates may serve as the main energy and carbon sources for fungi and bacteria, which have different requirements (Dohrmann & Tebbe 2005).

The phyllosphere (plant surface) community is relatively more exposed to the effects of UVR (and other environmental factors) compared to the soil or rhizospheric community. The phyllosphere microbiome is therefore dominated by only a few taxa compared to communities in the root zone. This observed reduction of diversity in the phyllosphere community is attributed to the drastic and more frequent changes in the

environment of the phyllosphere such as temperature, relative humidity, and solar radiation (Dohrmann & Tebbe 2005; Lynch 1990); thus, selection for tolerant taxa is stronger at this level.

Despite the fact that soil microbial communities are generally more abundant than phyllosphere communities, they are also more sensitive to environmental factors such as elevated levels of UV-B (Johnson et al., 2002); however, some species, including those that are part of the rhizosphere microbiome, differ in their sensitivity to UV-B radiation-induced damage (Arrage et al., 1993). There appear to be diverse mechanisms for UVR tolerance in bacterial species. For example, UVR tolerance in the plant pathogenic bacteria *Pseudomonas syringae* is conferred by the plasmid encoding the *rulAB* operon involved in DNA repair (Carzola et al., 2008). The production of an extracellular polysaccharide that can absorb UVR has been implicated in conferring UVR tolerance *Xanthomonas campestris* (Hugenholtz et al., 1998). The pigmentation of phyllospheric bacteria is another reported UV protection mechanism, specifically for exposure to UV-A radiation (320–400 nm). Most isolates of the bacterial phyllosphere community have been shown to produce pigments, which suggests that UVR protection is conferred by pigments important for survival in the phyllosphere. Indeed, the carotenoid compounds produced by *Erwinia herbicola* have been shown to play an important role in cellular protection against UV-A radiation (Whipps et al., 2008).

Non-motile gram-positive bacteria isolated from Antarctic soil were found to be tolerant to UVR owing to a melanin synthesis-protective mechanism (Bhattacharyya et al., 2012). The domain Archaea is also resistant to UV-B (Thummes et al., 2007), and there is a general trend of increased species diversity in environments with higher levels of UV-B exposure (Robson et al., 2005). The gram-negative bacterial community in Arctic soils experiences particular environmental stresses and nutrient limitations (Rinnan et al., 2005). Avery et al., (2003) conducted a study of the response of the rhizosphere microbial communities associated with populations of *Deschampsia antarctica* (a native vascular plant from Antarctica), which were exposed to UV-B, and found that radiation not only modified the overall growth of the plant but also the production of secondary metabolites. Furthermore, alterations in root metabolism may have an influence on the selection of different bacterial communities. Sundin & Jacobs (1999) found that most of the bacterial strains identified in the phyllosphere in *Arachis hypogaea* were gram-positive, with the genus *Bacillus* dominant, and showed

good tolerance to UV radiation based on comparison to two control species, *Pseudomonas aeruginosa* and *Pseudomonas syringae*, which are UV-sensitive and -tolerant, respectively.

While light does not penetrate easily into the soil, particularly at greater depths; however, UV-B radiation induces changes in soil microbial communities and biomass, as well as alters the populations of small invertebrates, and these changes have important implications for the soil nutrient cycle (Caldwell et al., 2007) given that rhizosphere microorganisms are needed to provide nutrients for plants and that microbial growth in turn depends on the plant root exudates. In summary, UVR exposure reduces the biomass of the roots resulting in less colonization of the microbial community, which in turn leads to low amounts of nutrients in the soil; thus, UVR has potential to disturb the soil community.

CO_2

Atmospheric CO_2 enrichment produces severe effects on terrestrial ecosystems and also interacts with the carbon cycle below ground. The main cause of these effects is the change in organic carbon dynamics. Previous studies have demonstrated reductions in pasture microbial decomposition rates after exposure to high concentrations of CO_2 (Hu et al., 2001; Van Ginkel et al., 2000). Hu et al., (2001) also suggested that a high level of CO_2 would result in decreased amounts of available N for microorganisms due to the consequent improvement of plant growth, thus reducing the degradation ability of the microorganisms. These effects of elevated atmospheric CO_2 concentrations on the dynamics of soil organic matter lead to indirect effects on the soil structure. The response of plants to elevated atmospheric CO_2 concentration has been well studied across diverse systems. Atmospheric CO_2 is closely associated with C availability in the soil; therefore, the detailed effects of this abiotic factor on microbial soil communities are the same as those discussed above in the section soil nutrients.

Temperature

Global warming decreases the moisture content in the soil, which limits the ability of microbial organisms to disperse, survive, and colonize soil spaces (Carson et al., 2010). Similarly, an increase in ambient temperature results in heating of the soil, which can modify the structure of the rhizosphere microbiome that established from interactions with a plant. Zogg et al., (1997) studied this effect *in vitro* by analysing the microbial communities through bacterial phospholipid profiles in soils subjected to prolonged changes in temperature from 5 to 25°C. Interestingly, both the kinetics of microbial respiration and the community structure varied across this wide range of temperatures. The authors suggested that changes in the composition of the microbial community following seasonal variations in soil temperature or smaller annual increases associated with global climate change have the potential to alter the decomposition of organic matter, which would in turn affect the bioavailability of carbon.

Mosier et al., (2015) recently investigated the effect of high temperatures on the expression of proteins using tandem mass tag technology-based proteomics in a microbial community located in an acidic mine drainage. Remarkably, a very clear correlation was observed between the different temperatures and the expression profile of proteins; in particular, those proteins involved in carbon use were repressed in two genotypes of *Leptospirillum*. Although this study was not conducted in a rhizosphere microbial community, it nonetheless demonstrates the importance of analyzing the effect of an abiotic factor such as temperature on community composition. Furthermore, this study indicates the value of adopting proteomic techniques to evaluate the expression pattern of proteins in other microbial communities with respect to the other main environmental parameters described herein.

SUMMARY AND PERSPECTIVES: TOWARD SUSTAINABLE AGRICULTURE

The rhizosphere microbiome consists largely of bacteria that benefit plant growth, which are better known as PGPR. Multiple mechanisms of plant growth promotion have been proposed based on PGPR, whether direct or indirect, including the production of siderophores, phytohormones, volatile compounds, or 1-aminocyclopropane-1-carboxylate deaminase, in addition to their capabilities of biocontrol and antagonism to

plant pathogens (Santoyo et al., 2015). Therefore, the potential of PGPR to the benefit of agricultural production has been amply demonstrated, but has not been sufficiently exploited and implemented, particularly in developing countries. Currently, pesticides, nitrogen fertilizers, and other chemicals are being overused and abused to enhance agricultural production. The long-term effect of fertilizers can produce positive effects on microbial soil and rhizosphere populations (Shi et al., 2010), while other drastically opposite to their use in agriculture (Compant et al., 2005). As the human population continues to grow, so will the demand for food. Currently, there are more than 7000 million people inhabiting our planet, and it is expected that the demand for food will double by 2050 (Tilman et al., 2002).

It is therefore essential to understand the abiotic and biotic interactions to best exploit the rhizosphere microbiome to benefit agricultural production. Likewise, understanding of the continuous environmental changes and impacts of anthropogenic effects on the environment and climate must be taken into account for future agricultural practices.

Some authors have proposed the need to manipulate the rhizosphere by generating transgenic plants that modulate signalling between plant root exudates and the rhizosphere microorganisms (Ryan et al., 2009; Chaparro et al., 2014), since the roots exudates are important to attract certain microbial species in the rhizosphere. However, this approach would require the release of a genetically modified organism, which is currently restricted by law in several countries. Another interesting option would be the generation of a microbiome-based bioinoculant, perhaps by selecting dozens or hundreds of bacterial and/or fungal species with proven plant growth-promoting and biocontrol activities. This bioinoculant could be employed by direct application to agriculture crops (such as other bioinoculants based on single or a few mixed species that are already in use and commercialized), in order to improve soil fertility and, consequently, crop production. Such an approach would avoid the use of chemicals that harm the environment and human and animal health, or genetically modified organisms, which are under a high level of scrutiny by some sectors of society and are thus tightly regulated. Finally, it is imperative to take the next steps as soon as possible to avoid food shortages, especially in the face of a changing climate. Toward this end, we need to take full advantage of the knowledge generated thus far and continue studying the abiotic factors affecting agriculture in order to effectively predict the consequences of environmental changes on productivity. In particular, the beneficial

interactions between plants and the rhizosphere microbiome should be further exploited by conducting basic research with the aim of achieving more sustainable global agriculture.

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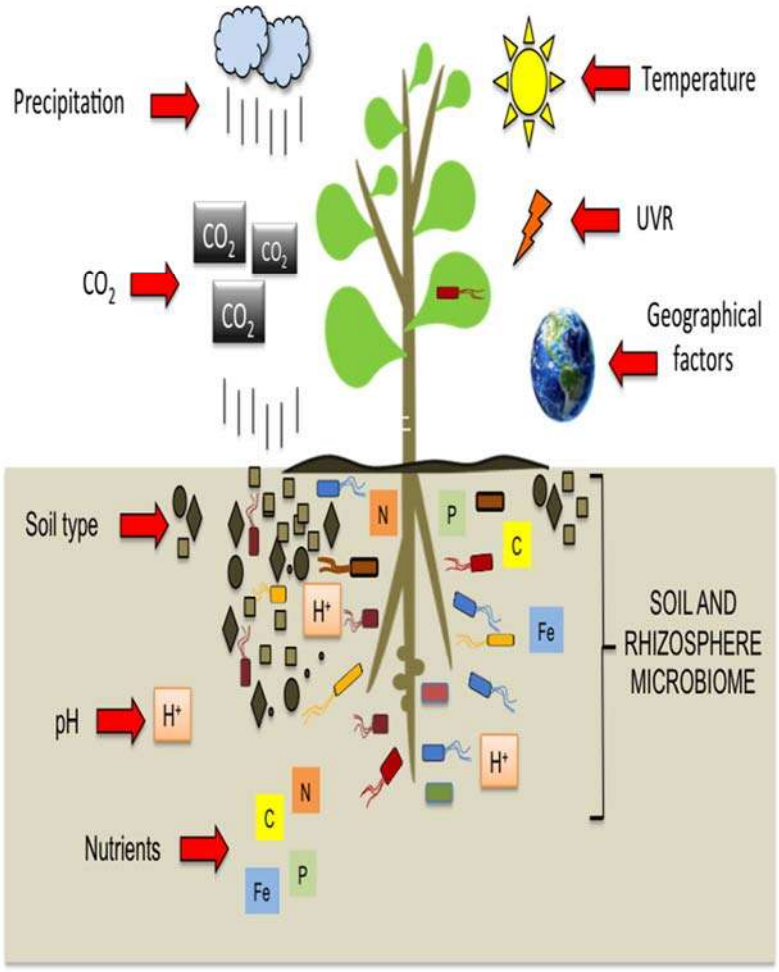

Figure legend

Figure 1. Summary of abiotic factors (rain precipitation, CO_2, soil type, pH, nutrients, temperature and ultraviolet radiation) directly affecting the bulk and rhizosphere microbiome and indirectly acting on plant growth and health. Abiotic factors such as rain precipitation, ultraviolet radiation and temperature might also affect microorganisms residing the phyllosphere (Superficial area of tissues like leafs). See text for details.

Table 1. Relevant works on the role of some abiotic factors modulating the soil and rhizosphere microbiome.

Abiotic modulating factor	Plant rhizosphere/type of soil	Microbiome profiling tecnique	Modulated or found Taxa, general features	Place of study	Reference
K, C, Ca	McMurdo Dry Valleys soils	Denaturing Gradient Gel Electrophoresis (DGGE), Terminal Restriction Fragment Length Polymorphism (T-RFLP) and 16S rRNA gene clone library construction	*Proteobacteria*, *Actinobacteria* and *Firmicutes* were dominant in all horizons. *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Gammaproteobacteria* were mainly found in permafrost interface	Antarctica	Stomeo et al. 2012
pH, C	Cacti rhizosphere (*Carnegiea gigantea* and *Pachycereus pringlei*) and bulk soil	Multiplexed pyrosequencing of the16S rRNA genes	Family *Desulfurococcaceae* was correlated with carbon and several classes of the phylum *Acidobacteria* with pH	Sonoran desert, Arizona	Andrew et al. 2012
Soil water content, C	Rice and tomato/ Yolo silt loam soil	Phospholipid Fatty Acid (PLFA) profiles	Species not detected. Soil water content and organic carbon availability are major determinants of the general microbial community composition	California	Drenovsky et al. 2004
DOM (Dissolved organic matter)	Tropical rain forest soil	Libraries of small-subunit ribosomal RNA genes (SSU rRNA)	*Gammaproteobacteria* and *Firmicutes* groups were increased while *Acidobacteria* were reduced	Costa Rica	Clevelandet al. 2007
Agricultural practices disturbances (Intense grazing, seasonal drought and fire)	Desert grassland	Carbon substrate utilization patterns in Biolog plates. Soil enzyme activity.	Species not specified. Fire and summer drought reduced soil microbial substrate utilization and enzyme activities. Winter drought, increased soil microbial diversity and activity.	Chihuahua	Liu et al. 2000
Temperature	Acid mine drainage (AMD) biofilms	FISH and Tandem Mass Tag (TMT)-based proteomics	*Leptospirillum* group III decreased with increasing temperature	Richmond Mine, California	Mosier et al. 2014
Temperature, atmospheric CO_2 and precipitation	Captina silt loam soil	Ribosomal DNA quantitative PCR (qPCR)	The relative abundance of *Proteobacteria* was greater in the wet soil. *Acidobacteria* abundance was greater in dry treatments. Fungal abundance increased in	National Ecological Research Park, Oak Ridge, Tennessee	Castro et al. 2010

			warm treatments		
Type of soil	Maize, sugarcane and Morrow Plots /three agricultural and boreal forest soils	DNA pyrosequencing	The most abundant bacterial groups in all four soils were the *Bacteroidetes*, *Betaproteobacteria* and *Alphaproteobacteria*. Forest soil is a rich phylum but less diverse of Archaeal species compared to the three agricultural soils	Brazil, Florida, Illinois and Canada	Roesch et al. 2007
pH	Typic Paleudalf soil	qPCR and bar-coded pyrosequencing	Relative abundance and diversity of bacteria were positively related to pH. The abundance of fungi was unaffected or weakly modulated by pH	Hoosfield Acid strip (Rothamsted Research, UK)	Rousk et al. 2010
Soil moisture, pH, electrical conductivity, soil organic matter, major nutrients and ions.	McMurdo Dry Valleys soils	Pyrosequencing of the 16S rRNA gene	*Acidobacteria* and *Actinobacteria* were prevalent at the organic carbon rich, mesic and low elevation sites, while *Firmicutes* and *Proteobacteria* were dominant at the high elevation, low moisture and biomass sites	Taylor and Wright Valleys (Antarctica)	Van Horn et al. 2013
pH	Multiple soil types	T-RFLP	Bacterial diversity was higher in neutral soils and lower in acidic soils, higly correlated with soil pH	North and South America	Fierer and Jackson 2006
Moisture,	Herbaceous species and pasture/Grassland	T-RFLP	Moisture had a comparatively higher impact on bacterial community, on fungal community soil N and C had a stronger effect	*Scotland,* UK	Singh et al. 2009
Phosphorus fertilization	Alfalfa/ loamy clay soil	DGGE and PLFA	The application of fertilizer was associated with shifts in the composition of fungal and bacterial communities without affecting their richness	Saskatchewan, Canada	Beaure gard et al. 2010
CO_2 and temperature	Rice/ tropical soil	Measurement of microbial biomass-C and soil enzyme activities	Elevated CO_2 significantly increased the mean microbial biomass carbon (MBC) content and soil enzyme activities and temperature	India	Das et al. 2011

Artículos de divulgación.

10.4 Diversidad de bacterias endófitas en raíces de tomate (*Physalis ixocarpa*) mediante el análisis de genes de la subunidad 16S de ARN ribosomal

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Diversidad de bacterias endófitas en raíces de tomate (*Physalis ixocarpa*) mediante el análisis de genes de la subunidad 16S de ARN ribosomal

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Resumen

La diversidad de bacterias endófitas fue estimada en raíces de plantas de tomate de cáscara (*Physalis ixocarpa*) por medio de la amplificación y secuenciación de genes ribosomales 16S. Se identificaron 16 unidades taxonómicas operacionales (OTUs) en una biblioteca de clonas mediante el análisis tipo Blast, incluyendo las clases Gammaproteobacteria, Betaproteobacteria, Actinobacteria, Bacilli y bacterias no cultivables. Los cinco géneros predominantes fueron *Stenotrophomonas*, *Microbacterium*, *Burkholderia*, *Bacillus* y *Pseudomonas*. Nuestros resultados sugieren que la diversidad bacteriana endófita dentro de las raíces de plantas de tomate pertenecen a géneros bacterianos asociados a la rizósfera y con gran potencial para llevar a cabo funciones de promoción del crecimiento vegetal.

Palabras clave: Endófitos, diversidad bacteriana, *Physalis ixocarpa*, rRNA 16S.

Abstract

The endophytic bacterial diversity was estimated in Mexican husk tomato plant roots by sequence homology analysis of the 16S rDNA genes. 16 OTUs (Operational Taxonomic Units) from the 16S rDNA root library were identified based on sequence analysis, including the classes Gammaproteobacteria, Betaproteobacteria, Actinobacteria, Bacilli and uncultured bacteria. The five predominant genera were *Stenotrophomonas*, *Microbacterium*, *Burkholderia*, *Bacillus* and *Pseudomonas*. Our results suggest that endophytic bacterial diversity in roots of tomato plants belong to bacterial genera associated to the rizhosphere with great potential to carry out the functions of plant growth promotion.

Keywords: Endophytes, bacterial diversity, *Physalis ixocarpa*, rRNA 16S.

Introducción

Existen diversas relaciones entre los microorganismos y las plantas. Puede haber asociaciones dañinas, neutrales y benéficas. Entre las asociaciones benéficas se pueden presentar aquellas donde las bacterias pueden colonizar el tejido interno de la planta. Es en este microambiente donde se da una estrecha relación bacteria-planta. Las bacterias endófitas pueden ser definidas como aquellas bacterias que colonizan los tejidos internos de la planta y no muestran signos externos de infección o efectos negativos en su hospedante (Schulz y Boyle 2006).

Las primeras evidencias de asociación entre microorganismos endófitos y plantas se originaron de observaciones en tejidos y hojas fosilizadas, lo que soporta la inferencia de que la asociación planta-endófito pudo haber ocurrido junto con la aparición de las primeras plantas en la tierra (Strobel 2004). El beneficio de las interacciones planta-bacteria que promueven la salud y desarrollo de la planta han sido objeto de estudio desde hace décadas. Algunos trabajos recientes también investigan su potencial para el mejoramiento de la biodegradación de contaminantes del suelo, síntesis de compuestos antibacterianos o antifúngicos, así como la producción de metabolitos o compuestos que promuevan el crecimiento vegetal (Rijavec et al., 2007; Berg et al., 2005; Brooks et al., 1994; Tan et al., 2006).

Distintos estudios han mostrado que la diversidad bacteriana endófita de plantas puede ser una subpoblación de los habitantes de la rizósfera (Germida et al., 1998). Así mismo, la gran mayoría de los estudios poblacionales bacterianos y de otros grupos se han enfocado en la rizósfera (Lindow y Brandl 2003; Kuiper et al., 2004; Berg et al., 2005). Se ha propuesto que de las aproximadamente 300,000 especies de plantas que existen en la tierra, cada planta individual es hospedera de diversas bacterias endófitas (Strobel et al., 2004).

Los endófitos bacterianos de plantas han sido estudiados en plantas de maíz, frijol, plátano, uva, trigo y papa, entre otras (Rosenblueth y Martínez-Romero 2006). La diversidad que se ha reportado depende en gran parte del tipo de especie vegetal que se ha analizado. Por ejemplo, se han reportado especies de las bacterias *Stenotrophomonas maltophilia* y

Microbacterium sp. en raíces de plantas de arroz, pastos y algodón (Sun 2008; Dalton et al., 2004; McInroy y Kloepper 1995). Así mismo, estos géneros han sido ampliamente estudiados por promover el crecimiento vegetal. Especies de otros géneros como *Pantoea* y *Cellulomonas* han sido aisladas y reportadas como endófitas de plantas de arroz, fríjol soya, uva y maíz (Elvira-Recuenco y Van Vurde 2000; Bulgari et al., 2009). Las especies de estos géneros han mostrado su capacidad para degradar compuestos tóxicos, así como se ha mostrado que especies del género *Pantoea* pueden inhibir el crecimiento de patógenos de plantas (Zinniel et al., 2002).

En el presente estudio, se realizó una caracterización de la comunidad endófita bacteriana que se encuentra en las raíces de plantas de tomate verde o de cáscara (*Physalis ixocarpa*) empleando la secuencia de genes que codifican para la subunidad 16S de ARN ribosomal (ARNr).

Materiales y Métodos

Muestreo de plantas y análisis físico-químico del suelo

Se colectaron diez plantas de *P. ixocarpa* Brot. de dos meses de edad y el suelo rizosférico de cada una de ellas en un campo agrícola en Salvatierra, Guanajuato, México (20°12'54"N,100°52'41"O, Altitud 1759 msnm). Las muestras fueron transportadas inmediatamente al laboratorio para su análisis. Las características físico-químicas del suelo se analizaron en el Laboratorio de Fertilidad de Suelos y Nutrición Vegetal en el INIFAP-México, y fueron las siguientes: textura franco-arcillosa, pH 7.9, el contenido de materia orgánica fue de 2.66%, 30.52% de arcilla, 12.1 ppm de N inorgánico.

Esterilización superficial de las raíces y extracción de ADN total

Una vez que se eliminaron las partículas del suelo rizosférico, las raíces se lavaron con agua destilada estéril. Las raíces se sumergieron en etanol al 70% durante 3 min, se lavaron con solución de hipoclorito de sodio fresco durante 5 min, se enjuagaron con etanol al 70% durante 30 segundos y finalmente se lavaron cinco veces con agua destilada estéril. Para confirmar que el proceso de esterilización de la superficie radicular se realizó correctamente, se inocularon alícuotas de 100 µL del agua destilada estéril utilizada en el enjuague final en caldo de cultivo Luria-Bertani (LB) y placas de agar nutritivo (AN). Las placas se examinaron en busca de crecimiento bacteriano después de incubación a 28°C durante 5 días, sin observarse colonias bacterianas. Las raíces de diez plantas (10 g en total de tejido vegetal) con superficie esterilizada se utilizaron para el aislamiento de ADN mediante el método descrito por Xie y colaboradores (1999).

Amplificación por reacción en cadena de la polimerasa de los genes 16S bacterianos

Los genes de la subunidad 16S de ARNr fueron amplificados utilizando los cebadores universales bacterianos FD1, 5'-CAGAGTTTGATCCTGGCTCAG-3' y Rd1, 5'-AAGGAGGTGATCCAGCC-3', correspondientes a las posiciones 8 a 28 y 1526 a 1542 del gen 16S de *Escherichia coli*, respectivamente (Weisburg et al., 1991). Se utilizaron las siguientes condiciones de PCR: una desnaturalización inicial a 95 °C durante 3 min, 30 ciclos de 1 min a 95° C para la desnaturalización, 1 min a 53 °C para el alineamiento y 2 min a 72 °C para la extensión; y un paso de extensión final a 72° C durante 5 min. Los productos de PCR se observaron en un gel de agarosa al 1% y se purificaron mediante el kit Wizard® SV Gel and PCR Clean-Up System (Promega, USA) de acuerdo al proveedor. Los fragmentos de PCR purificados fueron clonados en el vector pGEM-T Easy (Promega) y los productos de la ligación se utilizaron para transformar células de *E. coli* DH5 □ electrocompetentes para generar la biblioteca de genes ribosomales 16S. Se detectaron 146 clonas positivas en medio LB adicionado con ampicilina (100 µg/ml) con 80 mg/ml de X-Gal. El análisis de restricción de los plásmidos recombinantes del total de clonas se realizó con la enzima *Eco*RI para detectar los insertos. Una vez detectados los insertos en clonas positivas, se purificaron con un kit comercial (Promega, USA) y se secuenciaron mediante el uso de cebadores del vector (M13 "forward" y "reverse") en el Laboratorio Nacional de Genómica para la Diversidad, Irapuato Gto.

Análisis de las secuencias de los genes 16S ribosomales

La posibilidad de obtener secuencias quiméricas se analizó utilizando el programa CHIMERA_CHECK de la página web del Ribosomal Database Project (Maidak et al., 1999). Las secuencias consideradas como quimeras fueron depuradas y comparadas con la base de datos GenBank (NCBI) utilizando el programa BLASTN, para obtener las mejores identidades. Dichas secuencias fueron depositadas en el GenBank con los números de acceso HM216894-HM216910.

Resultados y Discusión

En este trabajo se analizó la diversidad bacteriana endófita en las raíces de plantas de tomate de cáscara mediante la amplificación y secuenciación de genes que codifican para la subunidad 16S del ARNr, con longitudes que varían entre 560 y 1450 pb. Este análisis sugiere que la biblioteca de raíces de tomate de cáscara (la cual consta de más de 140 clonas) contiene 16 OTUs, que incluye las clases Gammaproteobacteria, Betaproteobacteria, Actinobacteria, Bacilli y bacterias no cultivables (Figura 1). Los géneros predominantes fueron *Stenotrophomonas* y *Microbacterium*, ya que 20 clonas mostraron identidad con cada uno de dichos géneros, en particular con las especies *S. maltophilia y M. foliorum*. Otro género abundante fue *Pseudomonas* con 16 clonas, mientras que en el caso de *Burkholderia cepa-*

cia se encontró identidad en 14 clonas, *Bacillus licheniformis* con 9 y *Bacillus subtilis* con 8. Otras secuencias mostraron identidad con bacterias de los géneros *Pantoea*, *Xanthomonas*, *Cellulomonas* y bacterias no cultivables (Figura 2). Cabe destacar que los porcentajes de identidad fueron mayores al 98% en todas las secuencias encontradas.

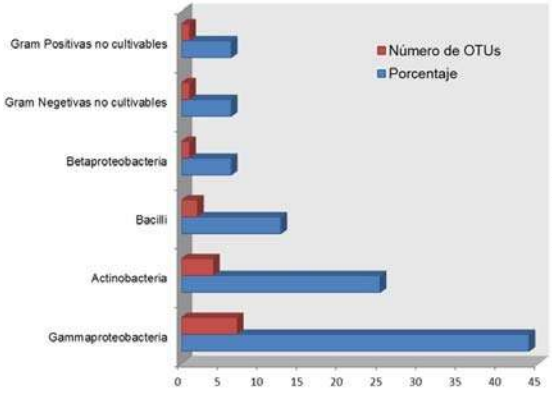

Figura 1. Diversidad de OTUs (unidades taxonómicas operacionales) y clases bacterianas encontrados en una biblioteca de genes ribosomales 16S aislados de raíces de plantas de tomate verde *Physalis ixocarpa*.

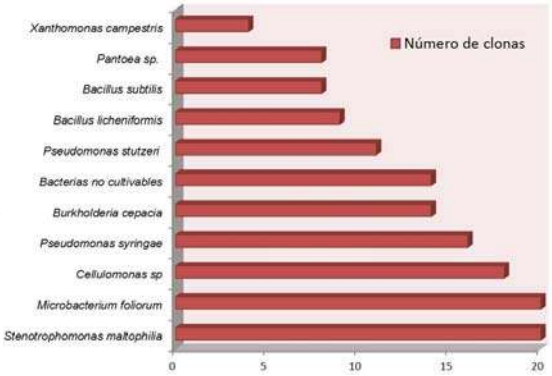

Figura 2. Diversidad de genes ribosomales 16S que corresponden a diferentes especies de bacterias endófitas en raíces de *Physalis ixocarpa*.

Nuestros resultados sugieren que el grupo más dominante está afiliado a las Gammaproteobacteria, lo cual es consistente con otros estudios (Chelius y Triplett 2001; Kaiser et al., 2001; Sun et al., 2008). Esta clase incluye siete de dieciséis OTUs encontrados en la población analizada, lo que representa casi el 45%. 41 clonas mostraron alta identidad con las especies *Stenotrophomonas* sp. y *S. malthophilia*. Las especies de *Stenotrophomonas* se han aislado o detectado como endófitos de las raíces del arroz (Dalton et al., 2004; Sun et al., 2008) y plantas de algodón (McInroy y Kloepper 1995). Algunos estudios reportan especies de *Stenotrophomonas* como promotoras del crecimiento vegetal, y que pueden suprimir el desarrollo de enfermedades por la secreción de algunos compuestos, tales como el antibiótico maltofilina (Jakovi et al., 1996). El segundo grupo más representado fue *Microbacterium* sp., que se ha reportado en asociación endófita con diferentes plantas y semillas de maíz (Zinniel et al., 2002; Conn y Franco, 2004; Rijavec et al., 2007). Por ejemplo, Conn y Franco (2004) reportaron varias especies de *Microbacterium* en un análisis de las poblaciones endófitas en las raíces de trigo (*Triticum aestivum* L.), siendo el género predominante en ese estudio.

Otras secuencias de ADNr 16S en nuestra biblioteca mostraron alta identidad con bacterias de los géneros *Pseudomonas*, *Burkholderia* y *Bacillus* (98-100%). Tales géneros se han estudiado ampliamente debido a su gama de productos metabólicos secundarios incluyendo antibióticos, compuestos orgánicos volátiles, antifúngicos, antivirales e insecticidas entre otros compuestos (Lodewyckx et al., 2001; Ryan et al., 2008). También se detectó la presencia de nueve clonas que tenían identidad con *Xanthomonas campestris*, un patógeno de diversas plantas (Bashan, 1982; Guevara y Maselli, 2005). Bashan y colaboradores en 1982 mostraron la supervivencia de *X. campestris* pv. *vesicatoria* en las semillas y raíces de plantas de chile sin mostrar síntomas de alguna enfermedad, aunque no se descarta la aparición de la enfermedad en algunas otras etapas de crecimiento. En este trabajo no se observó ningún síntoma de enfermedad en las plantas de tomate en el momento de la colecta. De acuerdo con diversos estudios, las poblaciones de endófitos pueden ser afectadas por factores biológicos o ambientales, tales como la edad de la planta, el tipo de tejido y el tiempo de muestreo (Siciliano et al., 1998; Araujo et al., 2001; Adams y Kloepper, 2002). El análisis de las funciones o actividades patogénicas en cepas de *X. campestris* endofíticas podría ser un interesante tema de investigación.

Pantoea y *Cellulomonas* son otros dos géneros que se encuentran en nuestra biblioteca como endófitos de las plantas de tomate. *Pantoea* es un residente endofítico de diferentes plantas, como el arroz, soya, uva y maíz (Elvira-Recuenco y van Vuurde 2000; Bulgari et al., 2009). Curiosamente, la cepa TR-5 de *P. ananatis,* que fue aislada a partir de semillas de maíz, inhibe *in vitro* el crecimiento del hongo *Lecanicillium aphanocladii* (Bulgari et al., 2009). Zinniel y colaboradores (2002) aislaron especies de *Cellulomonas* de tejidos vegetales y mostraron una adecuada capacidad de colonización y sobrevivencia.

Finalmente, nuestros resultados muestran que dentro de las raíces de plantas de tomate, *P. ixocarpa*, residen bacterias endófitas que podrían tener un gran potencial para el control biológico de enfermedades vegetales, así como realizar funciones que promuevan el

crecimiento y la salud de las plantas. Actualmente estamos llevando a cabo el aislamiento de estas bacterias cultivables para detectar la síntesis de fitohormonas y antibióticos en plantas de tomate verde.

Agradecimientos

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10.5 La guerra de los mundos

Cambio
DE MICHOACÁN

«Como cada eslabón lucha por su supervivencia, evidentemente surgen enemigos naturales que provocan un control inherente de poblaciones...»

La guerra
de los mundos

Rocío Hernández León

«Con un billón de muertes ha adquirido el hombre su derecho a vivir en la Tierra y nadie puede disputárselo [...], pues no en vano viven y mueren los hombres».
Herbert George Wells

En una cadena alimenticia cada individuo obtiene la energía necesaria para vivir de otro ser vivo. Como cada eslabón lucha por su supervivencia, evidentemente surgen enemigos naturales que provocan un control inherente de poblaciones. Este mecanismo biológico es un método que regula plagas y se basa precisamente en las enemistades naturales que existen entre los organismos.

Existen diferentes tipos de control. Por ejemplo, si una plaga tiene un enemigo natural pero éste no cuenta con una población suficiente para controlarla, se puede recurrir al aumento de los individuos enemigos. Si dicho enemigo estuviese en peligro de extinción, los esfuerzos se enfocarían en mantenerlo para que pudiera competir con la plaga.

Los más temerarios han propuesto introducir enemigos no nacidos en el medio, pero enemigos al fin, a un hábitat totalmente diferente al suyo donde una plaga esté generando dificultades. Sin embargo, existen algunos problemas con este tipo de enfoques. En Estados Unidos se trató de controlar el *cardoso nativo*, una maleza muy agresiva que ponía en riesgo grandes extensiones de tierras de cultivo, con la introducción del *picudo negro* (*Larinus planus*), un gorgojo externo que se alimenta de este tipo de plantas.

Los investigadores encontraron que el *picudo* tiene poca efectividad sobre el cardo no nativo que se suponía controlaría. Por el contrario, el gorgojo sí atacó al *cardo nativo*, una especie relativamente poco frecuente que sólo se encuentra en el oeste de Colorado y Utah del este.

Otra operación fallida fue el programa que trató de reintroducir a los lobos mexicanos para que vivieran en estado silvestre en su hábitat del suroeste de Estados Unidos, donde se habían extinguido en la década de 1930. Como resultado, los ganaderos se han visto afectados económicamente desde que se reintrodujo a los lobos en el área, pues la región ha perdido su valor dado que los lobos mexicanos han matado cientos de cabezas de ganado.

En muchos de los casos los científicos han visto en el biocontrol una alternativa amigable con la naturaleza de mantener las poblaciones de la manera más «conveniente» para los humanos pero en algunos de estos casos existe una evaluación incompleta del riesgo ecológico que se corre. Otro problema que existe es que los organismos vivos, a través del proceso de evolución, pueden lograr una mayor resistencia a los agentes biológicos como bien lo describe Wells en su novela, *La guerra de los mundos*:

«Los gérmenes de las enfermedades han atacado a la humanidad desde el comienzo del mundo, exterminaron a muchos de nuestros antecesores prehumanos desde que se inició la vida en la Tierra. Pero en virtud de la selección natural de nuestra especie, la raza humana desarrolló las defensas necesarias para resistirlos».

Es importante por lo tanto, que este método se aplique adecuada y responsablemente, con una mayor supervisión y que la plaga sea controlada pero no exterminada, pues todos los organismos son parte de una serie de interacciones complejas que aún no terminamos de comprender y que forman el ecosistema.

Crear un proyecto específico con base en lo estudiado para cada situación, para cada plaga, para cada agente y mantener verdaderamente un control que no se nos salga de las manos. Dentro de los estudios que se realizan actualmente en todo el mundo, incluyendo a México, se está evaluando la capacidad que ciertas bacterias tienen de competir con organismos dañinos para una gran variedad de cultivos que provocan grandes pérdidas económicas a los agricultores. Tal es el caso de *Bacillus thuringiensis*, una bacteria conocida por producir diversos compuestos que le permiten evitar el crecimiento de hongos como *Botrytis cinerea*, causante de pérdidas en muchas plantas de interés agrícola. De hecho, varios fungicidas que operan a base de *Bacillus* se están comercializando hoy en día.

Otras bacterias que han mostrado tener grandes capacidades para controlar diferentes plagas vegetales es la llamada *Pseudomonas fluorescens*. Esta bacteria puede producir una amplia gama de compuestos antibióticos que atacan a hongos, otras bacterias e incluso a virus dañinos para las plantas.

Con una adecuada investigación que los respalde, estos productos pueden ser la alternativa ideal al alto consumo de pesticidas que se lleva a cabo actualmente. Se trata de una alternativa amigable y responsable con la naturaleza.

10.6 Capítulo de libro

Promoción del crecimiento vegetal y biocontrol de fitopatógenos por bacterias del género *Pseudomonas*

Fronteras de la Biología del desarrollo de las plantas

Promoción del crecimiento vegetal y biocontrol de fitopatógenos por bacterias del género *Pseudomonas*

Rocío Hernández-León, Eduardo Valencia-Cantero y Gustavo Santoyo-Pizano

Diversos microrganismos causan enfermedades en cultivos de interés agrícola, lo que resulta en enormes pérdidas económicas. Una opción para combatir los patógenos de plantas son los tratamientos con agentes bacterianos, los cuales son una excelente alternativa a la utilización de productos químicos, inocua para el medio ambiente y la salud humana. Los agentes de biocontrol más conocidos pertenecen al género *Pseudomonas*. Cabe destacar que numerosas especies de *Pseudomonas* con actividad de biocontrol también promueven el crecimiento vegetal, lo que representa una opción ideal en la producción de bioinoculantes. En este capítulo se analizan diversos trabajos pioneros y recientes, sobre las características relevantes que presentan las especies de *Pseudomonas* para controlar el crecimiento de fitopatógenos y promover el desarrollo vegetal.

1.1. Introducción

Desde hace varias décadas se ha propuesto el uso de bacterias como agentes para el control de enfermedades en los cultivos. Otra característica deseable en estas bacterias es su capacidad de promover el crecimiento de las plantas PGPR (**P**lant **G**rowth-**P**romoting **R**hizobacteria, por sus siglas en inglés). A mediados de los años 50´s se publicó una revisión de trabajos sobre el control de enfermedades vegetales por medio de microorganismos antagonistas, considerando su empleo como agentes de biocontrol (Wood y Tveit, 1955). Investigaciones posteriores en los años 60´s y 70´s retomaron estas ideas de buscar y aislar microorganismos del suelo con capacidad de antagonizar a los fitopatógenos causantes de enfermedades en cultivos vegetales (Weinhold y Bowman, 1968; Burr *et al.*, 1978). Desde entonces, se ha incrementado la literatura donde se reporta el empleo de bacterias que al ser inoculadas en semillas y raíces de plantas, controlan el crecimiento de patógenos y/o además, promueven el crecimiento vegetal. Así, el género *Pseudomonas* proporcionó los

primeros aislados bacterianos con características de biocontrol y estimulación del crecimiento vegetal, y sin duda, siguen siendo el foco de atención de una gran cantidad de investigaciones, debido a que son habitantes comunes de suelos agrícolas (Compant *et al.*, 2005). Estudios recientes han mostrado que con el uso de bacterias PGPR, se puede reducir la utilización de fertilizantes químicos en una forma significativa (Adesemoye *et al.*, 2009; Adesemoye y Kloepper, 2009).

Kloepper y Scroth (1978), propusieron el término PGPR para designar a las bacterias que habitan la rizósfera de plantas con capacidad de promover significativamente el crecimiento vegetal. Tratando de identificar el mecanismo mediante el cual las *Pseudomonas* promueven dicho crecimiento, Kloepper *et al.* (1980a;1980b) encontraron que cepas de *Pseudomonas fluorescens* y *Pseudomonas putida* sintetizan compuestos quelantes del hierro, conocidos como sideróforos, por medio de los cuales podrían privar del hierro a los demás habitantes de la rizósfera, y por lo tanto, limitar su crecimiento. Desde entonces, se han sugerido diversos mecanismos, indirectos y directos, a través de los cuales las bacterias PGPR contribuyen al desarrollo de plantas y/o controlan el crecimiento de fitopatógenos (Ahmad *et al.*, 2008) (**Fig. 11.1**). En el presente capítulo, se revisarán las investigaciones recientes donde se han empleado bacterias *Pseudomonas* para el biocontrol y biestimulación vegetal.

11.2. El género *Pseudomonas*

Las *Pseudomonas* son bacterias con forma de bacilos, Gram-negativas que no forman esporas y junto con otros géneros como *Bacillus*, son usualmente encontradas en aislados del suelo (Compant *et al.*, 2005; Haas y Defago, 2005). Existen varias características que a continuación describiremos que les permiten estar presentes en diferentes tipos de suelo, incluyendo en los conocidos como suelos supresores de enfermedades, donde los cultivos sufren menos de lo esperado los efectos de los patógenos debido a las actividades de distintos microorganismos (Weller *et al.*, 2002; Mendes *et al.*, 2011). Las *Pseudomonas* presentan un crecimiento rápido y por lo tanto muestran buena colonización en la rizósfera. Lo anterior debido a que pueden utilizar diversos sustratos como

nutrientes y sobrevivir en condiciones adversas y estresantes para otras bacterias. Su capacidad para producir una gran cantidad de compuestos, como antibióticos, polisacáridos y sideróforos, también es crucial para el éxito de su colonización y ocupación de espacios. Así, el impacto que *Pseudomonas* ha tenido en los últimos años ha sido enorme, siendo uno de los géneros con mayor potencial para suprimir a los patógenos y las enfermedades que causan. A continuación detallaremos varios mecanismos de biocontrol y promoción del crecimiento vegetal de algunas cepas de *Pseudomonas* (**Tabla 11.1**).

11.3. Colonización y ocupación de nichos La rizósfera es un ecosistema altamente competente por espacios, nutrientes y protección de diversos factores bióticos y abióticos (Raaijmakers *et al.*, 2002). Las *Pseudomonas* son especialmente buenas colonizadoras de espacios en la rizósfera; en particular, donde existe poca disponibilidad de nutrientes. La síntesis de lipopolisacaridos por parte de rizobacterias es importante para colonizar espacios en la rizósfera; en el caso de *Pseudomonas* también es un factor relevante, aunque puede ser dependiente o específico de la especie o la cepa (Lugtenberg y Dekkers, 1999; Lugtenberg *et al.*, 2001). Se ha propuesto que el antígeno O de la cepa *P. fluorescens* WCS417Rr es importante en la colonización de la raíz de plantas de tomate y para penetrar los tejidos y vivir de manera endosimbiótica (Dekkers *et al.*, 1998b).

Figura 11.1. Efecto promotor del crecimiento vegetal en plantas de rábano. Se muestra una planta inoculada (izquierda) y otra no inoculada (derecha) con *Pseudomonas corrugata* SPB2184 productora de auxinas (Tomado de Lugtenberg y Kamilova, 2012).

Tabla 11.1. Mecanismos de biocontrol y PGPR de algunas especies de *Pseudomonas*. Abreviación del inglés: pyoluteorin (Plt), phenazine-1carboxylic acid (PCA), 2.4 diacetyl phloroglucinol (DAPG), pirrol nitrin (Prn), hydrogen cyanide (HCN), Volatile Organic Compounds (VOCs).

Especies o cepas	Planta/patógeno	Modo de acción	Referencia
Actividad promotora del crecimiento vegetal			
P. chlororaphis	Tabaco	2R, 3R-butanediol	Han *et al.* (2006)
P. lurida	Trigo	AIA, sideróforos	Mishra *et al.* (2009)
Pseudomonas sp. PsJN	Papa	Colonización	Frommel *et al.* (1993)
P. fluorescens	Papa	Colonización	Kloepper *et al.* (1980)
P. fluorescens CHAO	Tabaco	DAPG, PCA, Plt, Pm	Haas y Defago (2005)
P. fluorescens WCS365	Papa, trigo y jitomate	Recombinasa	Dekkers *et al.* (1998; 2000)
Mecanismos de biocontrol o acción antifúngica			

P. aeruginosa 7NSK2	Frijol/*Botrytis cinerea*	Ácido salicílico	Meyer y Höfte (1997)
Pseudomonas sp. B10	Papa/*Fusarium*	Sideróforos	Kloepper *et al.* (1980)
P. fluorescens ZUM80	*Fosarium oxysporum*	Sideróforos y otros	Valencia-Cantero *et al.* (2005)
	C. lindemuthianum	Sideróforos y otros	Santoyo *et al.* (2010)
	C. gloesporioides		
	P. cinnamomi		
P. fluorescens CHAO	Raíz negra/Tabaco	AIA, sideróforos	Haas y Defago (2005)
P. fluorescens pF29A	Hongo Take-all/trigo	Desconocido	Chapon *et al.* (2002)
P. putida 06909	*P. citrophthora*/Limón	Sideróforos	Steddom *et al.* (2002)
P. fluorescens Q8r1-96	Hongo Take-all/trigo	DAPG	Raaijmakers (2001)

La utilización de diversas fuentes de carbono, la motilidad, las respuestas quimiotácticas, entre otros factores, también son fundamentales para ocupar espacios y limitar el crecimiento de patógenos en la rizósfera (Lugtenberg *et al.*, 2001; Compant *et al.*, 2005). Por otra parte, un tema poco explorado es cómo los rearreglos genómicos que sufre *Pseudomonas* pueden estar involucrados en una mejor colonización en la raíz. Generalmente, los genomas bacterianos son sometidos a diversos rearreglos genómicos, ya sea por eventos de recombinación homóloga o de otro tipo sitio-específico (Orozco-Mosqueda *et al.*, 2009). Tal es el caso de la cepa con actividad de biocontrol *Pseudomonas fluorescens* WCS365, la cual requiere de una recombinasa sitio-específico para ser competitiva y poder colonizar las raíces de plantas de papa, trigo y tomate (Dekkers *et al.*, 1998;

Dekkers *et al.*, 2000). Se ha propuesto que en las bacterias donde se llevan a cabo rearreglos genómicos, podrían representar una ventaja adaptativa a cambios drásticos en el ambiente (Dybvig, 1993). Por medio de este mecanismo, se generarían subpoblaciones con diferentes arquitecturas genómicas, las cuales se adecuarían a estos cambios ambientales, permitiendo así la sobrevivencia de una parte de la población. De acuerdo a lo anterior, la cepa *Pseudomonas fluorescens* WCS365 llevaría a cabo rearreglos genómicos que le permitirían una mejor colonización en la rizósfera. Sin embargo, se requiere de más investigación para comprobar dicha hipótesis, ya que hasta el momento existen pocos trabajos que demuestran una relación directa entre la generación de ciertos rearreglos genómicos con adaptaciones ambientales como la rizósfera, que les confieran una ventaja adaptativa (Mavingui *et al.*, 1997; Romero y Palacios, 1997).

Específicamente, se ha propuesto que la síntesis de sideróforos por *Pseudomonas* puede ser un factor importante para colonizar las raíces de la planta. Los sideróforos son compuestos quelantes de hierro (Cornelis, 2010), como se mencionó antes y especialmente los producidos por *Pseudomonas* tienen una alta afinidad por el hierro, quelándolo y haciéndolo menos disponible para otros microorganismos, incluyendo los patógenos vegetales (Kloepper *et al.*, 1980a; Weller, 2007). Este mecanismo se considera una manera indirecta de promover el crecimiento vegetal por parte de *Pseudomonas*. En particular, las *Pseudomonas* sintetizan sideróforos en condiciones limitantes del hierro, siendo éste un factor que induce su expresión a nivel genético al igual que otros factores como el pH y la presencia de elementos traza: nitrógeno, fósforo y carbón (Duffy y Défago, 1999). La síntesis de sideróforos, se lleva a cabo principalmente en etapas exponenciales de crecimiento, siendo ésta la fase donde la población requiere más nutrientes para la división celular (Loper y Schroth, 1986; O´Sullivan y O´Gara, 1992). Asímismo, el complejo pseudobactina–Fe presenta una alta constante de estabilidad (Chen *et al.*, 1994), lo que sugiere que prácticamente toda molécula de pseudobactina excretada se una al Fe presente en el medio, donde dicho complejo actúa como un sistema de suministro de Fe (III) para la bacteria al ser introducido en la célula bacteriana (Koster *et al.*, 1995; Loper y

Henkels, 1999). Por lo tanto, en microambientes como la rizósfera, la síntesis de sideróforos les confiere a los organismos una ventaja competitiva por nutrientes y espacios (Loper y Henkels, 1999).

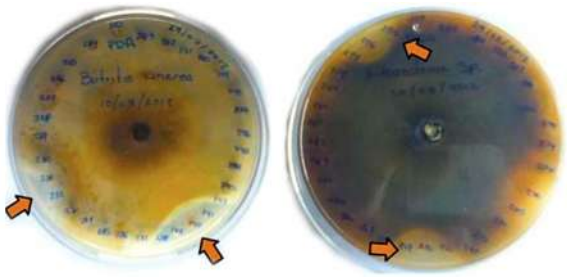

Figura 11.2. Búsqueda *in vitro* de actividades antifúngicas en cepas rizosféricas de *Pseudomonas* spp. contra *Botrytis cinerea* y *Rhizostonia solani*. Las flechas indican el halo de inhibición contra cada patógeno (Hernández-León, *et al.*, 2012. Resultados no publicados).

11.4. Mecanismos de biocontrol de fitopatógenos

Las *Pseudomonas* son agentes bacterianos naturales presentes en suelos supresores de enfermedades (Weller, 2007). Como mencionamos anteriormente, las diversas características genéticas y fenotípicas de *Pseudomonas* las hacen una opción importante de biocontrol en el campo debido a que presenta un rápido crecimiento y por lo tanto, es buena colonizadora de espacios en la rizósfera. Una razón para tal rapidez de crecimiento puede ser su capacidad de usar diversos sustratos como nutrientes y sobrevivir en diferentes condiciones que para otras bacterias serían un estrés. También la capacidad de producir diferentes metabolitos, como antibióticos, volátiles y sideróforos, son determinantes como agentes de biocontrol de patógenos (**Fig. 11.2**).

11.5. Producción de sideróforos

La síntesis de sideróforos por parte de *Pseudomonas* es una característica visible en algunos aislados; por ejemplo, en los medios de cultivo con concentraciones traza de hierro es posible observar un halo color verde-amarillento, el cual puede fluorescer bajo luz ultravioleta (Loper y Buyer, 1991; Budzikiewicz, 1993). Hace más de tres décadas se propuso que los sideróforos podrían estar involucrados en los mecanismos de biocontrol de fitopatógenos, así como en la promoción del crecimiento vegetal (Kloepper *et al.*, 1980a). Desde entonces ha sido ampliamente reconocido el papel de los sideróforos como agentes quelantes que privan a los patógenos del suelo del hierro, un elemento esencial para el crecimiento y sin el cual, su sobrevivencia se ve afectada (Loper y Henkels, 1999). Sin embargo, a diferencia de la propuesta anterior, algunos autores consideran que los sideróforos, en especial en condiciones de campo, no son un factor predominante en el biocontrol de patógenos debido a que las condiciones del suelo, principalmente la biodisponibilidad de hierro, así como el pH, podrían influir en los resultados (Loper y Buyer, 1991). Lo anterior es cierto; sin embargo, es bien conocido que *Pseudomonas* no sólo sintetiza sideróforos en respuesta a la presencia de patógenos, sino que ahora es aceptado que un gran número de factores influyen en las actividades antagónicas. Sin duda, más investigaciones son requeridas para determinar el papel de los sideróforos en el ambiente, y no sólo en el suelo, sino también en condiciones controladas como los tejidos de la planta, donde cepas de *Pseudomonas* son habitantes comúnes. Recientemente, Loaces *et al.* (2010) analizaron las bacterias endófitas de plantas de arroz (*Oryza sativa*) productoras de sideróforos, resaltando la presencia de un aislado de *Pseudomonas* con actividad antibacteriana.

El suelo sigue siendo un buen reservorio de una gran diversidad bacteriana, donde se espera encontrar usualmente cepas con características de nuestro interés. De este modo, en nuestro grupo de trabajo se aislaron *Pseudomonas* de la rizósfera de plantas de papa, las cuales presentaron una buena producción de sideróforos del tipo pseudobactina. En especial, se observó que la cepa ZUM80 lograba inhibir el crecimiento de fitopatógenos como *Fusarium oxysporum, Colletotrichum*

lindemuthianum, Colletotrichum gloesporioides y *Phytophthora cinnamomi*. Una mutante en la síntesis de sideróforos de la cepa *Pseudomonas fluorescens* ZUM80 presentó una inhibición diferencial a los patógenos en condiciones limitantes de hierro. Dicha mutante continuó inhibiendo el crecimiento de *Fusarium oxysporum*, pero no el de *Colletotrichum lindemuthianum, Colletotrichum gloesporioides* y *Phytophthora cinnamomi*. Los resultados anteriores permiten sugerir que probablemente algún otro metabolito, no regulado por hierro, podía inhibir el crecimiento de *F. oxysporum* (Valencia-Cantero *et al.*, 2005; Santoyo *et al.*, 2010).

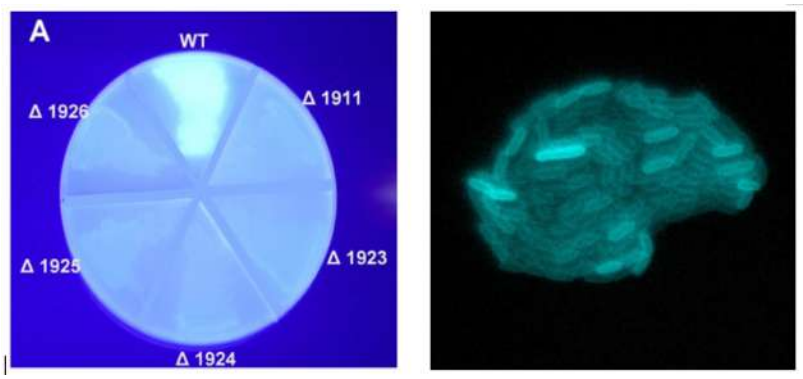

Figura 11.3. Células de *Pseudomonas* que sintetizan sideróforos fluorescentes bajo luz UV. (A) Cepa de *Pseudomonas* silvestre que produce sideróforos tipo pioverdina y mutantes deletadas en genes que codifican para péptido sintetasas no ribosomales (Δ1911-1926) responsables de la síntesis de pioverdina (Tomado de Owen y Ackerley, 2011 y ABCD Lab).

Los sideróforos no son solamente sintetizados por bacterias benéficas para la planta; sino que también los patógenos producen sus propios sideróforos, iniciándose así un combate por el hierro del medio. En este tipo de batalla las constantes de afinidad por la competencia del hierro juegan un papel relevante. Algunos autores han propuesto que la síntesis de sideróforos por parte de algunos patógenos de plantas, ya sean bacterias u hongos, son un determinante importante para la patogenicidad, aunque los ejemplos son escasos (Expert, 1999; Oide et al., 2006). Por otra parte, se sabe que para algunos otros patógenos los

sideróforos no son esenciales en la patogénesis mientras que otros como la bacteria fitopatógena *Pseudomonas syringae* pv. Tomato DC3000 es capaz de producir tres tipos de sideróforos (yersiniabactina, pioverdina y citrato que fluorescen bajo luz UV), los cuales son requeridos para el crecimiento en condiciones limitantes de hierro (Buell et al., 2003) (Fig. 11.3). En un estudio reciente de Jones y Wildermuth (2011) se generó una triple mutante carente de la producción e importación de los tres sideróforos que continúa siendo virulenta en experimentos con plantas de tomate. Sin embargo, la pregusta es ¿cómo adquiere el hierro la bacteria y sigue siendo patógena? Los autores proponen que *Pseudomonas syringae* es capaz de importar compuestos del tipo fierro-nicotinamina. Esto debido a que se ha reportado que diversas cepas de *Pseudomonas* pueden vivir como endófitos de plantas (Márquez-Santacruz et al., 2010); sin embargo, no ha sido explorado el papel que los sideróforos (y otros compuestos) juegan en esta estrecha interacción. También, sería interesante determinar si otros compuestos tipo antibiótico son sintetizados en este ambiente y si ellos son capaces de proteger la planta de invasiones de fitopatógenos.

11.6. Producción de antibióticos

Una gran cantidad de bacterias presentan la capacidad de sintetizar antibióticos, incluyendo *Pseudomonas*. El interés por los antibióticos que produce *Pseudomonas* y su efecto en la supresión de patógenos de plantas surgió a partir de los trabajos de Howell y Stipanovic (1979; 1980) donde se logró inhibir del crecimiento de diferentes hongos fitopatógenos en plantas de algodón inoculadas con los antibióticos producidos por la bacteria. Desde entonces se han estudiado diversas cepas de *Pseudomonas* con capacidad para sintetizar antibióticos y suprimir el crecimiento de patógenos, mejorando con ello la producción agrícola (Weller, 1988).

Pseudomonas sintetiza diversas moléculas con actividad antimicrobiana, tales como el ácido fenazina-1-carboxilico (PCA), 2,4 diacetil fluoroglucinol (DAPG),

pirrolnitrina (Prn), ácido cianhídrico (HCN) y pioluteorina (Plt), así como compuestos de tipo proteico (bacteriocinas) (Weller et al., 2002; Haas y Keel, 2003; Validov et al., 2005). Existen cepas de *Pseudomonas fluorescens* que sintetizan un amplio repertorio de compuestos. Por ejemplo, se sabe que la cepa CHAO produce más de 10 sustancias con actividades de biocontrol de patógenos y de promoción del desarrollo vegetal; por ejemplo DAPG, Plt, Prn, HCN, ácido indolacético y ácido salicílico entre otras (Haas y Defago, 2005).

Recientemente, se han descubierto alrededor del mundo nuevas cepas de *Pseudomonas* con gran potencial, mostrando que aún hay una gran cantidad de bacterias por descubrir en diversos ambientes (León et al., 2009; Mishra et al., 2009; Santoyo et al., 2010). Asímismo, herramientas como la metagenómica, conocida como el estudio del total del material genético dentro de una muestra, prometen descubrir determinantes genéticos con actividades de biocontrol o promoción del desarrollo vegetal sin necesidad de cultivar bacterias (Handelsman, 2004). En un trabajo reciente, Chung et al. (2008) aislaron del metagenoma de un suelo de bosque un conjunto de genes que codifican para una familia de policetidos sintasas tipo II, tales como la proteina acarreadora de acilos (ACP), ACP sintetasas, aminotransferasas y ACP reductasas. Un grupo de 40 Kb expresado en *E. coli* presentó actividad antifúngica y antibacteriana. Interesantemente, este grupo de genes mostró una alta homología con las secuencias genómicas de *Pseudomonas putida*. El aislamiento de secuencias metagenómicas es una buena oportunidad para descubrir genes de organismos no cultivables; sin embargo, hasta donde se sabe, *Pseudomonas* es un género de fácil aislamiento y cultivo en laboratorio, por lo que las técnicas clásicas de cultivo siguen siendo importantes. Más aún, si se desea tener con ellas cepas para producir algún tipo de bioinoculante. La modificación genética de cepas de *Pseudomonas* conocidas, también es una oportunidad por mejorar las funciones de bioconctrol o PGPR (Lecrere et al., 2005).

11.7. Respuesta sistémica inducida (ISR) en plantas

Al igual que diversas especies, *Pseudomonas* también induce la ISR en plantas. Existen diferentes reportes donde *P. fluorescens* EP1, P. putida 5-48 y *P. fluorescens*, protegen a las plantas de caña de azúcar y tomate de patógenos como *Colletotrichum falcatum*, *Ceratocystis fagacearum* y *Fusarium oxysporum*, respectivamente (Compant et al., 2005). Han et al. (2006) reportaron que el compuesto 2R, 3R-butanediol sintetizado por *P. chlororaphis* O6 es el responsable de inducir la ISR en tabaco. Interesantemente, 2R, 3R-butanediol sólo indujo dicha respuesta de manera específica contra el patógeno *E. carotovora* sub sp. carotovora SCC1, pero no con *Pseudomonas syringae* pv. tabaci. Cabe destacar que el 2R, 3R-butanediol promovió el crecimiento de las plantas de tabaco. De esta manera, los autores resaltan las similitudes entre la cepa de *Pseudomonas chlororaphis* O6 y la de *B. subtilis* GB03, debido a que ambas son PGPR e inducen respuesta sistémica en plantas, donde el 2R, 3R-butanediol juega un papel relevante en la interacción planta-bacteria. Es interesante notar que otros estudios reportan una cepa rizosférica de *Pseudomonas aeruginosa* que induce la ISR en plantas de forma dependiente de la producción de ácido salicílico en la rizósfera (de Meyer y Höfte, 1997).

11.8. Conclusiones y perspectivas

La mayoría de las investigaciones que se hacen sobre biofertilizantes se basan en bacterias rhizobias, las cuales pueden formar una estrecha simbiosis con plantas (Bashan, 1998). Las bacterias penetran las raíces y forman estructuras globulares conocidas como nódulos, en los cuales, las bacterias o bacteroides reducen el nitrógeno atmosférico para generar amonio, el cual es exportado hacia la planta. Los bacteroides obtienen de esta simbiosis nutrientes carbonados. Las características anteriores son importantes ya que de esta manera promueven el desarrollo de las plantas. En algunas investigaciones se ha propuesto que incluso se puede incrementar la producción y valor nutritivo de las semillas de consumo

humano mediante la sobreexpresión de genes involucrados en la fijación del nitrógeno (Peralta et al., 2004). Las bacterias rhizobias presentan una opción para promover el desarrollo vegetal, pero se ven limitadas por su capacidad de biocontrol. Hasta el momento han sido escasos los trabajos donde se han generado, principalmente por modificación genética, rhizobias con actividad antifúngica, aunque su capacidad simbiótica se afectó negativamente (Krishnan et al., 2007). Sin embargo, estas características de promover el crecimiento y protección vegetal las encontramos de forma natural en bacterias del género *Pseudomonas*. Bacterias como *Pseudomonas* no forman una simbiosis con las plantas del tipo de las rhizobias; aunque sí pueden penetrar los tejidos vegetales y establecerse como endófitos (Ryan et al, 2008; Márquez-Santacruz et al., 2010). Dentro de la planta también pueden jugar importantes papeles como PGPR y ejercer un control de patógenos, por diversos mecanismos (Compant et al., 2005; Ryan et al., 2008).

Las siguientes características presentadas por cepas de *Pseudomonas* son deseables en bacterias candidato para el biocontrol y PGPR: 1) rápido crecimiento, 2) capacidad para competir y producir compuestos antibióticos, 3) degradar y emplear varios sustratos como nutrientes, 4) sobrevivir en condiciones de estrés y 5) suprimir naturalmente a fitopatógenos. Las técnicas de modificación genética están muy avanzadas en *Pseudomonas*, lo que permitiría modificar el genoma de alguna de ellas e incrementar las funciones de PGPR y biocontrol. Es interesante resaltar también que el género *Pseudomonas*, el cual ha sido empleado como agente de biocontrol en diferentes estudios ha mostrado pocos efectos negativos contra otras bacterias PGPR (Weller et al., 2002). Finalmente, el uso y producción de bioinoculantes basados en bacterias del género *Pseudomonas* puede no ser la única opción, sino como últimamente se ha observado, lo sean las mezclas de bacterias con diferentes características y capacidades, las cuales podrían mejorar el desarrollo vegetal, así como el control de fitopatógenos. Se ha propuesto también que algunas especies de *Pseudomonas* pueden inducir lo que se conoce como "Tolerancia Sistémica Inducida" (Induced Systemic Tolerance o IST), donde las bacterias PGPR

promueven mecanismos de tolerancia a diversos estreses ambientales como escasez de agua. Sin embargo, este tema merece un análisis más detallado en otro ensayo. El empleo de *Pseudomonas* con capacidades IST mejoraría la habilidad de enfrentar factores ambientales y colonizar espacios en la rizósfera, lo que podría generar resultados más consistentes en experimentos en campo.

11.9. Referencias

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