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Efecto del estrés salino sobre los componentes lipídicos de la membrana en rizobacterias y su interacción con plantas de *Solanum Lycopersicum* L.

TESIS

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RESUMEN

Los suelos salinos son uno de los factores ambientales que más severamente afectan la productividad de los cultivos. En este trabajo evaluamos la capacidad de las bacterias benéficas *Bacillus* sp. CR71, *Bacillus* sp. E25, *Bacillus toyonensis* COPE52 y *Pseudomonas fluorescens* UM270 para promover el crecimiento y conferir tolerancia a estrés salino en plantas de jitomate (*Solanum lycopersicum* L.) crecidas en invernadero. También, se evaluó si los componentes lipídicos de membrana (fosfatidiletanolamina (PE), fosfatidilglicerol (PG), fosfatidilcolina (PC) y cardiolipina (CL)) de las bacterias se modificaron durante el estrés salino. Los resultados mostraron que las cuatro cepas son capaces de promover el crecimiento de las plantas y conferirles protección de las condiciones salinas de manera significativa, al restaurar algunos parámetros de crecimiento de las plantas inoculadas al nivel de las plantas crecidas sin estrés. Se evaluaron los mecanismos de promoción en las rizobacterias promotoras de crecimiento vegetal (PGPR´s, por sus siglas en ingles, Plant Growth Promoting Rhizobacteria), observándose que algunas capacidades; como la producción de ácido indol-3-acético (AIA), sieróforos y biofilm no se abolieron durante el estrés. Otro mecanismo indirecto, como el antagonismo hacia fitopatógenos, se redujo de manera dependiente de la cantidad de sal en el medio medio (100 y 200 mM de NaCl). Al analizar las proporciones de fosfolípidos y ácidos grasos presentes en las membranas de las bacterias, se encontró que las condiciones salinas favorecieron el incremento de fosfolípidos y ácidos grasos que incrementan la rigidez de la membrana confiriendo una mayor estabilidad y funcionalidad a la membrana. La máxima concentración de sal en el medio favoreció un incremento de un fosfolípido desconocido en las cepas CR71 y COPE52 de 17 % y 20 % respectivamente, además, se logró observar una disminución significativa de fosfatidiletanolamina (PE) para ambas cepas. Por el contrario, en *Bacillus* sp. E25 la adición de sal al medio condujo a un incremento en los niveles de PE en aproximadamente 74 %, mientras que el fosfolípido desconocido se redujo hasta 2 %. Por otro lado, el estrés salino generó un incremento del ácido palmitoleíco (16:1 Δ 9) y del ácido 15-metilhexadecanoico

(17:0i) en la cepa CR71, y de los ácidos grasos saturados palmítico (16:0) y esteárico (18:0) en la cepa E25.

Finalmente, el estrés salino incremento el contenido de los ácidos grasos ramificados 13-metiltetradecanoico (15:0i), 15-metilhexadecanoico, y del ácido palmítico en *Bacillus toyonensis*.

En el caso de *P. fluorescens* UM270, el estrés salino incrementó la acumulación del fosfolípido cardiolipina (2.5 %), triplicando la cantidad producida en comparación a las condiciones sin sal (7.5 %). Se construyeron dos mutantes, Δ*clsA* y Δ*clsB,* afectadas en genes que codifican para la enzima cadiolipina sintasa. Las mutaciones provocaron una disminución en la producción de este fosfolípido de 3.2 % en la mutante Δ*clsA* y de 3.5 % en la mutante Δ*clsB*. Se redujo la capacidad de promover el crecimiento y conferir tolerancia al estrés salino en plantas de *Solanum lycopersicum* L., durante ensayos en invernadero. Adicionalmente, se redujo la producción de AIA y se incrementó la producción de sideróforos.
En conclusión, los resultados indican que las cepas de *Bacillus* sp. CR71, *Bacillus* sp. E25, *Bacillus toyonensis* COPE52 y *Pseudomonas fluorescens* UM270 modificaron sus componentes lipídicos de membrana en respuesta al estrés salino, permitiendo mantener sus actividades de promoción de crecimiento vegetal en interacción con plantas de jitomate en suelos salinos.

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Palabras clave: Estrés salino; fosfolípidos; *Bacillus*; *Pseudomonas*; ácidos grasos; PGPB.

ABSTRACT

Saline soils are one of the environmental factors that most severely affect crop productivity. In this work we evaluate the capacity of the beneficial bacteria *Bacillus* sp. CR71, *Bacillus* sp. E25, *Bacillus toyonensis* COPE52 and *Pseudomonas fluorescens* UM270 to promote growth and confer tolerance to salt stress in tomato plants (*Solanum lycopersicum* L.) grown in greenhouses. Also, it was evaluated whether the membrane lipid components (phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC) and cardiolipin (CL)) of the bacteria were modified during saline stress. The results showed that the four strains are able to promote plant growth and provide them protection from saline conditions in a significant way, by restoring some growth parameters of inoculated plants to the level of plants grown without stress. The promotion mechanisms in plant growth promoting rhizobacteria (PGPR's, Plant Growth Promoting Rhizobacteria) were evaluated, observing that some capacities; As indole-3-acetic acid (IAA) production, sierophores and biofilm were not abolished during stress. Another indirect mechanism, such as antagonism towards phytopathogens, was reduced depending on the amount of salt in the medium medium (100 and 200 mM NaCl). When analyzing the proportions of phospholipids and fatty acids present in the membranes of bacteria, it was found that saline conditions favored the increase of phospholipids and fatty acids that increase the rigidity of the membrane, conferring greater stability and functionality to the membrane. The maximum concentration of salt in the medium favored an increase of an unknown phospholipid in the CR71 and COPE52 strains of 17% and 20% respectively, in addition, it was possible to observe a significant decrease in phosphatidylethanolamine (PE) for both strains. On the contrary, in *Bacillus* sp. E25 addition of salt to the medium led to an increase in PE levels by approximately 74%, while the unknown phospholipid was reduced to 2%. On the other hand, saline stress generated an increase in palmitoleic acid (16: 1 Δ 9) and 15-methylhexadecanoic acid (17: 0i) in strain CR71, and in saturated fatty acids palmitic (16: 0) and stearic (18: 0) in strain E25.

Finally, saline stress increased the content of the branched fatty acids 13-methyltetradecanoic (15: 0i), 15-methylhexadecanoic, and palmitic acid in *Bacillus toyonensis.*

In the case of *P. fluorescens* UM270, under saline stress conditions, the accumulation of the cardiolipin phospholipid increased (2.5%), tripling the amount produced compared to the conditions without salt (7.5%). Two mutants, Δ*clsA* and Δ*clsB*, affected in genes encoding the enzyme cadiolipin synthase were constructed. The mutations caused a decrease in the production of this phospholipid of 3.2% in the Δ*clsA* mutant and 3.5% in the Δ*clsB* mutant. The ability to promote growth and confer tolerance to saline stress in *Solanum lycopersicum* L. plants was reduced during greenhouse trials. Additionally, IAAproduction was reduced and siderophore production increased.

In conclusion, the results indicate that *Bacillus* sp. CR71, *Bacillus* sp. E25, *Bacillus toyonensis* COPE52 and *Pseudomonas fluorescens* UM270 modified their lipid membrane components in response to salt stress, allowing them to be maintain their plant growth promoting activities in interaction with tomato plants in saline soils.

Key words: Saline soils; Phospholipids; Fatty acids; PGPB.

1. INTRODUCCIÓN

Un ecosistema agrícola puede estar sujeto a diversos tipos de estrés, incluyendo los de tipo biótico y abiótico. Los tipos de estrés biótico incluyen: el ataque por virus, bacterias, hongos y herbívoros (Poschenrieder et al., 2006), una planta suceptible puede ser huésped para un patógeno virulento, o bien puede ser blanco de herviboros como;vertebrados o insectos (Freeman, 2008). Los tipos de estrés abiótio o ambiental, por otro lado, pueden limitar el crecimiento, desarrollo y producción de los cultivos agrícolas.

Entre estos factores se destacan el tipo de suelo; factor importante dado que los suelos están compuestos de arena, limo y arcilla, y su composición mineral determinará su estructura creando diferentes gradientes de pH, nutrientes y gases que de manera directa influyen en el desarrollo de las plantas, además de modular el microbioma asociado a la rizósfera de las mismas. El pH es otro factor que limita el crecimiento de las plantas al estar directamente relacionado con la disponibilidad de nutrientes mediante el control de las formas químicas de los compuestos presentes en el suelo (Santoyo et al., 2017). El estrés por sequía y temperaturas extremas, son factores que afectan el desarrollo de las plantas al deshidratar los tejidos y causar daño celular irreversible y la muerte (Bartels y Sunkar 2005). La presencia de metales pesados también causa serias amenazas al desarrollo de los cultivos al afectar el aparato fotosintético, además de generar especies reactivas de oxigeno que causan trastornos fisiológicos y la muerte (Tauuqeer et al., 2016).

Sin embargo, la salinidad del suelo es una de las principales tensiones abióticas que afectan negativamente a las prácticas agrícolas, se estima que actualmente 1 billón de hectáreas de suelos están afectadas por la sal, de las cuales aproximadamente 76 millones han sido inducidas por el hombre, provocando que las tierras cultivables en el mundo disminuyan entre 1 y 2 % por año (Hossain, 2019).

La exposición al estrés salino reduce el crecimiento de la planta debido a que las grandes cantidades de sodio tienen un impacto en la deficiencia y desequilibrio de nutrientes, y disrupción del estado de agua dentro de la planta, además de la

inhibición de muchas enzimas (del-amor y Cuadra-Crespo 2012; Yao *et al*., 2010; Zhu 2003).

Para contrarrestar los efectos negativos ocasionados por el estrés salino, varias estrategias se han desarrollado, incluyendo el uso de PGPR´s. Las cuales se definen como aquellas bacterias que habitan la rizósfera (parte del suelo que se encuentra influenciado por la raíz (Hilter 1904), de las plantas con las cuales interactúan mostrando un efecto positivo sobre los cultivos (Kloepper y Schoroth, 1978), teniendo la capacidad de establecer una relación simbiótica con la planta (Egamberdiyeva, 2007).

Algunas PGPR se han destacado por su capacidad para colonizar la rizósfera de las plantas y conferir un efecto a través de mecanismos directos e indirectos, los cuales pueden ser correlacionados por su habilidad para formar biofilm, quimiotaxis y producción de exopolisacáridos, ácido indol-3-acético (AIA) y aminociclopropano-1-carboxilato (ACC) desaminasa (Glick, 2015), entre otras. Las investigaciones sobre la interacción de PGPR con otros microorganismos y su efecto sobre la respuesta fisiológica de las plantas de cultivo bajo diferentes regímenes de salinidad del suelo están aún en la etapa inicial (Singh *et al*., 2011).

Sin embargo, para que las interacciones planta-microorganismo se lleven a cabo de forma correcta en situaciones de estrés es necesario que las bacterias mantengan la viabilidad celular, para esto las bacterias presentan una gran cantidad de mecanismos de halotolerancia entre los que podemos destacar; la modificación del perfil de ácidos grasos de membrana, expresión de proteínas de estrés a sal, acumulación de osmolítos y de trehalosa, producción de biofilm y modificación de los fosfolípidos de membrana. Todos estos mecanismos le permiten a la bacterias tolerar situaciones de estrés y de forma adicional les permitir ejercer actividades de promoción en interacción con plantas (Paul y Lade, 2014).

Pseudomonas y *Bacillus* son dos géneros bacterianos que presentan gran capacidad de adaptabilidad a condiciones de estrés (Santoyo et al., 2012; Orozco-Mosqueda et al., 2020). Resultando interesante conocer si la capacidad de

promoción de crecimiento ejercido por bacterias de estos géneros se mantiene en condiciones de estrés salino, además de intentar dilucidar qué mecanismos de protección presentan estas bacterias para mantener sus capacidades de promoción de crecimiento.

2. SUELOS SALINOS Y EL IMPACTO EN EL DESARROLLO Y CRECIMIENTO DE LAS PLANTAS.

La salinización consiste en la acumulación en el suelo de sales solubles en agua, que incluye iones de potasio (K^+), magnesio (Mg^{2+}), calcio (Ca^{2+}), cloruros (Cl^-), sulfatos (SO_4^{2-}), carbonato ($HCO3^-$) y sodio (Na^+) (Paul y Lade, 2014).

Se le llama suelo salino a aquel que presenta una conductividad eléctrica superior a 4dS/m (aproximadamente 40mM NaCl), en la zona de la raíz a 25°C y tiene 15 % de sodio intercambiable (Pierzynski et al., 2005).

Los procesos de salinización pueden ser primarios (causas naturales) y secundarios (actividades antropogénicas) (Ghassemi et al., 1995). Las principales causas de salinización primaria incluyen la intrusión de agua de mar hacia los mantos acuíferos, la deposición de sal oceánica transportada por el viento, la erosión de minerales, además en regiones áridas y semiáridas, la evapotranspiración favorece la aparición de suelos salinos (Safdar et al., 2019). Mientras que las causas secundarias las encabezan el riego de cultivos con agua salada, el uso de fertilizantes inorgánicos, el pastoreo excesivo y actividades de deforestación (Ondrasek et al., 2010).

El impacto que genera la salinidad para la mayoría de los cultivos agrícolas es considerable, debido a que las mayorías de las plantas son consideradas como glicófitas, es decir, que concentraciones superiores a 10 mM NaCl influyen en su desarrollo (Liang et al., 2018).

El estrés salino afecta el desarrollo de las plantas de varias maneras y el grado de inhibición en el crecimiento depende de diversos factores como la especie, etapa de la planta y la fuerza iónica de la salinidad (Yadav et al., 2019).

La exposición a la salinidad e inevitablemente al estrés salino puede reducir la germinación de las semillas, la longitud de la raíz, la altura de la planta, así como la cantidad y calidad de los frutos (Liang et al., 2014). El estrés osmótico es la primera condición adversa que enfrenta la planta y que reduce la capacidad de absorber agua (Horie et al., 2011), la toxicidad iónica ocurre más tarde cuando la cantidad de sal rebasa la capacidad de la planta a mantener la homeostasis iónica (Munns y Tester, 2008). El estrés osmótico junto con la toxicidad ejercida por los iones de sodio afecta el contenido de clorofila en la planta al incrementar la actividad de la enzima clorofilasa (Sevengor et al., 2011), la inhibición en la síntesis de clorofila resulta en la pérdida de la actividad fotosintética que conduce a la senescencia de las hojas, lo que da como resultado una disminución del rendimiento de los cultivos (Rengasamy, 2010).

Debido a ello, es que las plantas han desarrollado diversos mecanismos para adaptarse a estas condiciones adversas, como lo es el desarrollo de mecanismos de tolerancia o bien aprovechar las interacciones que se presentan dentro del entorno rizosférico.

3. MECANISMOS DE TOLERANCIA Y/O ADAPTACIÓN A LA SALINIDAD EN PLANTAS.

Las plantas han desarrollado varios mecanismos bioquímicos y fisiológicos en orden a sobrevivir en suelos con altas concentraciones salinas; estos mecanismos incluyen: homeostasis iónica y compartimentalización, toma y transporte de iones, biosíntesis de osmoprotectores y solutos compatibles, síntesis de poliaminas, generación de óxido nítrico, activación de enzimas y síntesis de compuestos antioxidantes y modulación de hormonas (Gupta y Huang, 2014).

Homeostasis y tolerancia a la sal. En las plantas se encuentran presentes dos tipos de transportadores de Na^+/H^+, localizados en la membrana vacuolar tipo H^+-ATPasa (V-ATPasa) y una pirofosfatasa vacuolar (V-PPasa). Bajo condiciones de estrés la sobrevivencia de la planta depende de la actividad de la ATPasa membranal que

evita que los iones de Na^+ lleguen al citoplasma favoreciendo la internalización en la vacuola (Reddy et al., 1992; Zhu, 2003). Existen además otros transportadores de la familia histidin cinasa que juegan un papel esencial en la tolerancia a la sal a través de la regulación del transporte de Na^+ y K^+ (Sairam y Tyagi, 2004).

Biosíntesis de osmoprotectores y osmolítos compatibles. Los osmolítos compatibles o solutos compatibles son compuestos orgánicos de bajo peso molecular que mantienen un equilibrio osmótico, sin interferir con el metabolismo celular, son un grupo de compuestos orgánicos diversos, incluye principalmente prolina, glicina, azúcar y polioles. La función de estos osmolítos es la de proteger la estructura y mantener el balance osmótico dentro de la célula, evitando su deshidratación y permitiendo un plegamiento correcto de proteínas y enzimas (Hasegawa et al., 2000).

Regulación antioxidante de tolerancia a la salinidad. La tolerancia a la salinidad está positivamente correlacionada con la actividad de enzimas antioxidantes, tales como superoxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPX), ascorbato peroxidasa (APX), y glutatión reductasa (GR) y con la acumulación de compuestos antioxidantes no enzimáticos, recientemente se han reportado un par de proteínas helicasas que funcionan en la planta mejorando o manteniendo la fotosíntesis y la maquinaria antioxidante (Gupta et al., 2005; Gill et al., 2013).

Síntesis de poliaminas. Las poliaminas juegan una variedad de papeles en el crecimiento y desarrollo normal de la planta; como regulación de proliferación celular, embriogénesis somática diferenciación y morfogénesis, ruptura de dormancia y germinación de la semilla, desarrollo de flor y fruto y senescencia. Bajo condiciones de estrés se han encontrado un incremento en la síntesis de poliaminas que ayudan en parte a continuar con los efectos benéficos que éstas realizan bajo condiciones normales (Gupta et al., 2013).

Funciones del óxido nítrico en la tolerancia a la salinidad. El óxido nítrico (ON) está involucrado en la regulación de varios procesos de desarrollo y crecimiento en planta, tales como; crecimiento radicular, respiración, cierre de estomas, floración,

muerte celular, germinación de la semilla y respuestas a estrés, esto último debido a que bajo estas condiciones el ON desencadena la expresión de genes que participan en procesos redox y ayudan en la activación de enzimas antioxidantes que a su vez suprimen la lipoperoxidación lipídica (Crawford, 2006; Bajgu, 2014).

Regulación de fitohormonas. El ácido abscísico (ABA) es una fitohormona implicada en aminorar los efectos causados por la salinidad implicado en la fotosíntesis, crecimiento, y translocación de asimilados. La relación positiva entre la acumulación del ABA y la halotolerancia ha sido parcialmente atribuida a la acumulación de K^+ y Ca^{2+} y solutos compatibles como prolina y azucares, en vacuolas de las raíces, que se contraponen a la toma de Na^+ y Cl^- (Gurmani et al., 2011). Otras hormonas de gran importancia en condiciones de estrés son el ácido salicílico y los brasinoesteroides (Fragnire et al., 2011; Clause y Sasse, 1998).

A pesar de que las plantas cuentan con los mecanismos anteriormente descritos, en muchas ocasiones estos resultan insuficientes para proteger el crecimiento y desarrollo de las plantas en condiciones de estrés, por fortuna las plantas se encuentran interactuando con microorganismos que habitan la rizósfera e impactan positivamente en el crecimiento de las plantas (Santoyo et al., 2017).

4. PAPEL DE LAS PGPR EN LA PROMOCIÓN DE CRECIMIENTO EN PLANTAS.

Las bacterias PGPR incluyen diversos géneros bacterianos de gran importancia, entre los que se encuentran; *Bacillus, Pseudomonas, Arthrobacter, Erwinia, Serratia, Azotobacter, Azospirillum, Burkholderia, Caulobacter,* y *Chromobacterium* (Santoyo et al., 2012; Numan et al., 2018).

Estas bacterias presentan distintos mecanismos para además de promover el crecimiento de la planta, también proteger a la misma de la infección causada por organismos fitopatógenos y condiciones medioambientales adversas; estos mecanismos se clasifican como directos e indirectos (Santoyo et al., 2012; Glick, 2014; Etesami y Maheshwari, 2018; Ferreira *et al.,* 2019; Gouda *et al.,* 2018).

Los mecanismos que son ejercidos directamente en la planta favoreciendo su crecimiento, se conocen como mecanismos directos, entre estos mecanismos se encuentran, la producción de ácido indol-3-acético, fitohormona que juega un papel crucial en el crecimiento y desarrollo de las plantas (Ostrowski y Jakubowska, 2008; Khan et al., 2016); la producción de la enzima ACC (ácido 1-aminociclopropano-1 carboxílico) desaminasa, la cual disminuye los niveles de etileno en la planta, al escindir el ACC en en α-cetobutirato y amonio, compuestos que pueden ser usados por las bacterias y las plantas como fuentes de energía (Glick, 2014); la solubilización de nutrientes, llevada a cabo a través de la liberación de iones para la adquisición de elementos esenciales como el hierro (Orosco-Mosqueda *et al.,* 2013), la síntesis de sideróforos, que facilitan la toma de hierro por la planta, además de restringirlo a los organismos patógenos(Santoyo *et al.,* 2010); la solubilización de fosfatos para que se encuentren en formas biodisponibles, se puede llevar a cabo por diversos mecanismos bacterianos como lo son la acidificación, quelación, reacciones de intercambio catiónico y la disolución de compuestos minerales (Hanaka *et al.,* 2019); la fijación de nitrógeno atmosférico para la promoción del crecimiento vegetal convirtiéndolo en amonio y nitratos (Garrido-Oter *et al.,* 2018); la producción de compuestos orgánicos volátiles (COV´s), como son el 2,3-butanediol y acetoína (Ryu et al., 2003), y dimetilhexadecilamina (DMHDA) (Castulo-Rubio et al., 2015).

Por otro lado, los mecanismos indirectos se presentan cuando el microorganismo protege a la planta, por lo que de manera indirecta mejora la salud y, por lo tanto, su desarrollo. Entre estos mecanismos se encuentran; la producción de enzimas líticas como quitinasas y glucanasas, las cuales tienen la capacidad de inhibir el crecimiento de hongos fitopatógenos (Won et al., 2019, Martinez-Absalón et al., 2014); la producción de biofilm que protege a las bacterias de las condiciones de estrés, además de participar directamente en los procesos de colonización en las raíces de las plantas (Kasim et al., 2016); la inducción del sistema de resistencia (ISR) en plantas como sistema de biocontrol (Shameer y Prasad, 2018); la producción de COV´s como el dimetil disulfuro (DMDS) (Rojas-Solis et al., 2018), y

DMHDA (Velázquez-Becerra et al., 2013) que inhiben el crecimiento de hongos fitopatógenos.

Resulta importante destacar que los mecanismos directos e indirectos, pueden llevarse a cabo incluso bajo condiciones de estrés abiótico, lo que les permite conferir resistencia a las plantas a diferentes tipos de adversidades.

5. MECANISMOS DE HALOTOLERANCIA EN BACTERIAS.

La salinidad tiene un impacto negativo en la abundancia, diversidad, composición y funciones de las bacterias presentes en el suelo (Shi et al., 2019). Para las bacterias, el establecimiento en altas concentraciones de sal es bioenergéticamente exigente porque deben mantener un equilibrio osmótico entre su citoplasma y medio circundante mientras se excluyen los iones de sodio al exterior de la célula (Jiang et al., 2007). Para contrarrestar estos efectos las bacterias han desarrollado diversas adaptaciones para lograr un equilibrio osmótico al mantener una concentración de sal citoplasmática similar a la de los medios circundantes (Figura 1) (Paul y Lade, 2014).

La primera respuesta de las células ante cambios en la presión osmótica es la absorción de potasio, además de la acumulación de solutos compatibles, entre los que se encuentran azucares y derivados, aminoácidos, polioles, betainas y ectoínas (Zeidler y Muller, 2018). Adicionalmente, estos solutos compatibles pueden ser sintetizados de *novo* o, si está presente en el medio, puede ser absorbido por los microrganismos. *Pseudomonas fluorescens* MSP-393, es capaz de sintetizar *de novo* los osmolítos; alanina, glicina, ácido glutámico, serina, treonina y ácido aspártico en el citosol, los cuales actúan como estabilizadores de proteínas que apoyan al correcto plegamiento de polipéptidos en condiciones desnaturalizantes (Paul y Nair, 2008; Street et al., 2006).

La producción de exopolisacaridos es otro de los mecanismos comúnmente utilizado por las bacterias para sobrevivir en condiciones de estrés, estos exopolisacaridos protegen a las bacterias del estrés hídrico y fluctuaciones en el

potencial del agua, al mejorar la retención de agua y regular las fuentes de carbono en el medio microbiano (Arun et al., 2017).

Otro mecanismo comúnmente empleado por las bacterias es la reticulación del peptidoglucano, las células estresadas con NaCl presentan un péptidoglucano más corto entre puentes peptídicos que las células no estresadas (Carniello, 2018).

Francius y colaboradores (2011), demostraron que la estructura del apéndice presente alrededor de las bacterias en condiciones de baja concentración de electrolitos actúa como una barrera protectora atenuando el impacto del intercambio iónico al bajar la restricción de la presión osmótica.

Bajo condiciones de estrés, las bacterias inician un programa de expresión génica en respuesta al estrés osmótico por altas concentraciones de NaCl, que se manifiestan como un conjunto de proteínas producidas en mayor número en respuesta al estrés (Numan et al., 2018). Un perfil transcripcional de la bacteria *Bacillus subtilis*, crecida en presencia de 1.2 M de NaCl mostró la inducción de 123 genes y la represión de 101, la mayoría de los genes inducidos ayudan a la bacteria a tolerar las condiciones de salinidad (Steil et al., 2003). Se ha demostrado que las proteínas de alivio al estrés ayudan a las bacterias a mantener el metabolismo celular, protegiendo así las propiedades que promueven el crecimiento en plantas en suelos salinos (Paul et al., 2006).

Otro de los mecanismos de gran importancia que les permite a las bacterias sobrevivir a condiciones de estrés, son los cambios que se presentan a nivel de membrana particularmente en los fosfolípidos y ácidos grasos, los cuáles en gran medida determinan la fluidez de la membrana siendo tal vez este parámetro el más importante para determinar la viabilidad celular (Figura 2)(Murínová y Dercová, 2014).

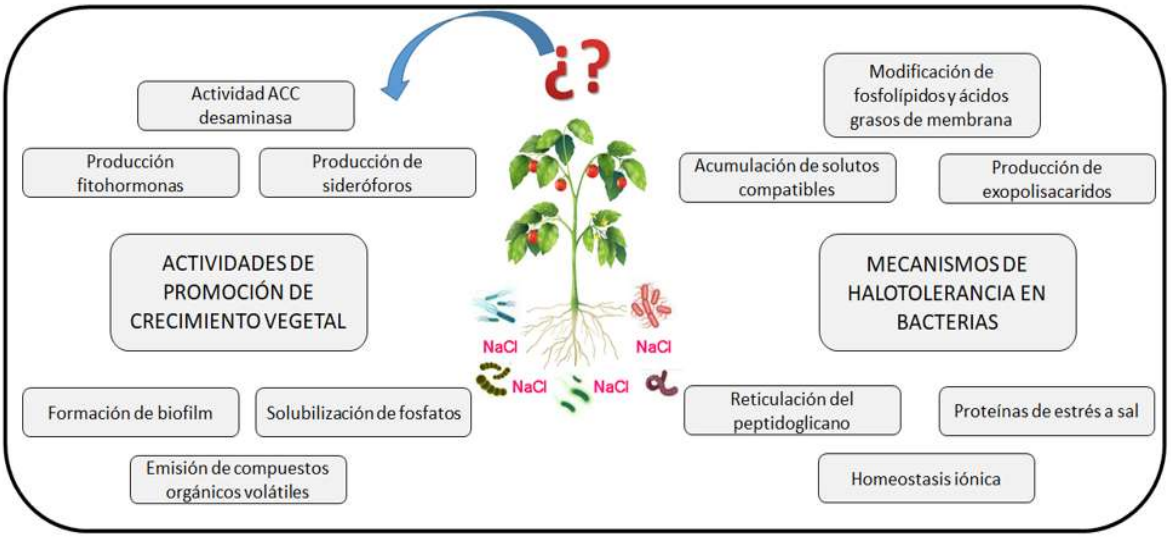


Figura 1. Los mecanismos de halotolerancia presentes en las bacterias en condiciones de salinidad, protegen las actividades de promoción de crecimiento.

Los fosfolípidos (conformados por una cabeza fosfato hidrofílica y dos colas hidrófobas) son los mayores componentes de las membranas bacterianas y cambios en su composición puede afectar procesos importantes como el metabolismo, la virulencia y las respuestas a estrés (Kunh et al., 2015). La composición fosfolípidica de las membranas bacterianas generalmente consiste de fosfatidilglicerol (PG), cardiolipina (CL), fosfatidiletanolamina (PE) y fosfatidilcolina (PC), estos fosfolípidos se clasifican como aniónicos (PG y CL) y zwitteriónicos (PE y PC). Adicionalmente, un conjunto de bacterias también posee derivados metilados de PE monometilfosfatidiletanolamina (MMPE), dimetilfosfatidiletanolamina (DMPE) (Lin y Weibel, 2016).

En la tabla 1 se muestra la composición de fosfolípidos en membranas de bacterias modelos ampliamente estudiadas.

Tabla 1. Composición mayor de FL (fosfolípidos) en membranas de diferentes bacterias modelo.

Bacteria	Porcentaje de FL totales en membranas (%)[a]			Referencia
	PE	PG	CL	
Bacterias Gram-Negativas				

Escherichia coli	80	15	5	Romantsov *et al*., 2007
Caulobacter crescentus	ND[b]	78	9	Contreras *et al*., 1978
Pseudomonas aeruginosa	60	21	11	Conrad y Gilleland 1981
Proteus mirabilis	76	13	6	Gmeiner y Martin 1976
Bacterias Gram-Positivas				
Bacillus subtilis	49	25	8	López *et al*., 2006
Staphylococcus aureus	ND[b]	50	32	Tsai et al., 2011
Streptococcus pneumonia	ND[b]	60	40	Trombe et al., 1979

[a] Porcentaje calculado de acuerdo al contenido de fosfato de los fosfolípidos extraídos de células en fase logarítmica.

[b] ND detectado

Los fosfolípidos de membrana pueden sensar cambios ambientales o señales de comunicación de otras células y apoyar diversas funciones celulares, incluyendo división celular, diferenciación, secreción de proteínas, entre otras (Li et al., 2019).

La PE es el fosfolípido más abundante en las membranas bacterianas, y comprende más del 70% de todos los fosfolípidos (Berg et al., 2006). Proporciona la presión lateral en la bicapa de la membrana bacteriana y mantienen la posición de los aminoácidos. Es un lípido que no forma bicapa debido a su conformación estérica, característica importante dado que la relación entre la bicapa y no bicapa varia en respuesta a los cambios ambientales (Murínová y Dercová, 2014). Por otro lado, la PC es un fosfolípido que tiende a formarse en la estructura de la bicapa, de modo que las membranas con mayor cantidad de PC tienden a ser más empaquetadas que aquellas que contienen mayores cantidades de PE, estos cambios favorecen un equilibrio en la bicapa y la no formación de poros (Paulicci et al., 2015).

Mientras que PG es importante en la síntesis de CL y juega un papel importante en la translocación de las proteínas a través de las membranas (Campo et al., 2004). Finalmente, el fosfolípido CL juega un papel fundamental en la adaptación de las bacterias al estrés ambiental, ya que en respuesta al estrés osmótico se promueve

la localización del transportador osmosensorial ProP, el cual al detectar una alta osmolaridad regula las concentraciones de osmolítos orgánicos en el citoplasma logrando mantener el balance osmótico en la célula (Romantov et al., 2007). Además, este fosfolípido cuenta con el mayor volumen de grupo cabeza de todos los fosfolípidos existentes, proporcionando mayor integridad estructural a la membrana, permitiendo que la bacteria presente un mejor funcionamiento celular en condiciones de estrés (Lewis y McElhabey, 2009).

Por otro lado, cambios en la composición de los ácidos grasos de membrana, son de las reacciones más importantes que presentan las bacterias para contrarrestar condiciones de estrés. La alteración de la relación de cadena larga a cadena corta de los ácidos grasos participa en la regulación de la fluidez de la membrana en condiciones adversas. El incremento en el tamaño de la cadena, así como el grado de saturación ocasionan una mayor rigidez en la membrana, confiriéndole mayor estabilidad en condiciones de estrés (Kotchaplai et al., 2017; Oh et al., 2012). Otro mecanismo lo representa la isomerización de los ácidos grasos insaturados de la posición *cis* a *trans*, condición que aporta mayor adaptabilidad de las bacterias a condiciones adversas (Tan et al., 2016; Eberlein et al., 2018). Adicionalmente, las condiciones ambientales desfavorables para la bacteria, alteran la proporción y el tipo de ramificación de los ácidos grasos que modulan la fluidez de la membrana (Kaiser et al., 2016; Sen et al., 2015), además de estimular la formación de ciclopropanos, los cuales se sugiere disminuye la permeabilidad de la membrana, siendo este un mecanismo importante para proteger a la bacteria de factores ambientales como presión, temperatura y salinidad (Guan y Liu, 2020).

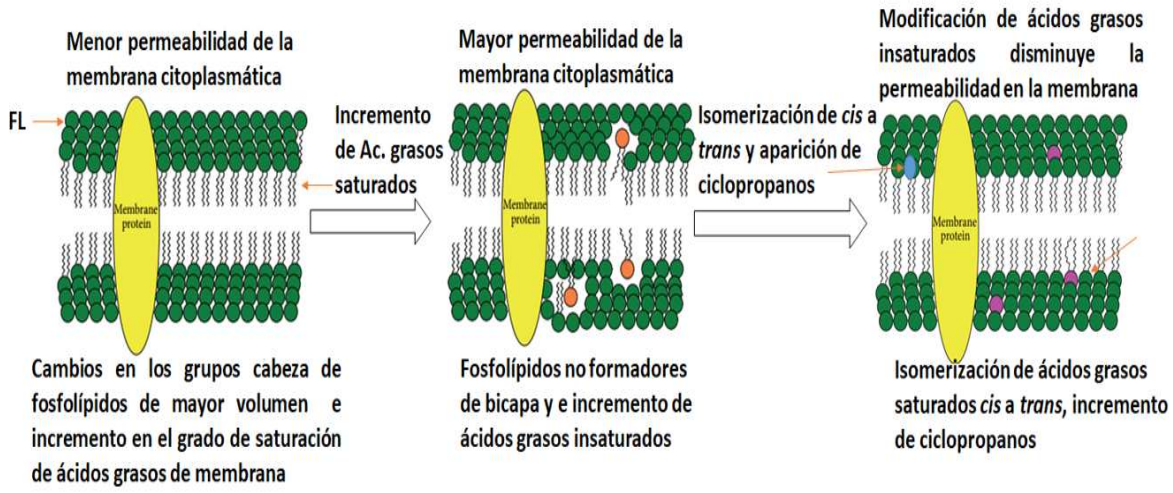


Figura 2. Mecanismos que incrementan la estabilidad en la membrana bacteriana bajo condiciones de estrés. (FL: Fosfolípidos).

La salinidad perturba la cantidad de los microorganismos beneficiosos asociados con la rizósfera de la planta, sin embargo, las PGPR tolerantes a la sal tienen sus propios mecanismos de halotolerancia y podrían proporcionar un beneficio significativo para las plantas cultivadas en suelos salinos, en términos de crecimiento y rendimiento.

6. JITOMATE (*Solanum lycopersicum* L. cv Saladette)

EL tomate o jitomate rojo (*Solanum lycopersicum* L.), pertenece a la familia de las *Solanaceae*, es la hortaliza de mayor importancia a nivel nacional, por el área total cosechada y el valor económico de la producción (Escobar y Lee, 2009).

La producción mundial en 2017 fue de 182 millones de toneladas, siendo México el principal exportador de jitomate; aportando el 24% de las exportaciones totales (TRADE MAP, 2018).

A nivel nacional se estimó, que para 2017 se produjeron 3,428 mil toneladas, siendo los principales estados productores en el ciclo primavera-verano: San Luis Potosí, Michoacán, Baja California, Zacatecas y Jalisco; mientras que otoño-invierno destacan los estados: Sinaloa, Sonora, Baja California Sur, Oaxaca y Michoacán (Figura 3) (SIAP, 2018).

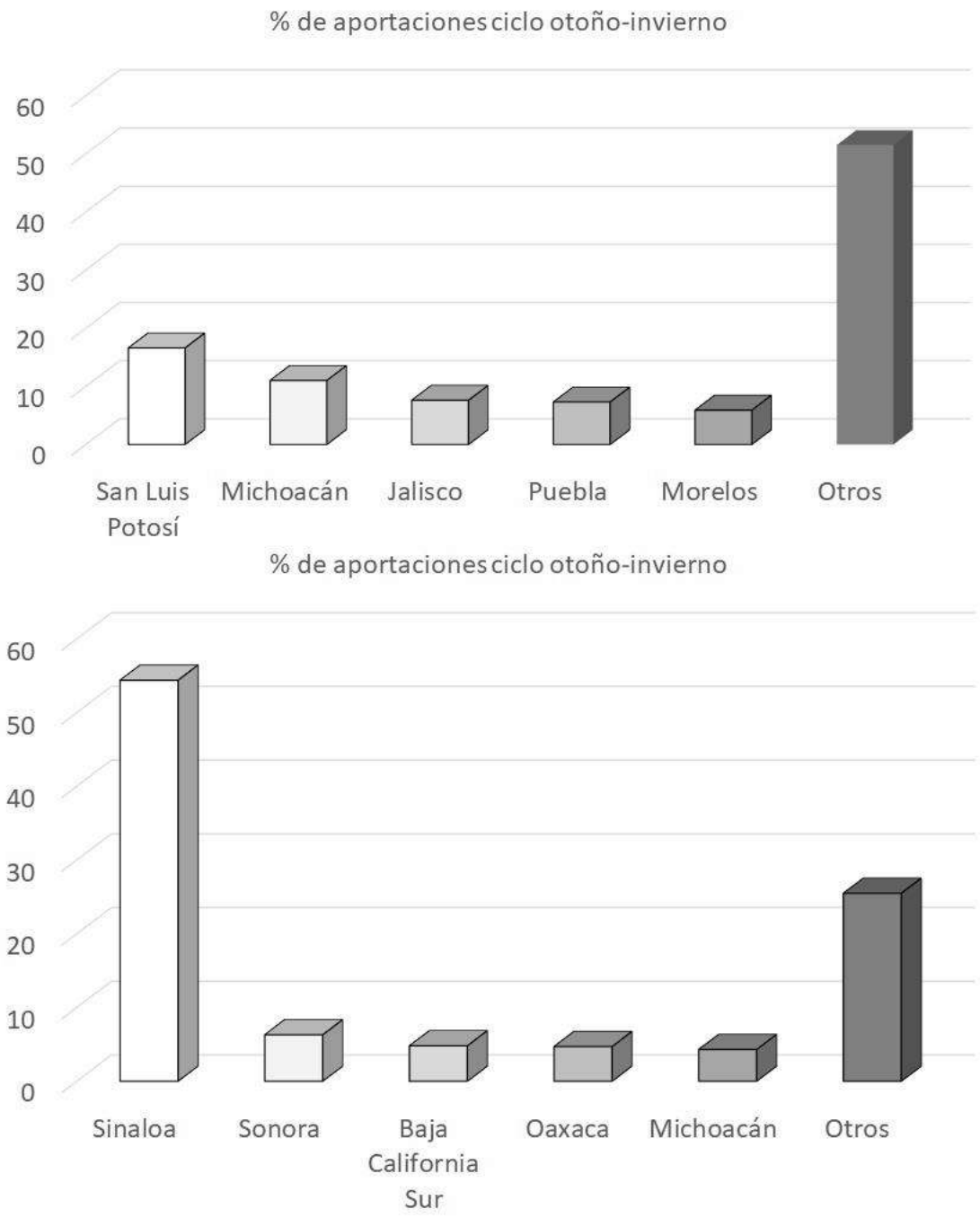


Figura 3. Principales estados productores de jitomate durante el año (SIAP, 2018).

Las principales variedades de jitomate que se producen en México son; saladette, bola y cherry, en 2015, la producción de tomate saladette representó el 79.9% del total de la producción nacional, el jitomate de bola el 16.5% y el jitomate cherry el

3.6%, siendo la variedad saladette en la cual destaca el estado de Michoacán (FIRA, 2016).

Por otro lado, los cultivos de jitomate, dependiendo de la variedad, puede ser moderadamente o altamente sensibles, a muchos tipos de estrés abiótico, incluyendo, sequía, temperaturas extremas, humedad excesiva, desequilibrio nutricional. Particularmente, a los cultivos de jitomate se les considera como plantas glicófitas, las cuales se ven obligadas a inducir sus propios mecanismos de tolerancia contra la salinidad a concentraciones de sal tan bajas como 10 mM NaCl (el grado de tolerancia depende del cultivar), para evitar efectos adversos sobre el crecimiento y rendimiento (Bui, 2003; Orcut y Nielsen, 2000).

Debido a esto es que resulta importante buscar métodos que permitan establecer los cultivos de jitomate en condiciones que no resultan óptimas para su establecimiento, adicionalmente el ciclo de vida de este cultivo permite utilizarlo como una planta modelo (Jaramillo et al., 2007).

7. ANTECEDENTES.

Estudios previos han demostrado que las PGPR tienen la habilidad de promover el crecimiento de plantas de cultivo y mejorar su nivel de tolerancia hacia condiciones salinas al mejorar la respuesta fisiológica de la planta.

El alivio al estrés salino por inoculantes PGPR se ha demostrado en arroz (Nautiyal *et al*., 2013; Mahmood et al., 2019), trigo (Egamberdiyeva, 2009; Acuña et al., 2019), maíz (Egamberdiyeva, 2007; Ferreira et al., 2018), algodón (Yao *et al*., 2010), y tomate (Mayak *et al*., 2004; Xu et al., 2014; Ali et al., 2014), entre otros.

Dentro de los géneros que mayor protección ejercen a los cultivos sometidos a estrés se destacan; *Arthrobacter, Erwinia, Serratia, Azotobacter, Azospirillum, Burkholderia, Caulobacter,* y *Chromobacterium*, destacándose a los géneros de *Bacillus* y *Pseudomonas* por su capacidad para conferir tolerancia a diversos tipos de cultivos sometidos a estrés salino (Numan et al., 2018).

Bacillus amyloliquefaciens (SQR9), contribuyó a la tolerancia a la sal en plantas de maíz al mejor su contenido de clorofila al colonizar e interactuar con las raíces de las plantas, adicionalmente SQR9 estimuló la producción de azúcares y enzimas antioxidantes dentro de los tejidos de las plantas (Chen et al., 2016).

Ansari et al., (2019), obtuvieron un aislado FB10 que posteriormente fue identificado como *Bacillus pumilus*, este aislado produjo una gran cantidad de biofilm, rasgo que le permitió colonizar de manera eficiente las raíces de plantas de maíz, la presencia de mecanismos adicionales presentados por esta cepa como la producción de AIA, actividad ACC desaminsa y la solubilización de fosfatos, permitió que plantas inoculadas con esta bacteria y sometidas a condiciones salinas presentaran un mejor crecimiento al compararlas con las plantas no inoculadas, adicionalmente las plantas inoculadas presentaron un incremento en la actividad de enzimas antioxidantes en condiciones de salinidad.

Recientemente, se obtuvieron aislados bacterianos de la planta halófita *Salicornia Europea* destacando el aislado ISE12 identificado como *Pseudomonas stutzeri*, el cual al ser inoculado en plantas de *Brassica napus* L. fue capaz de conferirle resistencia a condiciones de estrés salino además de promover el crecimiento de la planta en condiciones sin estrés, el mecanismo por el cual las plantas incrementaron diversos parámetros de crecimiento en condiciones salinas se basa en la activación de su sistema antioxidante y la reorganización de las paredes celulares (Szymańska et al., 2019).

La adición de consorcios bacterianos es otra forma de conferir resistencia a las plantas a condiciones de estrés, Samaddar y colaboradores (2019), co-inocularon a *Pseudomonas frederiksbergensi* OB139 y *Psedumonas vancouverensis* OB155 en plantas de pimiento rojo crecidas bajo diferentes concentraciones de NaCl, ambas cepas contaban con actividad ACC desaminasa por lo que en plantas inoculadas con el consorcio presentaron una reducción significativa en la cantidad de etileno, contribuyendo en la promoción de diversos parámetros de crecimiento.

Mientras que en cultivos de jitomate, recientemente, Kang y colaboradores (2019), demostraron que la interacción de *Leclercia adecarboxylata* con plantas de jitomate (*Solanum lycopersicum* L.) promueve el crecimiento de este cultivo bajo condiciones normales y les confiere tolerancia a condiciones salinas, este efecto se produce por la capacidad de la bacteria a producir ácido indol-3-acético, además de contar con la enzima ACC desaminasa, mecanismos responsables de promover de forma directa el crecimiento de las plantas de jitomate.

Por otro lado, Yoo y colaboradores (2019), obtuvieron dos aislados identificados como *Bacillus aryabhattai* H19-1 y *Bacillus mesonae* H20-5, encontrando que ambas cepas fueron capaces de promover el crecimiento y mejorar el contenido de clorofila al ser inoculadas en plantas de jitomate sometidas a estrés salino, la cepa H19-1 incrementó el contenido de carotenoides en planta, mientras que el aislado H20-5 incrementó de manera significativa el contenido de prolina y ácido abscísico en las plantas inoculadas, finalmente ambas cepas disminuyeron la fuga de electrolítos y aumentaron el contenido de Ca^{2+} a comparación de las plantas control.

Orozco- Mosqueda et al (2019), generaron mutantes en *Pseudomonas* sp. UW4, con el objetivo de evaluar el papel que juegan la enzima ACC desaminasa y la producción de osmolítos como trehalosa en conferir resistencia a plantas de tomate (*Lycopersicon esculentum* cv. Saladette), a condiciones de estrés salino, encontrando que únicamente la cepa silvestre, así como una cepa sobreexpresora de trehalosa (*OxtreS*) fueron capaces de promover el crecimiento y conferir resistencia a condiciones normales y a condiciones salinas respectivamente.

A pesar de que existen un gran número de reportes en los cuales se establece la importancia de las PGPRs, en aliviar cultivos sometidos a condiciones de estrés, en muy poco de ellos se busca dilucidar los mecanismos que les permite a las bacterias mantener sus actividades de promoción de crecimiento en condiciones que pudieran resultar adversas para su replicación y funcionamiento.

En uno de estos trabajos, Paulucci *et al.,* 2015 reportaron al aislado L115 (*Ochrobactrum intermedium*), que bajo concentraciones de 300 mM de NaCl, fue

capaz de incrementar la biomasa de plantas de *Arachis hypogaea* comparada con las plantas no inoculadas, dicha promoción se debió a la producción de IAA y sideróforos, actividad ACC desaminasa y reducción de nitratos. La cepa L115 modificó la composición de fosfolípidos, incrementando los niveles de fostatidilcolina y disminuyendo la síntesis de fosfatidiletanolamina, esto cambios favorecen un equilibrio en la bicapa y la no formación de poros, las bicapas lipídicas que se encuentran dentro de la membrana son cruciales en el mantenimiento estructural y la integridad funcional de la misma (Denich *et al*., 2003), el incremento de fostatidilcolina es un mecanismo de adaptación para mantener la estructura y función de la membrana bajo condiciones de estrés, al mejorar el plegado de proteínas de membrana.

En un trabajo más reciente Singh y Jha 2017, demostraron que la inoculación de *Klebsiella* sp. SBP-8 aminoró los efectos negativos de la sal sobre cultivos de trigo, al incrementar el contenido de prolina y disminuir la cantidad de malonialdehído en la planta, además de estimular la síntesis de enzimas antioxidantes, al buscar el mecanismo que le permite al aislado SBP-8 tolerar las condiciones de salinidad encontraron un incremento de ácidos grasos saturados de cadena larga en presencia de sal, cambios que ayudan a la membrana a mantener la integridad, fluidez y función bajo condiciones de estrés salino.

Por lo tanto, los fosfolípidos y ácidos grasos que conforman las membranas bacterianas cobran una relevancia importante y determinan procesos de interacción con plantas, bajo condiciones normales y condiciones de estrés, Vences-Guzman y colaboradores en 2008 reportaron que cepas de *Sinorhizobium meliloti* deficientes en la formación de fosfatidiletanolamina MAV01 y fosfatidilserina CS111, vieron afectada su capacidad para nodular en plantas de alfalfa (*Medicago sativa*), CS111 formó únicamente el 30% de los nódulos en comparación de la cepa silvestre *S. meliloti* 1021, mientras que MAV01 inició el proceso de nodulación 30 días después con respecto a la cepa silvestre, generando únicamente el 10% de los nódulos los cuales fueron deficiente en la fijación de nitrógeno.

En otro trabajo Lin y colaboradores (2015), demostraron que cepas de *Rhodobacter sphaeroides* deficientes en cardiolipina alteran la morfología de la célula generando una forma elipsoide, que directamente afecta la formación de biofilm, la complementación de las cepas mutantes restauró la morfología celular y aumentó la formación de biofilm, esta característica es de suma importancia durante los procesos de colonización de las plantas ya que facilita la unión de las células a la superficie de las plantas, además la formación de biofilm en condiciones de estrés es una estrategia importante adoptada por cepas bacterianas para su supervivencia en la rizósfera de la planta.

Para el siguiente proyecto se eligieron cepas de los géneros *Bacillus* y *Pseudomonas*, que han sido probadas con anterioridad en nuestro laboratorio.

Previamente, se demostró que *Pseudomonas fluorescens* UM270 fue capaz de promover el crecimiento de plántulas de *Medicago truncatula* en condiciones *in vitro*, a través de la producción de biofilm, sideróforos, proteasas, AIA y COV´s (Hernández-León et al., 20015). Por otro lado, *Bacillus toyonensis* COPE52 logró incrementar el crecimiento en plantas de arándano (*Vaccinium* spp. var. Biloxi) en condiciones de invernadero, presentando mecanismos de promoción de crecimiento como es la producción de AIA, actividad proteolítica y a la emisión de compuestos orgánicos volátiles como son el dimetil disulfuro y el 2,3-butanediol (Contreras-Pérez et al., 2019). Finalmente, se demostró que *Bacillus* sp. E25 y *Bacillus* sp. CR71, a través de la producción de los COV´s acetoína y DMDS fueron capaces de incrementar la longitud del tallo y raíz, además del contenido de clorofila y peso fresco de manera significativa, destacando que el consorcio entre ambas cepas potencializó el efecto de promoción (Rojas- Solis et al., 2018).

8. JUSTIFICACIÓN

La salinidad del suelo es uno de los principales factores abióticos que afectan las prácticas agrícolas. Para contender contra dicho estrés, las plantas tienen la capacidad de asociarse con un microbioma benéfico, incluyendo las bacterias promotoras de crecimiento vegetal. Las bacterias benéficas, a su vez, presentan diversos mecanismos de halotolerancia, incluyendo la modificación de fosfolípidos y ácidos grasos de sus membranas. Sin embargo, se desconoce si la capacidad de modular sus componentes membranales puede ser un factor importante para mantener (o mejorar) sus características benéficas, como lo son el antagonismo hacia potenciales fitopatógenos y la promoción del crecimiento vegetal en condiciones de salinidad.

9. HIPOTESIS.

Cepas de *Bacillus y Pseudomonas* bajo estrés salino modifican los fosfolípidos de membrana y mantienen sus actividades antifúngicas y promotoras del crecimiento vegetal.

10. OBJETIVOS

General

Evaluar el efecto del estrés salino sobre los fosfolípidos de membrana en diferentes cepas de *Bacillus,* y de *P. fluorescens* UM270 durante la interacción con *S. lycopersicum* L.

Específicos

1. Evaluar la halotolerancia (NaCl) de las cepas benéficas *Bacillus* sp. E25, *Bacillus* sp. CR71, *Bacillus toyonensis* COPE52 y *P*. *fluorescens* UM270.

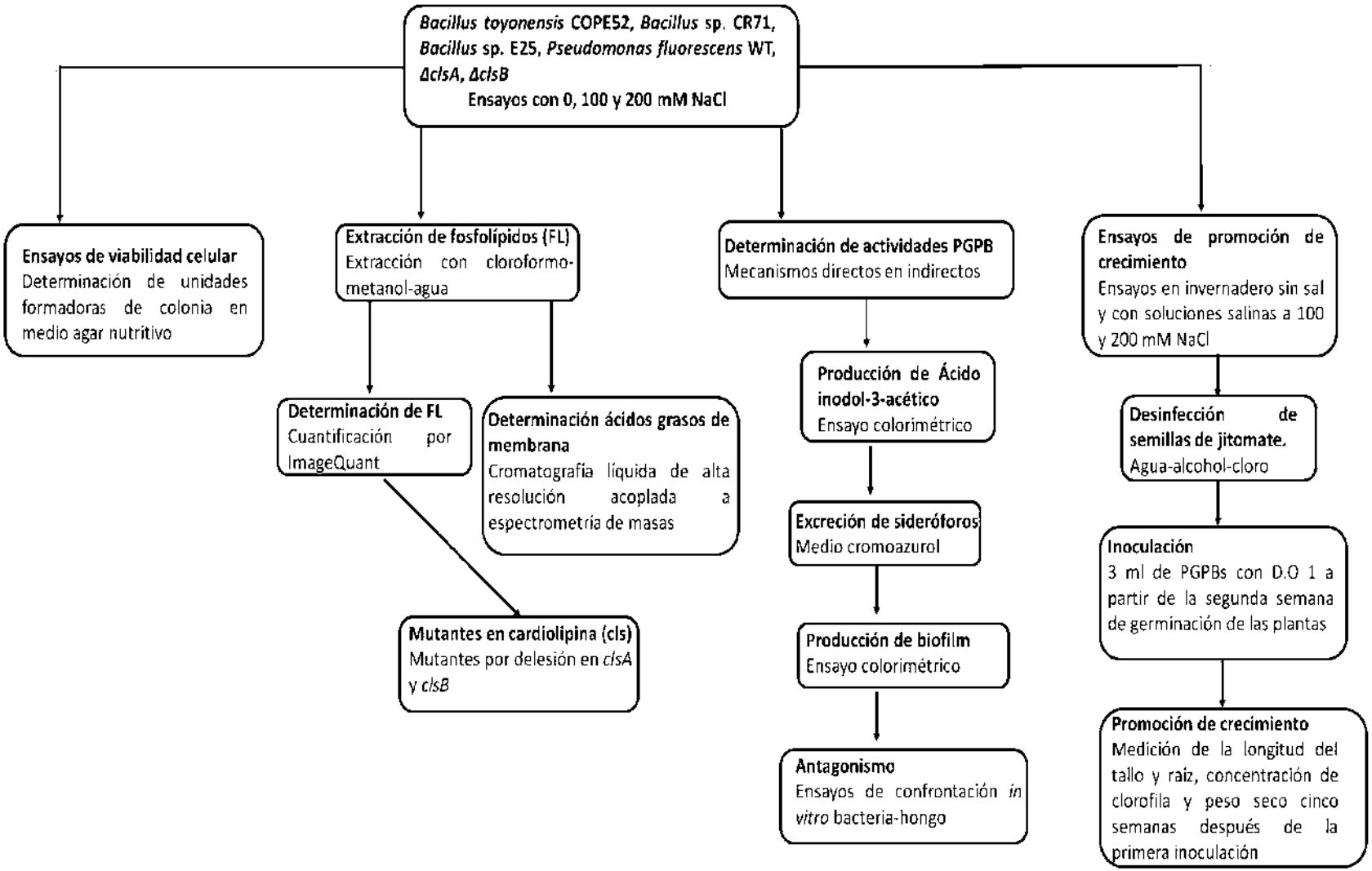
2. Determinar el efecto de la salinidad sobre los fosfolípidos y ácidos grasos de membrana en las cepas E25, CR71, COPE52 y UM270.

3. Evaluar el efecto de las mutaciones en los genes que codifican para cadiolipina sintasas (Δ*clsA* y Δ*clsB*) en *P. fluorescens* UM270 en la viabilidad celular bajo diferentes concentraciones de NaCl.

4. Analizar los mecanismos promotores del crecimiento vegetal (producción de ácido indol-3-acético, sideróforos, biofilm y antagonismo contra fitopatógenos) de las cepas E25, CR71, COPE52 y UM270 y las mutantes Δ*clsA,* Δ*clsB* de la cepa UM270 sometidas a condiciones de estrés salino.

5. Evaluar el efecto sobre el crecimiento de *S. lycopersicum* L de las cepas silvestres E25, CR71, COPE52, UM270, así como las mutantes Δ*clsA,* Δ*clsB* de la cepa UM270*,* en condiciones de estrés salino.

11. METODOLOGÍA



12. RESULTADOS

12.1 Capítulo 1

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ORIGINAL PAPER

Antifungal and Plant Growth–Promoting *Bacillus* under Saline Stress Modify their Membrane Composition

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Abstract
We explored the membrane lipid and fatty acid composition and plant growth–promoting (PGP) mechanisms of two bacterial endophytes, *Bacillus* sp. E25 and *Bacillus* sp. CR71, under saline stress, that is known to be a major detriment to crop yield. Effect of single and co-inoculation of the strains on root and shoot lengths, relative chlorophyll content, and plant biomass in tomato plants (*Lycopersicon esculentum* cv Saladette) grown in greenhouses was also evaluated. Our results show that single and co-inoculated tomato plants with bacilli strains E25 and CR71, growing under normal or saline stress conditions (100 and 200 mM NaCl), exhibited an increase in root and shoot lengths, chlorophyll content, and biomass parameters, compared with control plants (uninoculated). Additionally, these bacilli strains were antagonistic toward fungal pathogens *Fusarium oxysporum* and (to a greater extent) *Botrytis cinerea* in the presence or absence of NaCl. Interestingly, both endophytes maintained good PGP activities, producing compounds like indole-3-acetic acid (IAA), proteases, siderophores, and biofilm. Saline conditions led to changes in membrane phospholipid and fatty acid levels in both. Phosphatidylethanolamine biosynthesis and branched ($16:1^{\Delta 9}$; 17:0i) and unsaturated fatty acids increased in E25; CR71 showed increase in relative amounts of the same fatty acids and accumulated an unidentified lipid. *Bacillus* strains E25 and CR71 differentially modify their membrane phospholipid composition as a protective mechanism, potentially for maintaining PGP activities, under saline stress.

Keywords Plant growth–promoting bacteria · Soil salinity · Fatty acids · Phospholipids · Tomato plants · Antifungal action

1 Introduction

Salinity is one of the main environmental factors limiting the productivity of crop plants, thereby constituting a major global problem. It affects almost 1 billion ha of lands worldwide, which represents a little more than 6% of the planet's surface (Yensen 2008; Bui and Henderson 2003; Shrivastava and Kumar 2016). Soil is generally defined as saline if the electrical conductivity (EC) of the saturation extract (ECe) in the root zone exceeds 4 dS/m (corresponding to approximately 40 mM NaCl) at 25 °C (Pierzynski et al. 2005). Although the yield from most crop plants is reduced at this ECe, many other crops exhibit reduced yields at even lower ECes (Munns 2005; Jamil et al. 2011). Particularly, glycophytes like tomato plants (*Lycopersicon esculentum*) have been forced to employ tolerance mechanisms against salinity at salt concentrations as low as 10 mM NaCl (though the salt tolerance grade also depends on the cultivar), to avoid adverse effects on growth and productivity (Orcutt and Nilsen 2000; Bui and Henderson 2003). Such stressful growing conditions for crop plants constitute a challenge for food production. Therefore, innovative tactics should be explored to maintain the growth and production of fruits and vegetables under such conditions, especially since some countries have a predominance of soils with such salinity conditions.

Several strategies have been developed to counteract the adverse effects of saline soils in agricultural practices; one of them is the use of plant growth–promoting bacteria (PGPB) (Qadir and Oster 2004; Dimkpa et al. 2009; Saghafi et al. 2018). PGPB are commonly associated with crop plants and

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usually exert beneficial effects by stimulating plant growth and supporting plant health (Martínez et al. 2018). The promotion of plant growth by bacteria can occur by direct or indirect mechanisms. Direct promotion of plant growth occurs when a bacterium either facilitates the acquisition of essential nutrients or modulates the level of plant hormones. Indirect promotion of plant growth occurs when phytopathogenic activity is inhibited, thereby decreasing plant damage (Santoyo et al. 2012; Glick 2014). In some cases, PGPB use more than one mechanism to stimulate plant growth. For example, Orozco-Mosqueda et al. (2019) demonstrated that 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and disaccharide trehalose production in *Pseudomonas* sp. UW4 act synergistically to counteract the damaging effects of salt stress and allow the growth of tomato plants. Another mechanism of abiotic stress tolerance described in some PGPB is the modification of its membrane lipid (ML) composition. Cellular envelopes are the first barriers that confer protection to the bacterium against different environmental stresses. Therefore, their survival is determined by their capacity to adapt through the alteration of the lipid membrane composition (Aricha et al. 2004; Bakholdina et al. 2004). The ML composition can be tuned by the modification of existing ML, an example being the hydroxylation of ornithine lipids (OLs), which is important for resistance to stress conditions such as acidic pH or high temperatures in *Rhizobium tropici* (Vences-Guzmán et al. 2011). Alternatively, existing lipids can be degraded and lipids with new characteristics synthesized de novo to replace the old lipids (Sohlenkamp and Geiger 2016).

Additional mechanisms to stabilize membrane fluidity in bacteria involve changes in fatty acid (FA) composition, for example, changes in the ratios of saturated to unsaturated fatty acids, *cis* to *trans* unsaturated fatty acids, and branched to unbranched fatty acids. In addition, acyl chain length and presence of cyclopropane FA affect membrane fluidity (Ramos et al. 1997; Donato et al. 2000). *Ochrobactrum intermedium* L115 isolated from peanut rhizospheres was able to increase peanut shoot and root lengths as well as dry weight, by the production of indole-3-acetic acid (IAA) and siderophores, and supporting ACC deaminase activity. It showed tolerance to both high growth temperatures and 300 mM NaCl, apparently responding to these stresses through an increase in the degree of fatty acid unsaturation and phosphatidylcholine levels (Paulucci et al. 2015).

Species of the genus *Bacillus* are among the most common beneficial bacteria associated with crop plants, residing either endophytically or in the rhizosphere (Santoyo et al. 2019). For example, *Bacillus amyloliquefaciens* strain SQR9 is known to significantly promote the growth of maize seedlings and enhance chlorophyll content, compared with that in the control plants, after exposure to 100 mM NaCl. The underlying mechanisms could involve the enhancement of total soluble sugar content in plants, leading to decreased cell destruction and improved peroxidase/catalase activity and glutathione content for scavenging reactive oxygen species, as well as reduction in toxic Na levels in plants (Chen et al. 2016).

In this study, we hypothesize that the bacterial endophytes *Bacillus* sp. E25 and *Bacillus* sp. CR71 differentially modify their lipid and fatty acid membrane composition under saline stress, in order to potentially maintain their antifungal and plant growth–promoting mechanisms unaffected.

2 Materials and Methods

2.1 Growth Conditions for Bacterial Strains and Phytopathogenic Fungi

Bacillus sp. E25 and *Bacillus* sp. CR71 were grown at 30 °C for 24 h in Luria-Bertani (LB) medium, and routinely maintained at 4 °C. Strains E25 and CR71 were isolated as endophytes of tomato plants, and their draft genome sequences are available at the GenBank (E25 accession number CP031749.1; CR71 accession number CP031748.1). The fungal plant pathogens *Botrytis cinerea* and *Fusarium oxysporum* were inoculated and maintained on potato dextrose agar (PDA) at 30 °C for 4–5 days in darkness and thereafter stored at 4 °C; additionally, the strains were stored in 20% glycerol at − 80 °C.

2.2 Evaluation of Plant Growth Promotion by *Bacillus* Strains in a Greenhouse Under Salt Stress Conditions

Greenhouse pot experiments with tomato plants (*Lycopersicon esculentum* cv Saladette) were performed with 0, 100, and 200 mM NaCl in sterile peat moss with perlite substrate. Greenhouse experiments were carried out in a manner similar to previous reports (Orozco-Mosqueda et al. 2019). Briefly, tomato seeds were germinated, and after 1 week, seedlings of the same size were selected and transplanted into pots (one plant per pot). The experimental design included eight groups of plants (treatments): (1) control plants (24 in number) without inoculants and watered with a 100 mM NaCl saline solution; (2) 24 plants inoculated with E25 strain and watered with a 100 mM NaCl saline solution; (3) 24 plants inoculated with CR71 strains and watered with a 100 mM NaCl saline solution; (4) 24 plants co-inoculated with both E25 and CR71 and watered with a 100 mM NaCl saline solution; (5) control plants (24) without inoculants and watered with a 200 mM NaCl saline solution; (6) 24 plants inoculated with E25 strain and watered with a 200 mM NaCl saline solution; (7) 24 plants inoculated with CR71 strains and watered with a 200 mM NaCl saline solution; and (8) 24 plants co-inoculated with both the strains and watered with a 200 mM NaCl saline solution. Throughout the experiment, the plants

were irrigated every third day with deionized water or saline solution, while constantly controlling the salt concentration by measuring electrical conductivity (Field Scout. Mod. 2265FS). Bacterial inoculants were applied every week with the exception of the control groups. Bacterial inoculants were adjusted at $A_{600} = 1.0$. After 5 weeks of plant growth, the effect of addition of each of the bacterial inoculants on the root length, aerial parts, fresh weight, dry weight, and chlorophyll concentration was evaluated. The chlorophyll concentration was measured in at least three leaves from each plant (Orozco-Mosqueda et al. 2019).

2.3 Determination of Plant Growth–Promoting Traits Under Salt Stress

2.3.1 Siderophore Production

The production of siderophores was evaluated in a chrome azurol S (CAS) agar medium (Santoyo et al. 2019) supplemented with 0, 100, and 200 mM of NaCl. All experiments were performed in triplicates.

2.3.2 Colorimetric Estimation of IAA (and Similar Compounds)

The IAA content was determined based on the method described by Patten and Glick (2002), with some modifications. Briefly, 25-ml flasks were inoculated, supplemented with a range of NaCl concentrations (0, 100, and 200 mM) and kept at 30 °C on a rotary shaker maintained at 150 rpm. Cells were then collected by centrifugation at 10,000×*g* for 15 min and 2 ml of Salkowski reagent was added to the supernatant. The absorbance corresponding to the pink auxin complex was recorded at 540 nm in a UV–VIS spectrophotometer (JENWAY 7305). The calibration plot was generated using dilutions of a standard total indole (Fluka, Switzerland) solution and the uninoculated medium with the reagent as a control. Experiments were performed in triplicates.

2.3.3 Protease Production

Proteolytic activity was determined by using skimmed milk agar (pancreatic digest of casein 5 g, yeast extract 2.5 g, glucose 1 g, 7% skim milk solution 100 ml, and agar 15 g dissolved in 1 L distilled water supplemented with 0, 100, or 200 mM NaCl). After 2 days of incubation at 30 °C, a clear zone around the cells, indicative of positive proteolytic activity, was observed (Hernández-León et al. 2015).

2.3.4 Biofilm Production

Biofilm formation capacity in bacteria was analyzed following the protocol by Wei and Zhang (2006). Briefly, analyzed strains were grown in LB medium, supplemented with or without salt (100 or 200 mM of NaCl), to an O.D. of 1 (A_{570}) and then diluted (1:1000) with fresh LB broth. A 0.5 ml aliquot of the diluted culture was then transferred to an Eppendorf tube. Bacteria were incubated without agitation for 24, 48, and 72 h at 30 °C and the produced biofilm was quantified at each time points. The biofilm was stained with 0.1% (*w*/*v*) crystal violet for 15 min at room temperature and then rinsed thoroughly with water to remove unattached cells and residual dye. Ethanol (95%) was used to solubilize the crystal violet dye. The absorbance of the solubilized dye (A_{570}) was measured with a UV–VIS spectrophotometer (JENWAY 7305). All experiments were performed in triplicates in at least two independent sets.

2.3.5 In Vitro Evaluation of Fungal Antagonism

The evaluation of fungal antagonism was performed by Petri dish bioassays, as previously reported (Hernández-León et al. 2015). Strains E25 and CR71 were co-inoculated simultaneously with the pathogenic fungi on PDA agar plates supplemented with 0, 100, or 200 mM NaCl. The bacterial strains were streaked onto plates in cross patterns, and a mycelial plug of 4 mm was deposited in the center of each of the quadrants formed. The plates were incubated in the dark at 30 °C (BOD incubator), and the mycelial growth diameter was measured at day 6. Antifungal effects of volatile organic compounds (VOCs) emitted by the *Bacillus* 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, along with a mycelial plug of *B. cinerea* and *F. oxysporum* (4 mm) in the other section. The plates were incubated in the dark at 30 °C, and mycelial growth diameter was measured at day 6. Both experiments were independently performed at least thrice. The percentage of growth inhibition was measured using the following formula: % of growth inhibition = [(Ac–Ab) / Ac] × 100, where Ac is the control mycelial area and Ab is the mycelial area with treatment.

2.4 Analysis of Membrane Components

2.4.1 Bacterial Growth for Lipid Extraction

The analysis of membrane components was carried out as previously reported (Rojas-Solis et al. 2020). Briefly, 25 ml cultures supplemented with 0, 100, or 200 mM NaCl were adjusted to an optical density (D.O.) of 0.1. After that, a 1 ml aliquot was added to a sterile tube with 0.5 μCi of [1-^{14}C] acetate (Amersham Biosciences). Then, bacterial cultures were incubated at 30 °C for 24 h while shaking. The cells of the cultures were harvested by centrifugation at 6000 rpm for 10 min at 4 °C; then, pellets were washed with water and resuspended in 100 μl water. Bacterial cells from the labelled

cultures were centrifuged for 1 min at 14,000 rpm, washed once, and resuspended in 100 μl water.

2.4.2 Extraction, Separation, and Analysis of Phospholipids

The extraction, separation, and phospholipid analysis was carried out as previously reported (Rojas-Solis et al. 2020). Briefly, bacterial lipids were extracted using a chloroform/methanol/water system. Three hundred seventy-five microliters of methanol:chloroform (2:1, *v*/*v*) was added to the suspended bacterial cells and the mixture was vortexed. After that, a 125 μl each of water and chloroform was added to obtain separation into two phases. The lower organic phase, containing the lipids, was transferred to a new tube and washed once with water, dried under N_2, and dissolved in a suitable volume of 1:1 chloroform/methanol (*v*/*v*). Lipid extract aliquots were spotted on HPTLC silica gel 60 plates (Merck, Pool, UK). Then, lipids were separated by two-dimensional thin-layer chromatography (TLC) using chloroform/methanol/water (140:60:10, *v*/*v*/*v*) as solvents for the first dimension and chloroform/methanol/glacial acetic acid (130:50:20, *v*/*v*/*v*) as solvents for the second dimension. Unlabelled and radioactive ML were visualized by iodine staining and exposure to autoradiography film (Kodak) and in a PhosphorImager screen (Amersham Biosciences), respectively. Individual lipids were quantified by using the ImageQuant software (Amersham Biosciences).

2.4.3 Analysis of Fatty Acids by Gas Chromatography

The analysis of fatty acids was carried out following a protocol as previously reported (Rojas-Solis et al. 2020). Briefly, fatty acyl methyl esters (FAME) were prepared from total lipid extracts with 10% methanol- BF_3 (Sigma) (Morrison and Smith 1964), using tridecanoic acid (C13:0) as an internal standard and analyzed using a PerkinElmer Clarus 600 gas chromatography (GC) system coupled to a Clarus 600T mass spectrometer. The gas chromatograph was equipped with an Elite-5MS column from Perkin Elmer (length 30 m; inner diameter 0.2 mm; film thickness 0.32 mm). GC conditions were as follows: 250 °C injector temperature; 300 °C detector temperature; and hydrogen as the carrier gas. The column oven temperature was programmed at 140 °C for 6 min, and then increased by 10 °C/min to 240 °C for 5 min, and finally to 250 °C for 5 min. Fatty acids were identified by comparing retention times to commercial standards (Sigma Chemical Co., USA) and by MS fragmentation in the EI mode, with an electron energy set to 70 eV.

2.5 Statistical Analysis

The results were analyzed using the STATISTICA 8.0 software, and analysis of variance and Duncan's multiple range test for mean comparison were used for multiple comparisons ($P < 0.05$). The fungus antagonism experiments were statistically analyzed by Student's *t* test ($P < 0.05$).

3 Results

3.1 Plant Growth Promotion in Greenhouse by *Bacillus* Strains

Greenhouse experiments were carried out to analyze the influence of single and co-inoculation of bacilli strains on the growth of tomato plants under control and saline conditions (100 and 200 mM NaCl) (Fig. 1). The results indicate that inoculation with the individual strains or with both *Bacillus* strains showed growth-promoting capacities (increase in plant root and shoot length, total dry weight, and chlorophyll content) under control conditions (no salt stress). Interestingly, the co-inoculation of both strains exhibited an additive effect on root length and chlorophyll content.

When 100 mM NaCl was added, the PGP effect of inoculation with *Bacillus* sp. E25 on the root and shoot lengths was maintained. On the other hand, inoculation with *Bacillus* sp. CR71 under these conditions increased both root and shoot lengths and total biomass, compared with uninoculated plants (control). The co-inoculation of both strains (E25 + CR71) promoted each of the evaluated parameters. In addition, in trials with 200 mM NaCl, inoculation with the CR71 strain significantly increased plant root and shoot lengths and total dry weight (plant biomass). Plants inoculated with *Bacillus* sp. E25 exhibited a beneficial effect in all four parameters (total dry weight, chlorophyll content, and root and shoot lengths). Finally, the co-inoculation (E25 + CR71) showed beneficial effects on tomato plants under saline stress similar to single inoculation with these strains.

3.2 Antagonism Assays Against Fungal Plant Pathogens

The antagonistic action toward the phytopathogens *B. cinerea* and *F. oxysporum* was tested under salt stress (and non-saline control conditions), either by action of diffusible (direct co-inoculation on Petri dishes) or VOCs (inoculation on divided Petri dish plates) produced by E25 and CR71 strains. In general, both endophytes showed a significant inhibitory effect on the *B. cinerea* mycelial growth diameter during direct co-inoculation bioassays. An even greater antagonistic effect was observed under saline conditions corresponding to 100 and 200 mM NaCl (58 and 63%, respectively) compared with control experiments without salt. However, when confronted with *F. oxysporum*, only strain CR71 significantly inhibited mycelial growth at 0, 100, and 200 mM NaCl, while E25 showed minimal antagonistic effect (Table 1).

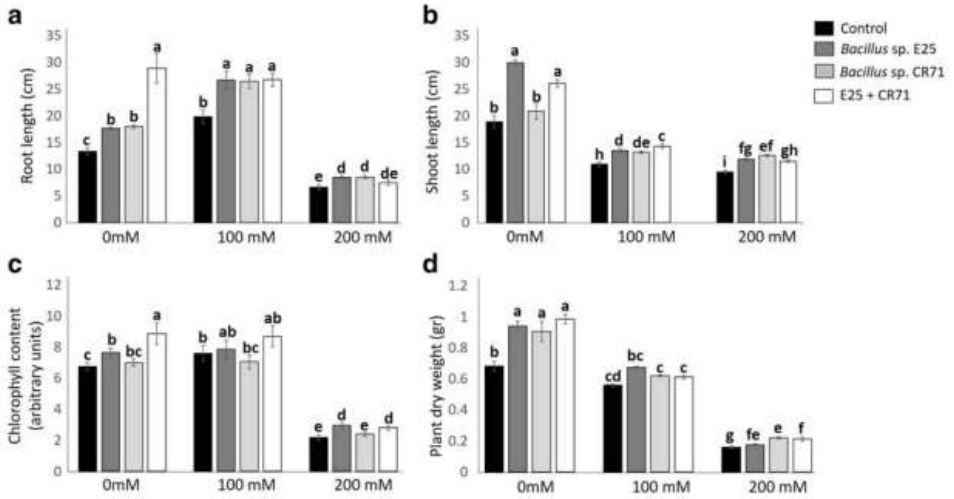

Fig. 1 Evaluation of tomato (*Lycopersicon esculentum* cv Saladette) growth–promoting effects by single and co-inoculation with *Bacillus* sp. E25 and *Bacillus* sp. CR71 strains. Images show the root length (**a**), shoot length (**b**), chlorophyll concentration (**c**), and total dry weight (**d**) of tomato plants. The bars represent the mean ± SE. Letters indicate significant differences in the mean values, estimated according to Duncan's multiple range test ($P < 0.05$)

The VOCs emitted by strain E25 significantly restricted the growth of the gray mold phytopathogen *B. cinerea* by 39% in non-saline conditions, while in bioassays supplemented with 100 and 200 mM NaCl, mycelial growth was inhibited by 14 and 21%, respectively. CR71 showed similar inhibitory action against *F. oxysporum*. When both strains were independently inoculated on divided Petri dish plates, only a slight antagonism was observed against *F. oxysporum* (Table 2).

3.3 Biocontrol and Potential PGP Traits of *Bacillus* sp. E25 and *Bacillus* sp. CR71

The bacilli strains E25 and CR71 employ diverse direct and indirect mechanisms to promote the growth of crop plants. Here, we evaluated several beneficial activities in saline conditions, including siderophore excretion, protease activity, and biofilm formation, in addition to the production of IAA (and similar compounds) as a direct mechanism of growth promotion (Table 3).

Table 1 Assessment of antifungal activity against *Botrytis cinerea* and *Fusarium oxysporum* exerted by diffusible compounds produced by *Bacillus* sp. CR71 and *Bacillus* sp. E25 grown on PDA

Treatments	Inhibition (%)		
	0 mM NaCl	100 mM	200 mM
Bacillus sp. E25			
Botrytis cinerea	36.45 ± 1.16 *	58.10 ± 1.82 *	63.44 ± 2.6 *
Fusarium oxysporum	1.46 ± 0.51	19.59 ± 0.26 *	2.90 ± 0.34
Bacillus sp. CR71			
Botrytis cinerea	45.79 ± 1.6 *	57.12 ± 1.5 *	65.95 ± 4.4 *
Fusarium oxysporum	9.52 ± 0.63*	19.25 ± 0.19 *	9.47 ± 0.13*

Fungal growth diameter is presented as the mean of at least three independent replicates compared with the control experiment (without bacterial inoculation). Statistically significant growth inhibition was observed between treatment and control experiment (without bacterial inoculum) marked by asterisks; Student's *t* test $P < 0.05$

Table 2 Assessment of antifungal activity against *Botrytis cinerea* and *Fusarium oxysporum* exerted by volatile organic compounds (VOCs) produced by *Bacillus* sp. CR71 and *Bacillus* sp. E25 grown on PDA

Treatments	Inhibition (%)		
	0 mM NaCl	100 mM	200 mM
Bacillus sp. E25			
Botrytis cinerea	39.65 ± 1.13 *	14.28 ± 0.89 *	21.55 ± 0.71 *
Fusarium oxysporum	7.69 ± 0.7*	7.33 ± 0.36	4.04 ± 0.8
Bacillus sp. CR71			
Botrytis cinerea	27.58 ± 0.32 *	20.89 ± 1.6 *	20.68 ± 1.81 *
Fusarium oxysporum	6.92 ± 0.26	0	1.65 ± 0.24

Fungal growth diameter is presented as the mean of at least three independent replicates compared with the control experiment (without bacterial inoculation). Statistically significant growth inhibition was observed between treatment and control experiment (without bacterial inoculum) marked by asterisks; Student's *t* test $P < 0.05$

Table 3 Summary of plant growth–promoting traits shown by *Bacillus* sp. E25 and *Bacillus* sp. CR71 in saline conditions. IAA production was measured in μg indoles/ml. Siderophores and protease secretion were measured as halo zone diameter around the bacterial colony (mm). Biofilm was measured as absorbance at 570 nm (O.D.)

Strain	Saline condition NaCl (mM)	IAA in similar compounds	Siderophore	Protease	Biofilm
Bacillus sp.E25	0	31.18 ± 1.5	13.5 ± 0.2	25.9 ± 0.5	0.027 ± 0.005
	100	20.51 ± 1.9 *	12.37 ± 0.2 *	23.5 ± 0.7 *	0.028 ± 0.005
	200	20.46 ± 1 *	9.5 ± 0.2 *	20.7 ± 0.5 *	0.026 ± 0.005
Bacillus sp. CR71	0	24.09 ± 1.5	17.75 ± 1.1	24.2 ± 0.5	0.031 ± 0.006
	100	26.22 ± 1.3	19.62 ± 0.5	22.6 ± 0.2 *	0.033 ± 0.007
	200	24.03 ± 1.4	11.75 ± 0.8 *	19.2 ± 0.5 *	0.032 ± 0.005

Data are mean values of three independent experiments. Asterisks (*) indicate significant differences relative to controls, according to Duncan's multiple range test ($P < 0.05$)

Strain E25 produced IAA and similar compounds (31.18 μg/ml) in control conditions. However, adding salt to the medium (100 and 200 mM NaCl) led to a decrease in IAA concentration to 20.51 and 20.46 μg/ml, respectively. CR71 initially produced 24.09 μg/ml of the same and did not undergo significant changes at increasing salt concentrations. Siderophore production was visualized by the formation of an orange halo in the two strains. *Bacillus* sp. E25 showed a decrease in siderophore production in a salt concentration-dependent manner, whereas *Bacillus* sp. CR71 only showed differences at the higher (200 mM) salt concentration. Although both strains were characterized by protease activity, the latter was found to diminish depending on the salt concentration in the medium. Finally, biofilm formation was quantified in both bacilli and was found to be unaffected by increasing salt concentrations in the growth medium. These results demonstrate that, although both strains exhibit a decrease in their activities in the presence of salt, these mechanisms are only moderately decreased, suggesting their potential ability to promote growth under stressful situations.

3.4 Effect of Salinity on Phospholipid Metabolism

Once the E25 and CR71 strains showed good PGP activities, we wanted to explore whether the strains made any modifications in their membrane composition. These changes could be helpful to these strains for tolerating the toxic effects of salt, and for potentially maintaining, as far as possible, their beneficial effects on the growth of tomato plants.

First, the viability of *Bacillus* sp. E25 and CR71 was evaluated at salt concentrations of up to 200 mM NaCl, and no significant decrease in the growth of both was observed (Supplementary Table 1). However, on exploring the ML composition of the bacteria by TLC analysis, we observed that the presence of salt in the growth medium induced substantial changes in ML composition in both strains. Under all conditions, the predominant ML were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), and an unknown lipid (NI) (Table 4 and Fig. 2). The unknown lipid migrated in a manner similar to phosphatidylcholine (PC), but could not be stained with Dragendorff's reagent, thereby ruling out the possibility that it is PC. The addition of salt (200 mM NaCl) to the growth medium of E25 caused an increase in PE concentration from 64.22 to 76.49%. In contrast, under the same conditions, a decrease in NI concentration from 7.26 to 2.42% was observed. In *Bacillus* sp. CR71, addition of salt (100 or 200 mM) to the medium led to a significant increase in the NI concentration, from 10.43% (no salt) to 17.43 and 17.64%, respectively, whereas the PE concentration decreased from 59.13% (no salt) to 46.3% in presence of 200 mM NaCl.

3.5 Effect of Salinity on Fatty Acid Composition

The FA compositions of strains E25 and CR71 are shown in Tables 5 and 6. For strain E25, the major FAs were 13-methyltetradecanoic acid (15:0i) and palmitic acid (16:0) in control conditions. Branched FAs showed the greatest changes in response to increasing salt concentrations: the relative amount of 13-methyl tetradecanoic acid (15:0i) decreased from 27.95 to 13.17% and 12.18% with 100 and 200 mM NaCl, respectively. In addition, the amount of 15-methyl hexadecanoic acid (17:0i) decreased from 14.47 to 7.05% and 10.38% in the presence of 100 and 200 mM NaCl, respectively. Finally, 11-methyl heptadecanoic acid (18:0i) content decreased under salt concentrations of up to 200 mM NaCl, from 11.83 to 8.7%.

On the other hand, the relative amount of the saturated FA stearic acid (18:0) increased from 10.63 to 26.94% and 22.21% on exposure to 100 and 200 mM NaCl, respectively. Similarly, the presence of 200 mM NaCl was observed to lead to an increase in relative content of the FA palmitic acid (16:0) 20.62 to 34.05%. In addition, the amount of the branched FA 11-methyl heptadecanoic acid (18:0i) increased from 11.83 to 22.57% at 100 mM NaCl. The growth conditions for E25 caused a shift in the ratio of branched to saturated FA, which decreased in all experimental conditions.

With regard to the strain CR71, the major FAs detected in control conditions without salt were palmitic acid (16:0), stearic acid (18:0), and myristic acid (14:0). The FA composition changed in response to increasing salt concentrations. The saturated FAs showed a reduction in the presence of

Table 4 Effect of salinity stress on the incorporation of [^{14}C] acetate into phospholipids of *Bacillus* sp. E25 and *Bacillus* sp. CR71 strains

	PL (%)	Growth conditions		
		0 mM NaCl	100 mM NaCl	200 mM NaCl
Bacillus sp. E25				
	PE	64.4 ± 5.7	63.5 ± 6.0	74.6 ± 4.6 *
	PG	24.6 ± 3.2	26.6 ± 5.6	18.6 ± 4.6
	NI	7.2 ± 2.2	8.1 ± 0.3	2.4 ± 0.9 *
	CL	3.8 ± 0.8	1.8 ± 0.4	4.4 ± 2.2
Bacillus sp. CR71				
	PE	59.3 ± 0.6	51.6 ± 4.2	46.3 ± 2.7 *
	PG	26.8 ± 1.8	28.6 ± 2.7	34.0 ± 5.9
	NI	10.4 ± 1.5	17.4 ± 3.1 *	17.6 ± 4.8 *
	CL	3.5 ± 0.7	2.4 ± 0.9	2.1 ± 0.4

PL phospholipids, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *CL* cardiolipin, *NI* no identified. Values represent means ± SE of three independent experiments. Asterisks (*) indicate significant differences relative to controls, according to Duncan's multiple range test ($P < 0.05$)

100 mM NaCl, with the relative amount of palmitic acid (16:0) and stearic acid (18:0) decreasing from 25.32 to 18.52% and 22.97 to 17.73%, respectively. The addition of 200 mM NaCl in the medium caused a decrease in the degree of saturation of the FAs, along with a decrease in 13-methyl tetradecanoic acid (15:0i) content from 10.43 to 6.86%. The relative amount of palmitic, stearic, and myristic acids decreased from 25.32 to 7.54%, 22.97 to 5.36%, and 16.97 to 12.64%, respectively. Conversely, increasing amounts of salt in the medium favored an increase in the relative amounts of branched-chain and unsaturated FAs. At 100 mM NaCl, the relative amounts of palmitic acid (16:1 Δ 9) and 13-methyl tetradecanoic acid (15:0i) increased from 12.43 to 17.23% and 10.43 to 18%, respectively. Similarly, on exposure to 200 mM

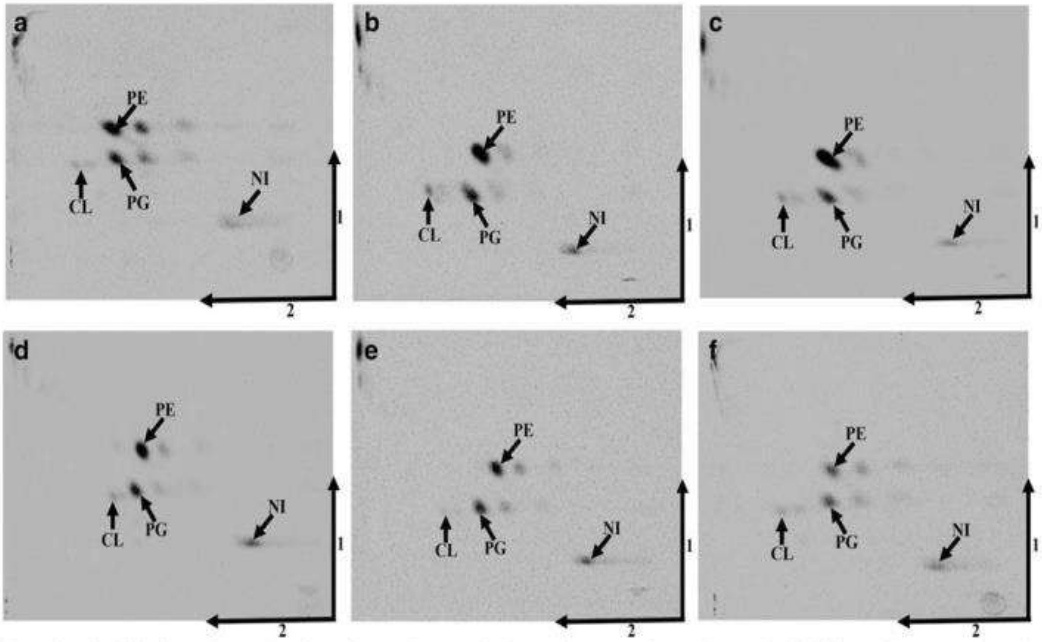

Fig. 2 Phospholipids of *Bacillus* sp. E25 and *Bacillus* sp. CR71 growing in complex LB medium supplemented with different salt concentrations. E25 with 0 (**a**), 100 (**b**), and 200 (**c**) mM of NaCl; and CR71 with 0 (**d**), 100 (**e**), and 200 (**f**) mM of NaCl

Table 5 Effect of salinity stress on fatty acid composition of *Bacillus* sp. E25

Fatty acid (%)	Growth conditions		
	0 mM	100 mM	200 mM
Saturated			
Palmitic acid (16:0)	20.62 ± 0.56	18.33 ± 1.31	34.05 ± 1.97*
Stearic acid (18:0)	10.63 ± 0.54	26.94 ± 1.54 *	22.21 ± 5.04 *
Sum of saturated FA	31.25	45.27	56.26
Unsaturated			
Palmitoleic acid (16:1 Δ 9)	14.51 ± 0.16	11.94 ± 1.32	12.47 ± 3.03
Branched			
13-methyl tetradecanoic acid (15:0i)	27.93 ± 0.87	13.17 ± 0.19 *	12.18 ± 1.11 *
15-methyl hexadecanoic acid (17:0i)	14.47 ± 0.31	7.05 ± 2.35 *	10.38 ± 0.40 *
11-methyl heptadecanoic acid (18:0i)	11.83 ± 1.40	22.57 ± 0.24 *	8.7 ± 0.89*
Sum of branched FA	54.23	42.79	31.26
U/S[a]	0.46	0.26	0.36
B/S[b]	1.73	0.94	0.55

Lipids were extracted and total lipid fatty acids were converted to methyl esters and analyzed by GC as described in the text. Percentage of each fatty acid is relative to total fatty acids defined as 100%. Values represent means ± SE of three independent experiments. Asterisks (*) indicate significant differences relative to controls, according to Duncan's multiple range test ($P < 0.05$)

[a] Ratio between sums of unsaturated and sums of saturated fatty acids

[b] Ratio between sums of branched and sums of saturated fatty acids

NaCl and palmitic acid (16:1 Δ 9) and 15-methyl hexadecanoic acid (17:0i) content increased from 12.43 to 32.6% and 11.87 to 35%, respectively.

In summary, changing growth conditions of the CR71 strain caused a shift in the ratio of saturated to unsaturated FAs (U/S[a] in Table 3), which increased from 0.19

Table 6 Effects of salinity on fatty acid composition of *Bacillus* sp. CR71

Fatty acid type (%)	Growth conditions		
	0 mM	100 mM	200 mM
Saturated			
Myristic acid (14:0)	16.97 ± 0.09	15.07 ± 4.20	12.64 ± 0.37 *
Palmitic acid (16:0)	25.32 ± 1.84	18.52 ± 2.02 *	7.54 ± 1.43 *
Stearic acid (18:0)	22.97 ± 0.96	17.73 ± 1.52 *	5.36 ± 0.06 *
Sum of FA saturated	65.26	51.32	25.64
Unsaturated			
Palmitoleic acid (16:1 Δ 9)	12.43 ± 0.09	17.23 ± 0.33 *	32.6 ± 1.27 *
Branched			
13-methyl tetradecanoic acid (15:0i)	10.43 ± 1.16	18 ± 4.13 *	6.86 ± 0.33 *
15-methyl hexadecanoic acid (17:0i)	11.87 ± 1.60	13.44 ± 1.2	35 ± 0.79 *
Sum of FA branched	22.3	31.44	41.86
U/S[a]	0.19	0.33	1.2
B/S[b]	0.34	0.61	1.6

Lipids were extracted and total lipid fatty acids were converted to methyl esters and analyzed by GC as described in the text. Percentage of each fatty acid is relative to total fatty acids defined as 100%. Values represent means ± SE of three independent experiments. Asterisks (*) indicate significant differences relative to controls, according to Duncan's multiple range test ($P < 0.05$)

[a] Ratio between sums of unsaturated and sums of saturated fatty acids

[b] Ratio between sums of branched and sums of saturated fatty acids

to 0.33 and 1.2 in the presence of 100 and 200 mM NaCl, respectively. The ratio of branched-chain FAs (B/S in Table 3) also changed, increasing from 0.34 to 0.61 and 1.6 in 100 and 200 mM NaCl-containing médium, respectively.

4 Discussion

Soil saline conditions inhibit the growth of crops, resulting in a diminished production. One strategy to overcome this damaging effect is to inoculate plants with PGPB, either of rhizosphere or endophytic origin. The ability of several *Bacillus* species to promote the growth of crop plants, as well as to antagonize phytopathogens, has been widely documented (Mendis et al. 2018). Several advantages over other genera have been reviewed, including their excellent rhizosphere and endophyte colonization capacity, high tolerance to harsh abiotic conditions, and of course, their ability to sporulate, which facilitates their commercial distribution and storage for long periods of time (Santoyo et al. 2012, 2017).

Previously, we have showed that strains E25 and CR71 have beneficial effects when inoculated to tomato plants (*Lycopersicon esculentum* cv Saladette), in addition to antagonizing the mycelial growth of *B. cinerea* (Rojas-Solis et al. 2018). Here, it is showed that single and co-inoculation of tomato plants with these strains promote plant growth under saline conditions (mainly at 100 mM NaCl). The ability of both the strains to promote the growth of tomato plants could be attributed to the production and emission of diverse volatile organic compounds (VOCs), including IAA, acetoin, and 2,3-butanediol (Rojas-Solis et al. 2018). Mohamed and Gomaa (2012) reported that *Bacillus subtilis* and *Pseudomonas fluorescens* maintained the capacity to produce IAA and siderophores in salt stress, which supported the growth of radish plants (*Raphanus sativus*). Both of these abilities (IAA and similar product production and siderophores) are present in CR71 and E25 strains, along with their capacity to produce biofilms, which is a conserved response within the genus *Bacillus* when grown under stress (Bais et al. 2004; Kasim et al. 2016).

Interestingly, the antagonistic activity of strains E25 and CR71 against phytopathogens *B. cinerea* and *F. oxysporum* (two of the fungi that cause the greatest economic losses to tomato crops) was evaluated under saline conditions and by evaluating the action of diffusible (direct co-inoculation) or VOCs (inoculation on divided Petri dishes) produced by the *Bacillus* strains. The antagonism exerted against both phytopathogens could be mainly due to production of volatile *S*-containing compounds, such as dimethyl disulfide (DMDS), which is produced by both the strains (Rojas-Solis et al. 2018). The produced siderophores could be another inhibitory mechanism against the fungi tested here, since such antagonistic activity of these iron-chelating compounds has been previously reported in *Burkholderia cepacia* XXVI, in particular, toward the fungal pathogen *Colletotrichum gloeosporioides*, the causal agent of anthracnose in mangoes (De los Santos-Villalobos et al. 2012). However, the contribution of other diffusible compounds in PGP activities of both bacterial endophytes could not be excluded. Current global analysis of these compounds is being carried out by high-performance liquid chromatography (HPLC) mass spectrometry.

Growing in saline conditions is a stressful situation for bacteria. So, when *Bacillus* sp. E25 and *Bacillus* sp. CR7 were subjected to such harsh conditions, it was hypothesized that both bacilli would modify their metabolism, including the synthesis of membrane phospholipids (ML) and fatty acids (FA). Such mechanisms to salt tolerance have been previously reported, since bacterial cells have to induce adaptive response to environmental saline conditions to maintain viability. Such adaptations include modifications of ML and FA to regulate and control membrane fluidity (Ramos et al. 1997; Härtig et al. 2005).

Here, it was observed that the strain E25 modified its ML profile in the presence of 200 mM NaCl by increasing phosphatidylethanolamine (PE) and decreasing an unknown lipid. PE provides lateral pressure to bacterial membrane bilayers and maintains the position of amino acids in proteins. It is a nonbilayer-forming lipid because of its steric conformation (small glycerol group and large acyl chain volume). The ratio between bilayer- and nonbilayer-forming lipids varies in response to environmental changes (Dowhan et al. 2008). On the other hand, the CR71 strain modified the phospholipid composition under the tested conditions by increasing the unknown lipid content and decreasing PE synthesis. This unknown lipid migrated similar to phosphatidylcholine (PC), but did not stain with Dragendorff's reagent, which is specific for betaine groups, meaning that the accumulated lipid is not PC. Paulucci et al. (2015) showed that *O. intermedium* L115 exhibited increased PC levels under high temperature and salinity (37 °C and 300 mM NaCl), and suggest that this increase of PC could indicate an important adaptive mechanism for maintaining membrane structure and function under stress conditions. In other studies, changes in the amount of cardiolipin (CL) in the membranes have been described as an important mechanism for bacterial adaptation to environmental stress (Ti-Yu and Douglas 2016). Our observations indicate that in the two strains studied here, neither PC or CL are involved in the osmotic stress response. Apparently, different bacterial species have found different ways to modify their membranes in response to saline stress. In fact, recent results with the plant growth–promoting bacterium *B. toyonensis* COPE52 show that this strain increased the relative amount of branched-chain fatty acids and accumulates an unknown membrane lipid, while PE levels are decreased under saline growth conditions (Rojas-Solis et al. 2020).

The other adaptive mechanism often used by bacteria is alteration of the fatty acid components of ML. For example, changing the ratio of long- to short-chain fatty acids could regulate membrane fluidity under adverse conditions (Murínová and Dercová 2014). Thus, *Bacillus* sp. E25 increased the proportion of saturated chain FA in the presence of 100 or 200 mM NaCl. The increase in the degree of saturation in FA in E25 correlates with earlier reports under salinity conditions, where bacterial cells have been shown to increase the amount of saturated FA, leading to increase in membrane rigidity to counteract the fluidity and permeability caused by saline conditions (Chihib et al. 2005). On the other hand, *Bacillus* sp. CR71 increased the proportion of unsaturated and branched-chain FA in the presence of NaCl. This modification increases membrane fluidity and permeability under saline conditions (Murínová and Dercová 2014). These studies, along with the present observations, highlight the importance of diverse complementary salt-adaptive mechanisms for the survival of bacteria.

5 Conclusions

In conclusion, *Bacillus* sp. E25 and *Bacillus* sp. CR71 strains promote the growth of tomato plants (*Lycopersicon esculentum* cv Saladette) in greenhouses under saline stress. In addition, both *Bacillus* strains responded differently to NaCl stress conditions by modifying their membrane composition in markedly different manners. These changes could potentially confer protection to their plant growth–promoting mechanisms. Future research priorities include identifying the unknown lipid in strain CR71 and generating mutant cell lines without/reduced major membrane lipids, such as phosphatidylethanolamine, in order to explore its more specific role during plant growth–promoting activities as endophytic bacterial strains.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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12.2 Capítulo 2

Bacillus toyonensis COPE52 modifies lipid and fatty acid composition, exhibits antifungal activity, and stimulates growth of tomato plants under saline conditions

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Abstract

Salinity is one of the most important factors that limit the productivity of agricultural soils. Certain plant growth-promoting bacteria (PGPB) have the ability to stimulate the growth of crop plants even under salt stress. In the present study, we analysed the potential of PGPB *Bacillus toyonensis* COPE52 to improve the growth of tomato plants and its capacity to modify its membrane lipid and fatty acid composition under salt stress. Thus, strain COPE52 increased the relative amount of branched chain fatty acids (15:0i and 16:1Δ9) and accumulation of an unknown membrane lipid, while phosphatidylethanolamine (PE) levels decreased during growth with 100 and 200 mM NaCl. Importantly, direct and indirect plant growth-promoting (PGP) mechanisms of *B. toyonensis* COPE52, such as indole-3-acetic acid (IAA), protease activity, biofilm formation, and antifungal activity against *Botrytis cinerea*,

remained unchanged in the presence of NaCl *in vitro*, compared to controls without salt. In a greenhouse experiment, tomato plants (*Lycopersicon esculentum* 'Saladette') showed increased shoot and root length, higher dry biomass, and chlorophyll content when inoculated with *B. toyonensis* COPE52 at 0 and 100 mM NaCl. In summary, these results indicate that *Bacillus toyonensis* COPE52 can modify cell membrane lipid components as a potential protecting mechanism to maintain PGP traits under saline-soil conditions.
Keywords: *Bacillus,* Salinity, Fatty acids, Phospholipids, Plant Growth Promotion, Bacterial Endophytes.

1. Introduction.

Soil salinity is one of the major abiotic stresses that adversely affect agricultural practices, constituting a major global problem and affecting almost 1 billion ha worldwide [1, 2]. For instance, salinity has caused a loss of approximately 65% in wheat yield in moderately saline soils [3], because it affects almost all developing aspects of plant biology including, germination, vegetative growth and reproductive stages [4, 5]. Particularly, glycophyte plants like tomato plants (*Lycopersicon esculentum*) are forced to induce their tolerance mechanisms against salinity at salt concentrations as low as 10 mM NaCl (the salt tolerance grade also depends on the cultivar), to avoid adverse effects on growth and productivity [1, 6]. To counteract the negative effects of agricultural practices in saline soils, several strategies have been developed, including selection of genotypes resistant to salt stress, genetic engineering of hypertolerant saline plants, vegetative bioremediation, utilization of better irrigation management strategies, and the use of plant growth-promoting bacteria (PGPB) [7-9]. Various PGPB have been isolated and characterized by their ability to improve the growth of plants under salt stress conditions [10]. The PGPB contain their own saline

stress tolerance mechanisms and in turn, can induce varying degrees of salt tolerance during interaction with plants through ion homeostasis, accumulation of osmolytes or by reducing ethylene and reactive oxygen species [11].

During growth in saline soils, PGPB and plants interact beneficially to survive. In some cases, PGPB exhibit synergistic effects of two plant growth mechanisms to counteract the salt stress in plants. For example, Orozco-Mosqueda and colleagues [12], demonstrated that 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and disaccharide trehalose production in *Pseudomonas* sp. UW4, act synergistically to counteract the damaging effects of salt stress and allow the growth of tomato plants.

Another mechanism of tolerance described in some PGPB is the modification of its membrane lipid components. For example, the bacterial strain L115 belonging to the genus *Ochrobactru*m, modifies the degree of unsaturation of fatty acids and increased its phosphatidylcholine levels under salt stress (and high temperature) growth conditions, allowing it to maintain growth promoting effects of *Arachis hypogaea* plants [13]. It is important to mention that the survival of bacteria in response to environmental changes is often determined by their capacity to adapt by altering the composition of the lipid bilayer. There are two types of lipid modifications: One in which the lipids can be modified to obtain a membrane with different properties. For instance, Vences-Guzman [14], described that the hydroxylation of ornithine lipids (OLs) is important for *Rhizobium tropici* to resist stress conditions such as acidic pH or high temperatures. In the second type, existing lipids are degraded and lipids with new characteristics are synthesized *de novo* replacing the old lipids [15]. Additional mechanisms to stabilize membrane fluidity in bacteria involve changes in fatty acid (FA) composition of membrane lipids such as the phospholipids (PL) [16].

Another genus of bacteria widely studied for being a promoter of plant growth under conditions of salt stress is *Bacillus* [17]. *Bacillus amyloliquefaciens* H-2-5 was able to enhance the growth of Chinese cabbage, radish, tomato, and mustard plants and additionally, strain H-2-5-mediated mitigation of short-term salt stress when tested on soybean plants that were affected by sodium chloride. H-2-5 did this by regulating gibberellin, abscisic acid, jasmonic acid, salicylic acid and increasing plant proline levels [18].

In the case of the *Bacillus toyonensis,* there are few studies describing it as a PGPB [19-21]. In fact, the bacterial endophyte strain COPE52 of *B. toyonensis* presents diverse mechanisms of growth promotion in blueberry plants, such as the production of indoleacetic acid, protease activity, and the emission of volatile compounds like acetoin, 2,3-butanediol and dimethyl disulphide, that can act as potential plant growth-promoting mechanisms [21]. However, their beneficial activities have not been analysed under conditions of saline stress.

In this study, we analysed the lipid and fatty acid composition of the bacterium *B. toyonensis* COPE52 while in growth under salt stress, as well as its growth promoting mechanisms during inoculation in tomato (*Lycopersicon esculentum* 'Saladette') plants.

2. Materials and methods

2.1. Bacterial strain and growth conditions.

B. toyonensis COPE52 was previously isolated and characterized [21]. Strain COPE52 was grown at 30°C for 24 h on Luria Bertani (LB), and routinely maintained at 4°C. The fungical plant pathogens *Botrytis cinerea* and *Fusarium oxysporum* were inoculated and maintained on potato dextrose agar (PDA) at 30°C for 4-5 days in darkness and maintained at 4°C.

2.2. Analysis of membrane components

2.2.1. Growth of the bacteria for lipid extraction

Twenty-five ml cultures with 0, 100, or 200 mM NaCl were adjusted to an optical density of 0.1. A 1 ml aliquot was transferred to a sterile tube and 0.5 μCi of [1-^{14}C] acetate (Amersham Biosciences). The cultures were incubated at 30°C while shaking for 24 h. The cells of the larger cultures were harvested by centrifugation at 6000 rpm for 10 min at 4°C. Pellets were washed with water and re-supended in 100 μl water. Cells from the labelled cultures were centrifuged for 1 min at 14,000 rpm, washed once and resuspended in 100 μl water.

2.2.2. Lipid extraction, separation, and analysis of phospholipids.

Lipids were extracted using a chloroform/methanol/water extraction [22]. 375 μl methanol:chloroform (2:1) were added to the suspended cells and the mixture was vortexed. Then, 125 μl of water and 125 μl chloroform were added to obtain a separation into lower and upper phase. The lower phase, containing the lipids, was transferred to a new tube and washed once with the water, dried under N_2, and dissolved in a suitable volume of chloroform/methanol 1:1 (v/v). Aliquots of the lipid extracts were spotted on HPTLC silica gel 60 plates (Merck, Pool, UK). Lipids were separated by two-dimensional TLC using chloroform/methanol/water (140:60:10, v/v/v) as solvents for the first dimension and chloroform/methanol/glacial acetic acid (130:50:20, v/v/v) as solvents for the second dimension. Unlabelled membrane lipids were visualized by iodine staining and radioactive membrane lipids were visualized by exposition to autoradiography film (Kodak) and in a PhosphorImager screen (Amersham Biosciences), respectively. Individual lipids were quantified using ImageQuant software (Amersham Biosciences).

2.2.3. Analysis of fatty acids by GC

Fatty acyl methyl esters (FAME) were prepared from total lipid extracts with 10% Methanol-BF_3 (Sigma) [23] using tridecanoic acid as an internal standard and analysed using a Perkin Elmer Clarus 600 gas chromatography system coupled to a Clarus 600T mass spectrometer. The gas chromatograph was equipped with a column Elite 5-MS de Perkin Elmer (length 30 m; inner diameter 0.2 mm; film thickness 0.32 mm). Gas chromatograph conditions were as follows: 250°C injector temperature, 300°C detector temperature and hydrogen as the carrier gas. The temperature was programmed at 140°C for 6 min and then increased by 10°C/min to 240°C for 5 min and finally to 250°C for 5 min. Fatty acids were identified by comparing retention times to commercial standards (Sigma Chemical Co., St. Louis, MO, USA) and by MS fragmentation in the EI mode with the electron energy set to 70 eV.

2.3. Determination of the plant growth-promoting traits under salt stress

2.3.1 Siderophore production.

The production of siderophores was evaluated on Chrome Azurol agar (CAS) medium [17] amended with 0, 100, 200 mM of NaCl. All experiments were performed in triplicate.

2.3.2. Colorimetric assay for the IAA determination.

The IAA (Indole-3-acetic acid) content was determined based on the method described by Patten and Glick [24] with some modifications. Briefly, 25 ml flasks were inoculated, supplemented with a graduated series of NaCl concentrations (0, 100, 200 mM NaCl) at 30°C on a rotary shaker at 150 rpm. Cells were then collected by centrifugation at 10,000 *g* for 15 min and 2 ml of Salkowski reagent were added to the supernatant. The absorbance of the pink auxin complex was read at 540 nm in a UV–Vis Spectrophotometer (JENWAY 7305). The calibration plot was constructed using dilutions of a standard total indole (Fluka, Switzerland) solution and the uninoculated medium with the reagent as a control. Experiments were performed in triplicate.

2.3.3. Proteases production.

Proteolytic activity was determined by using skimmed milk agar (pancreatic digest of casein 5 g, yeast extract 2.5 g, glucose 1 g, 7% skim milk solution 100 ml, agar 15 g dissolved in 1 L distilled water, with 0, 100 or 200 mM NaCl). After 2 days incubation at 30°C, a clear zone around the cells indicated positive proteolytic activity [25].

2.3.4. Biofilm production.

Biofilm formation capacity in bacteria was analysed following the protocol by Wei and Zhang [26]. Briefly, testing strains were grown in LB medium, supplemented with or without salt (100 or 200 mM of NaCl) to an O.D. of 1 and then diluted (1:1000) with fresh LB broth. A 0.5 ml diluted culture was transferred to an Eppendorf tube. Bacteria were incubated without agitation for 24, 48, and 72 h at 30°C and the biofilm was quantified at each time points. The biofilm was stained with 0.1% (w/v) crystal violet for 15 min at room temperature and then rinsed thoroughly with water to remove unattached cells and residual dye. Ethanol (95%) was used to solubilize the dye that had stained the biofilm cells. The absorbance of the solubilized dye (A_{570}) was determined with a UV–Vis Spectrophotometer (JENWAY 7305). All experiments were carried out in triplicate.

2. 3. 5. *In vitro* evaluation of fungal antagonism

The evaluation of fungal antagonism was performed as previously reported on Petri dish bioassays [25]. Bacterial isolate COPE52 was co-inoculated, simultaneously with the pathogenic fungi on PDA agar plates, amended with 0, 100 or 200 mM NaCl. Pathogenic fungal strains have been previously tested and analysed in previous studies [25]. The bacterium strain 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 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 *Bacillus* 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* and *Fusarium oxysporum* (4 mm) was inoculated. The plates were incubated in the dark at 30°C, and mycelial growth diameter was measured at day 6. All experiments were carried out in triplicate. The percentage of growth inhibition was measured using the following formula: % of growth inhibition = [(Ac–Ab) /Ac] ×100, where Ac is the control mycelial area and Ab is the mycelial area with treatment.

2.4. Evaluation of plant growth promotion by *Bacillus toyonensis* COPE52 in a greenhouse under salt stress conditions.

Greenhouse pot experiments with tomato plants (*Lycopersicon esculentum* 'Saladette') were performed with 0, 100, and 200 mM NaCl in sterile peat moss. Greenhouse experiments were carried out as previously reported [12]. Briefly, tomato seeds were germinated, and after one week, seedlings of the same size were selected and transplanted into pots (one plant was transplanted in each pot). The experimental design included the following treatments: 24 control plants (1 plant per pot) without NaCl and/or COPE52 and other 24 plants were inoculated with COPE52 strain under normal conditions (no salt addition). Another set of plants were irrigated with 100 (24 plants) or 200 mM NaCl (24 plants) and without bacterial inoculants, while other two groups of 24 plants each, were inoculated with COPE52 strain and watered with a 100 or 200 mM NaCl. Throughout the experiment, the plants were irrigated every third day with deionized water or saline solution, while constantly controlling the salt concentration by measuring electrical conductivity (Field Scout. Mod. 2265FS).

Bacterial inoculants, adjusted to 1×10^8 Colony Forming Units (CFU)/mL, were applied every week with exception of the control groups. After 5 weeks of plant growth, the effect of adding the bacterial inoculum on the root and shoot length, fresh weight, weight dry and chlorophyll concentration was evaluated. The chlorophyll concentration was measured in at least three leaves from each plant [12].

2.5. Statistical analysis.

The results were analysed using Statistica 8.0 software, and analysis of variance and Duncan's test for mean comparison was used for multiple comparisons ($P < 0.05$). The fungus antagonism experiments were statistically analysed by Student's *t*-test ($P < 0.05$).

3. Results.

3.1. Effect of salinity on phospholipid metabolism and fatty acid composition.

The cell viability of *Bacillus toyonensis* COPE52 was evaluated under salt concentrations of up to 200 mM NaCl. Here, no significant decrease in the growth of COPE52 was observed (Supplementary Table 1). Thin layer chromatography (TLC) analysis revealed that the presence of salt in the growth medium induced substantial changes in membrane lipid (ML) composition. The predominant membrane lipid was phosphatidylethanolamine (PE), followed in descending order by phosphatidylglycerol (PG), an unknown lipid (NI), and cardiolipin (CL) under non-saline conditions (Table 1 and Figure 1). The unknown lipid NI migrated as phosphatidylcholine (PC), but it did not stain with Dragendorff reagent, so we exclude that it is PC (not shown). ML patterns for COPE52 were qualitatively similar for all experimental conditions, but quantitative changes were observed for individual ML. The addition of salt to the growing medium of COPE52 caused an increase in NI from 11% to

21% with 100 mM, and with 200 mM NaCl the NI increased from 11.5% to 20%. A decrease was observed for PE under saline conditions from 60% to 49% with 100 mM NaCl, and from 60% to 47% with 200 mM of NaCl.

The FA composition of COPE52 strain is shown in Table 2. The major FA was stearic acid (18:0), palmitoleic acid (16:1 Δ 9) and 14-methyl pentadecanoic acid (16:0i) in control conditions without salt. The FA composition changed in response to increasing salt concentrations: with 100 mM NaCl, the relative amount of stearic acid (18: 0) decreased from 30.3% to 16.09%, whereas myristic acid (14: 0) increased from 10.87% to 16.23% and the branched chain FA 13-methyl tetradecanoic acid (15: 0i) and 15-methyl hexadecanoic acid (17: 0i) increased from 7.89% to 12.07%, and from 5.81% to 16.09 % respectively. At 200 mM NaCl, 14-methyl pentadecanoic acid decreased from 18.58% to 7.84%, while palmitoleic acid (16: 1 Δ 9) declined from 19.21% to 8.44%, myristic acid (14: 0) and stearic acid (18: 0) declined from 10.87% to 6.47% and from 30.03% to 12.28% respectively. On the other hand, palmitic acid increased from 7.61% to 16.46%, as did the branched chain FA 13-methyl tetradecanoic acid (15: 0i) and 15-methyl hexadecanoic acid (17: 0i) whose percentages increased from 7.89% to 24.24%, and from 5.81% to 24.27%, respectively. In summary, changing growth conditions applied to COPE52 caused a shift in the ratio of saturated to branched chain FA (B/S in Table 2), which increased from 0.66 to 1.02 under 100 mM NaCl condition and from 0.66 to 1.6 in 200 mM NaCl.

3.2. Biocontrol and potential PGP traits of *Bacillus toyonensis* COPE52

The potential of biocontrol and plant growth-promoting determinants was analysed in COPE52, such as siderophore excretion, protease activity and biofilm formation, in addition

to the production of IAA (Table 3). *Bacillus toyonensis* COPE52 did not produce siderophores, and the production of IAA was maintained without significant difference with or without NaCl, while the biofilm formation was slightly increased in the presence of additional salt, specifically at 200 mM of NaCl. The proteolytic activity was the only activity that suffered a decrease (Table 3).

The antagonism experiment in no-salt conditions showed that the strain COPE52 inhibits the growth of the mycelium of *Botrytis cinerea* through diffusible and volatile compounds, by 10.89 % and 18.12 %, respectively. This degree of antagonism was maintained in saline conditions with 100 mM NaCl (10.71 %), but not at 200 mM during the direct co-inoculation experiment (3.06 %). Interestingly, volatile compounds of COPE52 were able to significantly inhibit the diameter of the *B. cinerea* mycelium with or without salt. With respect to the phytopathogen *F. oxysporum*, the strain *B. toyonensis* COPE52 restricted the diameter of the mycelium only through diffusible compounds and under control conditions (8.23 %) and with salt at 100 mM (10.61 %) (Table 4). These results indicate that strain COPE52 maintains its biocontrol and PGP in saline conditions, mainly at the concentration of 100 mM.

3.3. Effect of *Bacillus toyonensis* COPE52 inoculation on tomato plants under salt stress.

To further evaluate the plant growth-promoting capacity of *B. toyonensis* COPE52, tomato plants were inoculated with strain COPE52 under three different conditions (0, 100 and 200 mM NaCl) (Figure 2A). The inoculation of COPE52 significantly improved the root and shoot length, as well as the chlorophyll concentration, while the dry weight remained unchanged in control conditions. In the case of plants subjected to salt stress at 100 mM, it was observed that the length of the root and the stem were significantly increased. The

chlorophyll concentration was similar but interestingly, in the presence of COPE52 the biomass (dry weight) of the tomato plants under these salinity conditions was increased. At 200 mM, the strain of *B. toyonensis* alone increased the length of the root significantly (Figure 2). This result suggests that COPE52 have the ability to promote the growth of tomato plants under saline conditions (100 mM of NaCl).

Discussion

Bacteria regulate the fluidity of their membrane in response to several abiotic factors, including saline conditions [27]. Here, we analysed the phospholipid (PL) and fatty acid (FA) concentrations in the plant growth-promoting endophytic bacterium *Bacillus toyonensis* COPE52, and how its direct and indirect PGP mechanisms remain active under salt stress conditions.

Phospholipids are a major component of bacterial membranes, and changes in membrane lipid composition can affect important cellular processes such as metabolism, stress response, antimicrobial resistance, and virulence [28]. The COPE52 strain modified the phospholipids composition under tested conditions by increasing an unknown lipid (NI) and decreasing phosphatidylethanolamine (PE) synthesis. This unknown lipid migrated similarly to PC, but did not stain with Dragendorff reagent which is specific for betaine groups, meaning that the accumulated lipid is not PC. Paulicci and collaborators [13], showed that *Ochrobactrum intermedium* L115 presents with increased PC levels under high temperature and salinity (37°C plus 300 mM NaCl), and this increase of PC could indicate an important adaptive mechanism for maintaining the structure and function of the membrane under stress conditions. However, changes in the amount of CL in the membranes, is perhaps the most

important mechanism for bacterial adaptation to environmental stress [29]. For example, CL accumulates in *E. coli* cells in response to osmotic stress, which in turn promotes the polar localization of transporter ProP. ProP is denoted an osmosensory transporter because it is activated by increasing osmolarity and regulates the concentrations of organic osmolytes as proline in the cytoplasm [15]. López et al [30], described a CL-deficient *Bacillus subtilis* strain, finding that this mutant is unable to grow in high salt concentrations. This result clearly indicates that the presence of CL in the membranes of *B. subtilis* is important for high salinity adaptation. The other adaptive mechanism often used by bacteria is alteration of membrane fatty acids (FA) [31]. *Bacillus toyonensis* COPE52 increased its proportion of branched chain FA in the presence of 100 and 200 mM NaCl. The increase in the proportion of branched FA increased the fluidity and permeability of the membrane in saline conditions [32], therefore, it is important to present other mechanisms that allow the cells to adapt to salinity. Haque and Russell [33], determined changes at the level of FA in seven strains of *Bacillus cereus*, finding that strain BR2853-5, when grown in a medium with NaCl (7 %, w/v), increases to more than double the amount of the branched FA 13-methyl tetradecanoic acid (15: 0i). Such a report is consistent with the results obtained in this study. However, the changes that occur at the level of membrane FA in bacteria are generally conditioned by the type of stress to which they are subjected [34], that determined the composition of fatty acids of *Bacillus thuringiensis* under triclosan stress, where results showed increased concentrations of myristic acid, palmitoleic acid, palmitic acid and linoleic acid in the treatment samples with triclosan, as opposed to results in COPE52 wherein, the unsaturation level decreased. This indicates that the observed adaptative mechanism presented at the FA level are different under several stress conditions. It is important to mention that, myristic acid increased its production at 100 mM NaCl, but decreased when COPE52 cells were

subjected to 200 mM NaCl. It has been observed with other bacilli strains that myristic acid similarly decreases its proportion as cell grow in media supplemented with salt (200 mM) (Rojas-Solís et al., 2020. Unpublished results). An explanation for this result is not known at this point.

The ability of several *Bacillus* species to antagonize phytopathogens, as well as promoting the growth of crop plants has been widely documented [35]. However, there are few reports about the beneficial effects exerted by strains of *B. toyonensis* on plants [19-21]. For example, Lopes and colleagues [19] described *in silico*, several genes for the synthesis of antimicrobial compounds, including bacteriocins, non-ribosomal peptides and chitinases during an *in silico* analysis of the *B. toyonensis* BAC315 genome, but no experimental evidence was evaluated. Recently, the draft genome sequence of the *B. toyonensis* COPE52 was reported, which presented its stimulating growing effects on blueberry plants (*Vaccinium* spp. var. Biloxi) in a greenhouse experiment [21]. The authors also detected the production of indoleacetic acid and volatile compounds like acetoin, 2,3-butanediol, as potential direct plant growth-promoting mechanisms. Interestingly, the strain COPE52 also produced the volatile compound and dimethyl disulfide, which has been associated with antifungal action, in particular, against the grey mould phytopathogen *B. cinerea*. Similar results were observed here, as the strain COPE52 exhibited better antagonism against *B. cinerea* and *F. oxysporum* through emission of volatile compounds. The ability of *B. toyonensis* COPE52 to promote the growth of tomato plants under saline conditions, mainly observed in growing plants at 100 mM NaCl, could be attributed to production of IAA and the volatile compounds acetoin and 2,3-butanediol. Several studies have shown that these compounds are responsible of stimulating growth of Arabidopsis and Canola plants [36, 37]. In fact, overproduction of

Indole acetic acid in the rhizobacterium *Pseudomonas* sp. UW4 showed enhanced root elongation under gnotobiotic conditions [37]. In recent reports, the role of dimethyl disulfide as a modulator compound for the root system in Arabidopsis via auxin signalling pathway was elucidated [38].

Interestingly, the formation of a biofilm in *B. toyonensis* COPE52 was increased under saline conditions. Biofilm production has been proposed as a strategy adopted by bacterial strains for their successful biocontrol and survival in a plant rhizosphere and attachment to its roots. Biofilm production is a highly conserved mechanisms within the genus *Bacillus* under stressing growing conditions [39, 40]. In a report by Kasim et al [39], the biofilm production of 20 bacterial isolates, mostly belonging to Bacilli, was evaluated under different salt concentrations. The authors observed that a large number of bacteria increased the production of biofilm presence of salt in the medium. Similar results were observed here, since *B. toyonensis* COPE52 increases the amount of biofilm as the salt was increased in the medium. Thus, biofilm production is also a potential strategy for protection and survival against toxic effects of the salt. In addition, it has been suggested that biofilm production is important to perform better plant growth promoting activities in diverse rhizobacterial species [41].

Conclusion

The plant growth-promoting bacterial endophyte *Bacillus toyonensis* COPE52 modified the membrane profile of its PL, which potentially contributes to its adaptation to saline conditions and protection of plant growth-promoting mechanisms. Future research priorities include identifying the unknown membrane lipid NI and constructing COPE52 mutants in

genes involved in the synthesis of specific phospholipids and fatty acids production, in order to unveil specific roles of each component of the cell membrane.

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Table 1. Effect of salinity stress on the incorporation of (14C) acetate into phospolipids of COPE52.

PL(%)	Growth conditions		
	0mM NaCl	100mM NaCl	200mM NaCl
PE	60.07 ± 0.135	49.04 ± 2.0 *	47.26 ± 0.95 *
PG	24.38 ± 0.91	28.07 ± 4.08	30.80 ± 2.41
CL	4.01 ± 0.80	1.90 ± 0.62	1.91 ± 1.12
NI	11.53 ± 1.58	20.98 ± 5.51 *	20.01 ± 2.58 *

PL: phospolipids, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, CL: cardiolipin, NI: no identified. Values represent means ± SE of three independent experiments. * indicate that the means differ significantly with respect to the control value (0 mM) according to Duncan's multiple range test ($p < 0.05$)

Table 2. Effects of salinity on fatty acid composition of *Bacillus toyonensis* COPE52.

Fatty acid type(%)	Growth conditions		
	0 mM	100 mM	200 mM
Saturated			
Myristic acid (14:0)	10.87± 1.69	16.23 ± 1.61 *	6.47 ± 0.94 *
Palmitic acid (16:0)	7.61 ± 0.28	11.06 ± 4.40	16.46 ± 1.86 *

Stearic acid (18:0)	30.03 ± 0.59	16.09 ± 0.94 *	12.28 ± 0.70 *
Sum of FA saturated	48.51	43.38	35.21
Unsaturated			
Palmitoleic acid (16:1 Δ 9)	19.21 ± 1.97	13.27 ± 3.60	8.44 ± 1.21 *
Branched			
13-methyl tetradecanoic acid (15:0i)	7.89 ± 0.45	12.07 ± 1.48 *	24.24 ± 0.50 *
14-methyl pentadecanoic acid (16:0i)	18.58 ± 3.71	15.19 ± 1.49	7.84 ± 0.93 *
15-methyl hexadecanoic acid (17:0i)	5.81 ± 0.28	16.09 ± 5.48 *	24.27 ± 0.24 *
Sum of FA branched	32.28	43.35	56.35
U/S[a]	0.39	0.30	0.23
B/S[b]	0.66	1.02	1.6

Lipids were extracted and total lipid fatty acids were converted to methyl esters and analysed by GC as described in the text.

Percentage of each fatty acid is relative to total fatty acids defined as 100%. Values represent means ± SE of three independent experiments. * indicate that the means differ significantly with respect to the control value (0 mM) according to Duncan's multiple range test ($p < 0.05$).

[a] Ratio between sums of unsaturated and sums of saturated fatty acids

[b] Ratio between sums of branched and sums of saturated fatty acids

Table 3. Summary of plant growth promoting traits showed by *Bacillus toyonensis* COPE52 in saline conditions

Saline Concentration NaCl (mM)	IAA production (µg/ml)	Siderophore Halo zone diameter/colony diameter (mm)	Protease Halo zone diameter/colony diameter (mm)	Biofilm Absorbance at 570nm (DO)
0	24.08 ± 0.50	N.D	28.6 ± 1.6	0.022 ± 0.001
100	21.80 ± 1.84	N.D	25.2 ± 1.15 *	0.030 ± 0.001 *
200	26.61± 0.90	N.D	19.1 ± 0.33 *	0.032 ± 0.001 *

All data are mean values of three independent experiments, error bars indicate standard error. * indicate that the means differ significantly with respect to the control value (0 mM) according to Duncan's multiple range test ($p < 0.05$). N.D= Not determinated.

Table 4. Assessment of antifungal activity against *Botrytis cinerea* and *Fusarium oxysporum* by *Bacillus toyonensis* COPE52 through difused compounds and volatile organic compounds on PDA.

Treatments	Inhibition (%)		
	0 mM NaCl	100 mM	200 mM
Difused compounds			
Botrytis cinerea	11.49 ± 0.84 *	14.70 ± 2.4 *	-3.06 ± 0.29
Fusarium oxysporum	8.23 ± 1.8	10.61 ± 0.12 *	-8.31 ± 1.05

Volatile organic compounds			
Botrytis cinerea	15.51 ± 1.5 *	14.71 ± 0.61 *	10.71 ± 3
Fusarium oxysporum	0 ± 1	-2 ± 0.25	-3.7 ± 1.25

- Negative values against phytopathogens

Fungal growth diameter is presented as the mean of at least three independent replicates 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 $P < 0.05$.

Tabla S1. The effect of salt on growth of *Bacillus toyonensis* COPE52

	Growth conditions		
Strain	0 mM NaCl (CFU/mL)	100 mM NaCl (CFU/mL)	200 mM NaCl (CFU/mL)
B. toyonensis COPE52	8.0×10^8 ($\pm 1.53 \times 10^8$) a	4.33×10^8 ($\pm 1.45 \times 10^8$) a	5.33×10^8 ($\pm 1.76 \times 10^8$) a

The experiment was performed independently three times; the standard deviation is shown in parentheses (±). Letters indicate that the means differ significantly according to Duncan's multiple range test ($p < 0.05$).

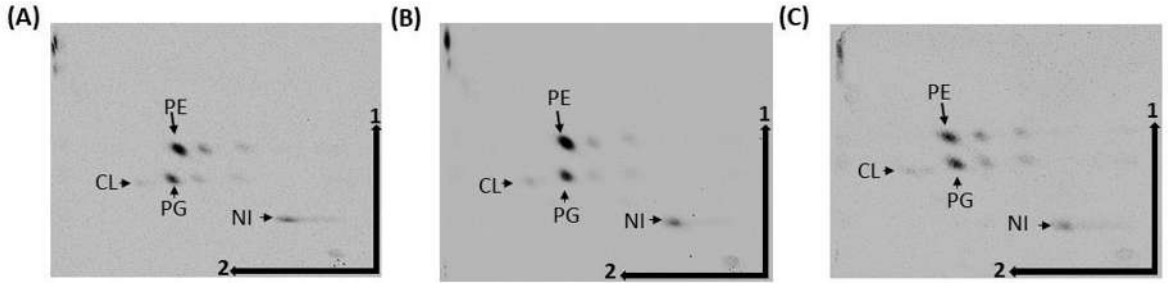

Figure 1. Membrane lipids of *Bacillus toyonensis* COPE52 growth in LB medium supplemented (or not) with different salt concentrations: 0 mM (A), 100 mM (B) and 200 mM NaCl (C).

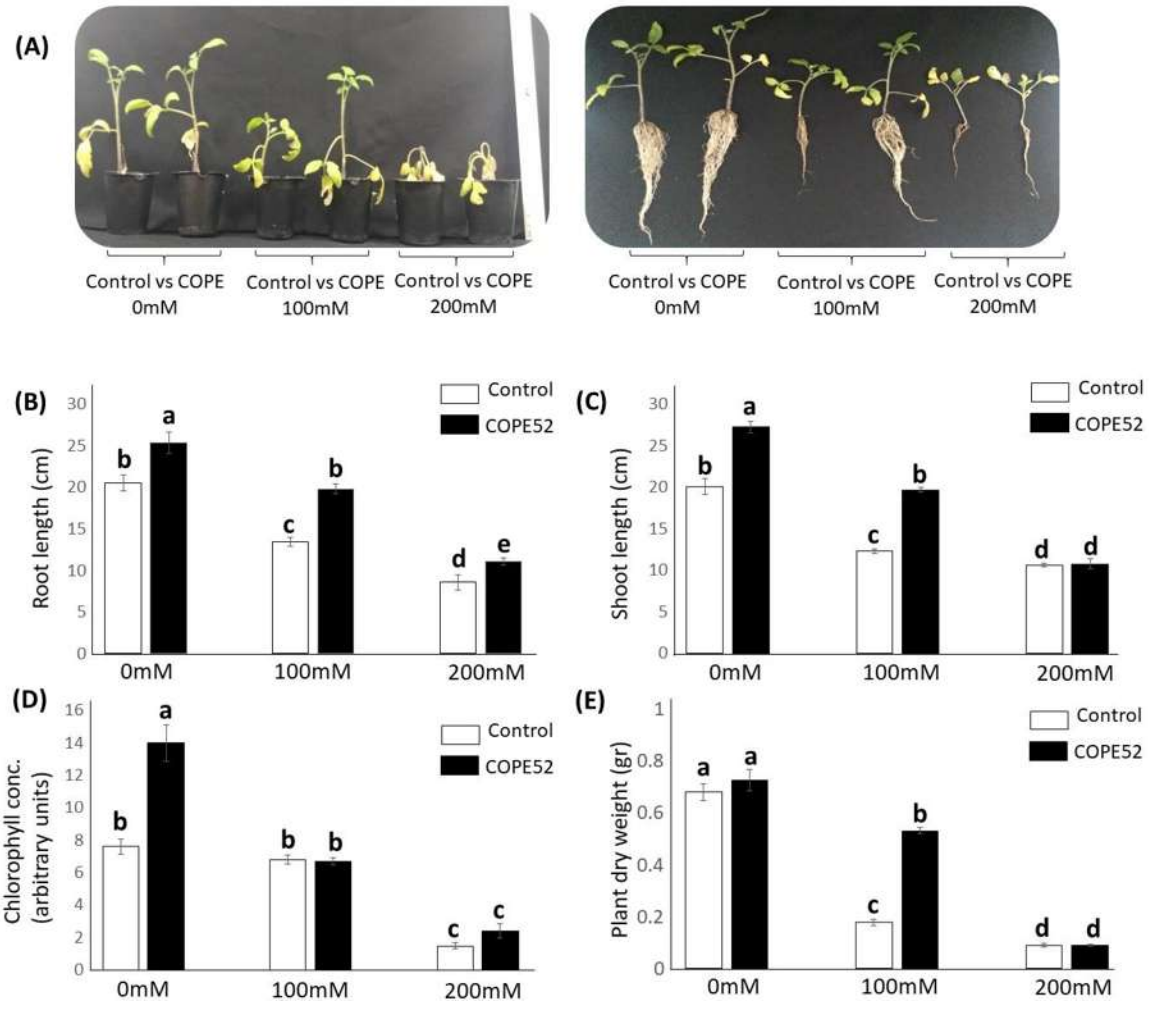

Figure 2. Effect of inoculation of isolate COPE52 on plant growth and biomass content under different treatments; 0 mM, 100 mM, and 200 mM NaCl. Representative plants of the experiment are shown in Panel (A). Graphics show the root length (B), shoot length (C), chlorophyll concentration (D) and total dry weight (E). The bars represent the values of the mean ± SE. Letters indicate that the means differ significantly according to Duncan's multiple range test ($p < 0.05$).

12.3. Capítulo 3

Pseudomonas fluorescens mutants reduced in cardiolipin synthesis, are affected in their ability to promote plant growth under normal and salt-stressed conditions

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Abstract

The membrane cardiolipin (CL) phospholipid plays a fundamental role in the adaptation of bacteria to multiple environmental conditions, including saline stress. Here, we constructed deletion mutants in two CL synthetase genes, *clsA* and *clsB*, in the rhizobacterium *Pseudomonas fluorescens* UM270, and evaluated their role on plant growth promotion under salt stress. *P. fluorescens* UM270 Δ*clsA* and Δ*clsB* mutants showed a significant reduction in CL synthesis, compared to the UM270 wild-type strain (58% Δ*clsA* and 53% Δ*clsB*), and were not affected in their growth rate, except when growing at 100 and 200 mM NaCl. Concomitant with the deletion of *clsA* and *clsB* genes, some physiological changes were observed in the UM270 Δ*clsA* and Δ*clsB* mutants, such as a reduction in the indole acetic acid. On the contrary, an increase in the siderophores biosynthesis and biofilm

production were observed. When each of the mutants, Δ*clsA* and Δ*clsB*, was inoculated in tomato plants (*Lycopersicon esculentum* cv Saladette) grown in the greenhouse, it was observed a reduction in root length only when growing at 200mM NaCl, but the shoot length, chlorophyll content and total plant dry weight parameters were significantly reduced either growing in normal or saline conditions (100 and 200 mM NaCl), compared to UM270 wild type-inoculated plants. In conclusion, these results suggest that CL synthesis in *Pseudomonas fluorescens* UM270 have important roles during promotion of tomato plant growth under normal and salt-stress conditions.

Keywords: Plant Growth-Promoting Bacteria; glycerophospholipids; siderophores; IAA.

Introduction

Salinity in soils is recognized as one of the main abiotic conditions that cause extensive losses to agricultural production worldwide (Flowers, 2004; Numan et al., 2018). A saline soil may contain salts like sulfate and chloride of Calcium (Ca), Magnesium (Mg), Sodium (Na), and Potassium (K) (Zaman et al., 2002), thus producing osmotic stress, affecting water balance, and ion homeostasis in plants. In addition, saline growing conditions alters the plant's hormonal status, disturbing transpiration, nutrient acquisition and the photosynthetic systems (Munns and Tester, 2008; Ilangumaran and Smith, 2017). However, in many regions around the world saline soils represent the only arable areas, therefore farmers require efficient strategies to cope with such stressful conditions to cultivate and generate good yields (Santoyo et al., 2019a). One strategy is making use of genetically modified plants (GMP) or salt-resistant genotypes (SRG) (Roy et al. 2014). However, not all regions in the world have easy access to such GMP or SRG, for example, developing countries. Therefore, a different

way to help plant to tolerate salt stress is to employ plant growth-promoting bacteria (PGPB) (Lugtenberg and Kamilova 2009; Numan et al., 2018). PGPB can be not only easy to isolate and characterize but are highly abundant in almost every soil or plant endosphere or rhizosphere-associated (Santoyo et al., 2019b).

Species of the genus *Pseudomonas* are among the most studied PGPB, since they have different mechanisms for promoting plant growth. For example, the production of phytohormones (e.g., indole-3-acetic acid, IAA), solubilization of nutrients in the rizhosphere or the production of stimulating diffusible or volatile compounds may directly induce plant growth (Glick, 2012). Other metabolites such as hydrogen cyanide (HCN), phenazines, siderophores, lipopeptides, 2,4-diacetylphloroglucinol (DAPG), as well as hydrolytic enzymes, such as, proteases, cellulase, chitinase and β-glucanase have shown to be effective to reduce/eliminate plant diseases caused by diverse microorganisms. This amazing diversity of compounds and enzymes is considered an indirect way to promote plant growth, as well as the ability to induce systemic resistance (ISR) in plants by *Pseudomonas* spp. (Raaijmakers et al., 2009; Weller, 2007).

Other mechanisms such as the production of biofilm and the ability to colonize in PGPB is also important to occupy spaces, thus restricting access to nutrients to potential pathogens, and at the same time, exercise direct mechanisms to promote plant growth in the rhizosphere or rhizoplane (Hernández-Salmerón et al., 2017; Rojas-Solís et al., 2016). However, under salinity conditions in soils, bacteria must keep their membranes stable and in turn, allow fluids and exchange of nutrients and metabolites, and ideally, keep PGP mechanisms active (Soltani et al., 2005). Thus, bacteria make use of specific mechanisms to adapt to environmental conditions (including salt stress), such as adjusting their membrane

phospholipid (PL) composition (Ramos et al., 1997; Kondakova et al., 2015). *Pseudomonas* spp. possesses four major PL types, including phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC) and cardiolipin (CL) (Kondakova et al., 2015; Geiger et al., 2013). CL is synthetized by cardiolipin synthase (*cls*) that catalyses the condensation of two PG molecules to yield CL and glycerol (Von Wallbrunn et al. 2002; Bernal et al. 2007). CL is a phospholipid plays an important role in cell membrane adaptation to saline stress (Murínová and Dercová, 2014; Romantsov et al., 2009; De Leo et al., 2009). For example, López et al. (2006) reported that a *Bacillus subtilis* strain deficient in producing CL (*clsA* mutant), was impaired when growing in elevated NaCl concentrations. Another major membrane lipid, like PE, has been shown to be important to the establishment of a nitrogen-fixing root nodule symbiosis in *Sinorhizobium meliloti* (Vences-Guzmán et al., 2008). In another work, a bacterial strain L115 could promote the growth of peanut (*Arachis hypogaea*) and tolerate high growth temperature and salinity by modifying the fatty acid unsaturation degree and increasing phosphatidylcholine levels (Paulucci et al., 2015).

To our knowledge, even though it is known that membrane lipid components are important for survival under osmotic stressful conditions, a specific role has not been associated with plant growth promoting activities and coding genes for membrane components in any non-symbiotic bacteria. Therefore, here we report the generation of two mutants in the synthesis of cardiolipin to be able to assign the role of this important membrane phospholipid in plant growth-promoting activities in the rizhospheric strain *P. fluorescens* UM270 under normal and salt-stress conditions.

Materials and methods.

Bacterial strains, media and growth conditions

Pseudomonas fluorescens UM270 strains were grown at 30°C for 24 h on Luria Bertani (LB), and routinely maintained at 4°C. *Escherichia coli* strains were grown on Luria-Bertani medium at 37°C. Antibiotics were added to the medium in the following concentrations when required (in micrograms per milliliter): 200 for neomycin, 100 for carbenicillin, for *P. fluorescens* and 100 for carbenicillin, 50 for kanamycin, and 20 for tetracycline for *E. coli*.

Construction of the *clsA* and *clsB* deletion mutants in *P. fluorescens* UM270

The genome sequence of *P. fluorescens* UM270 was searched for the presence of genes encoding putative cardiolipin synthases (GenBank accession number: JXNZ00000000.1). Two sequences of *P. fluorescens* UM270, with access numbers: KIQ59265.1 (*cls*A) and KIQ56391.1 (*clsB*), which encode the putative CL synthase genes *clsA* and *clsB*, were cloned, sequenced and mutated. Oligonucleotide primers CLSA01 (5´-ACTGGAATTCCCTGCGCCGGGGTCAGGCAGACGCGAA-3´) and CLSA02 (5´-ACTGGGATCCCTGGGACGCGCGGGCGGTCAAAGCTTC-3´) were used in a PCR to amplify the upstream region (~1kb) of the putative *clsA* gene from UM270, introducing EcoRI and BamHI sites into the PCR product. Similarly, primers CLSA03 (5´-ACTGGGATCCTAAGATCGTCTACGCCGCGTCCAGCCT-3´) and CLSA04 (5´-ACTGTCTAGAAACCTCGAGCAGTACTGCAAGACGCTG-3´) were used to amplify the downstream (~1kb) putative *clsA* gene from *P. fluorescens*, introducing BamHI and XbaI sites into the PCR product. After digestion with the respective enzymes, PCR products were cloned as EcoRI/BamHI or BamHI/XbaI fragments into pUC19 to yield the plasmids

(pUCD04) and (pUCD05) respectively. Then, the BamHI/XbaI fragment from (pUCD05) subcloned into (pUCD04) to yield (pUCD06). Plasmid (pUCD06) was digested with EcoRI and XbaI to subclone the regions usually flanking the *clsA* gene into the suicide vector pK18*mobsacB* (Schäfer *et al*., 1994) to yield (pK18D01). Via diparental mating using *E. coli* S17-1 as a mobilizing strain, pK18D01 was transfered into the UM270 wild-type strain. Transconjugants were selected on LB medium containing neomycin and nalidixic acid to select for single recombinants in a first step. The plasmid pK18*mobsacB* contains the *sacB* gene (Selbitschka *et al*., 1993), which confers sucrose sensitivity to many bacteria. Growth of the single recombinants on 12.5 % (w/v) sucrose will select for double recombinants and the loss of the pK18*mobsacB* vector backbone from the bacterial genome. PCR analysis confirmed that deleted the gene *clsA* was deleted. Similarly, a *clsB* deletion mutant was constructed. Oligonucleotide primers CLSB01 (5´-ACTGGAATTCCGTCGCGGCTGTCGTTCATCAGCAGCA-3´) and CLSB02 (5´-ACTGGGATCCGCGATTGTAGAAACGCAACTCCACCCC-3´), were used in a PCR to amplify about 1.0 kb of genomic DNA upstream of the putative *clsB* gene from UM270, introducing EcoR1 and BamHI sites into the PCR product. Similarly, primers CLSB03 (5´-ACTGGGATCCGGTGGACGACTGGGTCAGCATCGGCTC-3´) and CLSB04 (5´-ACTGTCTAGAGCAGTACTTCGATGACACCACCGGCCC-3´) were used to amplify about 1.0 kb of genomic DNA downstream of the putative *clsB* gene from *P. fluorescens*, introducing BamHI and XbaI sites into the PCR product. After digestion with the respective enzymes, PCR products were cloned as EcoRI/BamHI or BamHI/XbaI fragments into pUC19 to yield the plasmids pUCD07 and pUCD08 respectively. The UM270 BamHI/HindIII fragment from pUCD08 was subcloned into pUCD07 to yield pUCD09. Plasmid pUCD09 was digested with EcoRI and XbaI to subclone the regions usually flanking

the *clsB* gene into the suicide vector pK18*mobsacB* (Schäfer *et al*., 1994) to yield pK18D02. Via diparental mating using *E. coli* S17-1 (Simon *et al*., 1983) as a mobilizing strain, pK18D02 was introduced into the UM270 wild-type strain. Subsequent steps were performed as described above (similar for Δ*clsA*) for the construction of the *clsB* mutant. PCR and sequencing analysis confirmed genes *clsA* and *clsB* were deleted from UM270 genome, generating strains Δ*clsA* and Δ*clsB*. The bacterial strains and plasmids used here and their relevant characteristics are shown in Table 1.

Survival experiments on salt stress

For evaluating the survival of UM270 and mutants (Δ*clsA* and Δ*clsB*) on saline stress, strains were cultured in different concentrations of salt (0, 100 and 200 mM NaCl) in LB medium (supplemented with the respective NaCl concentrations). Growth was evaluated at an optical density (OD) at 590 nm (OD590), and determined starting off on an OD=0.1, monitoring every 5 hours the bacterial growth with a spectrophotometer.

Determination of lipid composition of *P. fluorescens* UM270 and derivative mutants

The lipid compositions of *P. fluorescens* UM270 wild-type and mutant strains were determined following labeling with [1-^{14}C] acetate (Amersham Biosciences). Twenty-five ml cultures with 200 mM NaCl were adjusted to an optical density of 0.1. A 1 ml aliquot was transferred to a sterile tube and 1 μCi of acetate was added. The cultures were incubated at 30°C while shaking for 24 h. The cells of the larger cultures were harvested by centrifugation at 6000 rpm for 10 min at 4°C. Pellets were washed with water and re-suspended in 100 μl water. Cells from the labelled cultures were centrifuged for 1 min at 14,000 rpm, washed once and resuspended in 100 μl water.

Quantitative analysis of lipid extracts

Lipids were extracted using a chloroform/methanol/water extraction (Bligh and Dyer 1959). 375 µl methanol: chloroform (2:1) were added to the suspended cells and the mixture was vortexed. Then, 125 µl of water and 125 µl chloroform were added to obtain a separation into lower and upper phase. The lower phase, containing the lipids, was transferred to a new tube and washed once with the water, dried under N_2, and dissolved in a suitable volume of chloroform/methanol 1:1 (v/v). Aliquots of the lipid extracts were spotted on HPTLC silica gel 60 plates (Merck, Pool, UK). Lipid were separated by two-dimensional TLC using chloroform/methanol/water (140:60:10, v/v/v) as solvents for the first dimension and chloroform/methanol/glacial acetic acid (130:50:20, v/v/v) as solvents for the second dimension. Unlabelled membrane lipids were visualized by iodine staining and radioactive membrane lipids were visualized by exposition to autoradiography film (Kodak) or to a Phosphor Imager screen (Amersham Biosciences). Individual lipids were quantified using Image Quant software (Amersham Biosciences).

Determination of the plant growth-promoting traits under salt stress

The IAA (Indole-3-acetic acid) content was determined based on the method described by Patten and Glick (2002), with some modifications. Briefly, 25 ml flasks were inoculated, supplemented with a graduated series of NaCl concentrations (0, 100, 200 mM NaCl) at 30°C on a rotary shaker at 150 rpm. Cells were then collected by centrifugation at 10,000 *g* for 15 min and 2 ml of Salkowski reagent were added to the supernatant. The absorbance of the pink auxin complex was read at 540 nm in a UV–Vis Spectrophotometer (JENWAY 7305). The calibration plot was constructed using dilutions of a standard total indole (Fluka, Switzerland) solution and the uninoculated medium with the reagent as a control. Experiments were performed in triplicate.

The production of siderophores was evaluated by growing wild-type and mutant strains on Chrome Azurol agar (CAS) medium supplemented (or not) with 0, 100 and 200 mM NaCl. All experiments were performed in triplicate.

The biofilm formation capacity in UM270 wild-type and mutant derivative strains was analysed following the protocol by Wei and Zhang (2006). Strains were grown in LB media, supplemented (or not) with salt (100 or 200 mM of NaCl) to an O.D. of 1 and then diluted (1:1000) with fresh LB broth. A 0.5 ml diluted culture was transferred to an Eppendorf tube. Bacteria were incubated without agitation for 72 h at 30°C and the biofilm was quantified in this time. The biofilm was stained with 0.1% (w/v) crystal violet for 15 min at room temperature and then rinsed thoroughly with water to remove unattached cells and residual dye. Ethanol (95%) was used to solubilize the dye that had stained the biofilm cells. The absorbance of the solubilized dye (A_{570}) was determined with a UV–Vis Spectrophotometer (Jenway 7305). All experiments were performed in triplicate at least twice, independently.

Evaluation of plant growth promotion by UM270, Δ*clsA* and Δ*clsB* strains under normal and salt stress conditions

Inoculating experiments of wild-type UM270, Δ*clsA* and Δ*clsB* strains in tomato plants (*Lycopersicon esculentum* 'Saladette') under a greenhouse pot experiment were performed according to Rojas-Solís et al. (2018). Briefly, the experiments were carried out in pots (6 cm tall × 5 cm wide) with sterile peat moss (Sphaigne, Canada), with or without irrigation with a salt solution of 100 and 200 mM NaCl. First, tomato seeds were germinated *in vitro*, and after 1 week, seedlings of the same size were selected and transplanted into pots (one plant was left in each pot). Bacterial inoculants dissolved in sterile deionized water were applied every week after pot transplantation according to the experimental design, which also included treatments without bacterial inoculations. The concentration of bacterial inoculants

was adjusted such that their optical density at 600 nm was 1 (~0.75–1×10^8 UFC). Throughout the experiment, the plants were irrigated every third day with deionized water or saline solution, while constantly controlling the salt concentration by measuring electrical conductivity (Field Scout. Mod. 2265FS). Each of the experimental treatments; Control, Control + NaCl and Control + each of the three strains (either UM270 WT, Δ*clsA* and Δ*clsB*) included 12 plants. The effect of each of the bacterial inoculants on the root length, shoot length, total dry weight, and chlorophyll content was evaluated after 5 weeks of plant growth. The chlorophyll concentration was measured in three leaves from each plant, as previously reported (Orozco-Mosqueda et al., 2013).

Results

Effect of salinity on phospholipid metabolism of *P. fluorescens* UM270, Δ*clsA* and Δ*clsB*

It was evaluated the synthesis and percentages of diverse membrane phospholipids in *Pseudomonas fluorescens* UM270 strain under normal and NaCl stress growth conditions. First, it was detected that wild-type UM270 strain, significantly increased the production of CL at 200 mM of NaCl, while a reduction in phosphatidylcholine (PC) was observed. Other phospholipids like phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) remained unchanged when strain UM270 was growing in presence of NaCl (Table 2, Figure 1). Interestingly, when Δ*clsA* and Δ*clsB* mutants were subject to salt stress (200 mM NaCl), a significant redution in CL synthesis was observed (58% Δ*clsA* and 53% Δ*clsB*), while an increase in the synthesis of PG and PC was noted (Table 3). These results suggest that *Pseudomonas fluorescens* UM270 increased the CL synthesis under salt stress, and that *clsA* and *clsB* genes are important for the biosynthesis of such phopsholipid (CL).

Growth of Δ*clsA* and Δ*clsB* mutants under normal and saline conditions

Given that the CL phospholipid play an important role in cell membrane adaptation to different stress environmental conditions, it was investigated the role of *clsA* and *clsB* genes in salt stress growth (100 and 200 mM NaCl) in *P. fluorescens* UM270 and derivative Δ*clsA* and Δ*clsB* mutants (Figure 2). Deletion of the *clsA* or *clsB* genes did not affect the growth in medium without salt stress; however, a similar and significant growth reduction was observed in Δ*clsA* and Δ*clsB* mutants when growing at 100 and 200 mM NaCl, particularly, during the exponential phase. This result suggests that *clsA* or *clsB* genes, which codes for CL synthesis, are important for growth of *P. fluorescens* UM270 under salt stress conditions.

Plant growth-promoting activities of *Pseudomonas fluorescens* UM270 and derivative Δ*clsA* and Δ*clsB* mutants

The deletion of the *clsA* or *clsB* gene in *P. fluorescens* UM270 had important changes in the physiology of the mutant strains, particularly related with some plant growth-promoting activities evaluated here. For example, both Δ*clsA* and Δ*clsB* mutants presented a reduction in the indole acetic acid (IAA) production. This is, under control conditions (whitout salt), the UM270 wild-type strain produced 47.97 µg/ml of IAA, while the Δ*clsA* and Δ*clsB* mutants produced 20.45 µg/ml and 25.20 µg/ml of IAA, a 53.37 and 52.53% less, respectively. When IAA production in mutant strains was analyzed under salt stress, the negative effect was more pronounced (Table 4), reducing the IAA production to 2.78 µg/ml for Δ*clsA* and 2.63 µg/ml Δ*clsB* at 200 mM of salt.

On the contrary, the synthesis of siderophores was increased in both mutants (Table 4 and Supplementary Figure 1). Both Δ*clsA* and Δ*clsB* mutants presented wider siderophores halo

production either on normal CAS medium or CAS-Salt supplemented with 100 or 200 mM NaCl. The biofilm production is increased in both mutants (Δ*clsA* and Δ*clsB*) when submitted to normal conditions (no salt) compared to WT strain; however, in salt stress conditions did not present a significant differences between the three strains.

Tomato growth-promoting experiments

Greenhouse experiments were carried out to analyze the inoculating effect of the UM270 wild-type and the mutants in CL on the growth of tomato plants under control and saline conditions (100 and 200 mM NaCl). For parameters were evaluated in the tomato plants, including root and shoot length, chlorophyll content and total plant dry weight (biomass). First, the inoculation of *P. fluorescens* UM270 WT increased the four parameters analyzed in the tomato plants, these are root and shoot length, chlorophyll content and biomass content, either under normal or a circumstance of stress caused by salt (100 or 200 mM NaCl). When the Δ*clsA* and Δ*clsB* mutants were inoculated in *Lycopersicon esculentum* grown in the greenhouse, it was observed a significant reduction in the root length (only when growing at 200mM NaCl), while the shoot length, chlorophyll content and total plant dry weight were significantly reduced, either growing in normal or saline conditions (100 and 200 mM NaCl), compared to UM270 wild type-inoculated plants. This result suggests that a mutation on either of the *cls* genes, important for the biosynthesis of the membrane cardiolipin phospholipid, affects the natural plant growth promoting traits of *P. fluorescens* UM270 (Table 5, Figure 3).

Discussion

The wild-type bacterium *P. fluorescens* UM270 is a salt-tolerant strain (up to 4% w/v of NaCl) (Hernández-Salmerón et al., 2016); however, CL plays an essential role in the

adaptation of many bacterial species to environmental stresses, including salinity (López et al., 2006; Romantsov et al., 2007; López et al., 2016). In the present study, we analyzed the role of the CL levels in the response to the saline stress in *P. fluorescens* UM270 CL-reduced productive strains, where *cls* genes have been deleted aiming to determine if mutants lose the ability to promote growth in tomato plants (*Lycopersicon esculentum* cv Saladette). The genome annotation of *P. fluorescens* UM270 (Hernández-Salmerón et al., 2016), facilitated the amplification and removal the sequences of putative *clsA* and *clsB* genes, the fact that the mutants presented a marked reduction in the CL content (58% Δ*clsA* and 53% Δ*clsB*), indicated out that both sequences were involved in the CL synthesis in *P. fluorencens* UM270. When analyzing the cell growth, the mutant strains showed a slightly reduced growth when placed in liquid medium added with NaCl, presented during the exponential phase, these results contrast a little with what has been reported, which mention that CL deficiency causes delayed growth that occurs until the stationary phase (Cronan, 2003; Czolkoss et al., 2016). Has been reported, CL its accumulates in *E. coli* cells in response to osmotic stress and promotes the polar localization of ProP, an osmosensor transporter that senses a high osmolality and regulates the concentrations of organic osmolytes in the cytoplasm (Romantov et al., 2007). Aditionally, the structure of the CL that consists of a quadruple-chained anionic amphiphile phospholipid composed of two 1,2-diacyl phosphatidate moieties esterified to the 1- and 3-hydroxyl groups of a single glycerol molecule, whose polar headgroup thus contains two phosphodiester moieties, that confer a larger headgroup volume comparate with the rest phospholipids, allow the increase CL to improve of the structural integrity of the cell membrane, allowing the bacterium to present a better cellular functioning under stress conditions (Lewis and McElhabey, 2009; Hoch, 1984; Murinová and Dercová, 2014). Interestingly, López et al. (2006), reported that a *Bacillus*

subtilis strain deficient in producing CL, was unable to grow in high salt concentrations. This result indicates that the presence of CL in the membranes of is important for salinity adaptation, nevertheless in our study we observed that the absence of CL in the *P. fluorescens* UM270 membranes, although important for the adaptation to salinity, is not essential for growth under this condition.

When evaluating mechanisms for the promotion of plant growth in the wild strain, as well as in the mutants we found that these traits are differentially modified in the different strains, the mutant strains showed a reduction in the production of AIA compared to the wild strain, Otherwise, when evaluating the production of siderophores where the mutants showed an increase in said activity, a possible explanation of the observed results is a decrease in the expression of the stationary phase transcription factor (RpoS), because both mechanisms are contrasted to what was established by Saleh and Glick (2001), where they reported to strains *Enterobacter cloacae* CAL2 and *Pseudomonas putida* UW4 that were genetically transformed with the *rpoS* gene of *P. fluorescens*, the mutant strains overexpressed this gene, synthesizing more AIA and lower siderophores production compared to unprocessed bacteria, the same behavior was reported years later by Sun et al. (2009), in a mutant strain of *Burkholderia phytofirmans* PsJN deficient in ACC deaminase activity, this presented a higher production of AIA and a lower amount of siderophores, so when analyzing the RpoS expression levels found an overexpression of this factor. That is why in our case, by decreasing the amount of AIA and increasing the production of siderophores, we suggest that both mechanisms are regulated by this transcriptional factor.

Another activity of great importance to reduce saline stress conditions is the ability of bacteria to form biofilm, the biofilm formation are a central mechanism that bacteria use to

adapt to changes in their environment, during his formation the bacteria secrete a layer of extracellular polymeric substances that encapsulates cells and protects them from environmental stress (Lin et al., 2015). In this study the biofilm formation did not present significant changes between the three conditions analyzed, surprisingly the biofilm formation increased in the CL-deficient mutants in comparation with the wild-type strain in conditions without salt, there results are different as report by Lin and collaborators (2015), which mention that CL-deficient mutants of *Rhodobacter sphaeroides* generate ellipsoid-shaped cells that directly reduce biofilm formation. This in the first report in which different PGP activities in mutants deficient in CL are evaluated.

Few studies evaluate changes at the level of membrane phospholipids in bacteria and how these modifications alter the interaction that bacteria present with plants, Vences-Guzmán and collaborators (2008) reported that PE-deficient *Sinorhizobium meliloti* strains affect their ability to nodulate in alfalfa plants. Additionally, the number of reports decreases further when plant-microorganism interactions are evaluated under stress conditions.

Particulary, the salinity is one of the main environmental factors limiting the productivity of crop plants, constituting a major global problem, affecting almost 1 billion ha worldwide, which represents a little more than 6% of the planet's surface (Yensen 2008; Bui 2013; Shrivastava and Kumar 2016). Inhibition of plant by salinity is considered to be due to toxic effects of the NaCl, to the ability of the root system to control entry of ions to the shoot and to slowing down water uptake of plants (Lambers, 2003). Specifically, glycophyte plants like tomato plants (*Lycopersicon esculentum*) are forced to induce their tolerance mechanisms against salinity at salt concentrations as low as 10 mM NaCl (the salt tolerance grade also depends on the cultivar), to avoid adverse effects on growth and productivity (Bui 2003;

Orcutt and Nielsen 2000). We evaluate the capacity for growth promotion and protection against saline stress in tomato (*Lycopersicon esculentum* 'Saladette') plants exerted by *P. fluorescens* UM270 wild-type and mutant strains. Only, the wild strain of UM270 was able to protect tomato plants from the saline maximum condition evaluated, allowing root and shoot growth in a similar way to plants grown in conditions without salt (control condition), in this conditions the root is the most important trait to contrarest for salt stress because roots are in direct contact with the soil and absorb its water for shoot supply (Jamil and Rha, 2004). The protective effect exerted by the wild strain can be attributed to the fact that this strain was the maximum producer of IAA, this hormone has a profound effect on plant growth and development, including the promotion of root formation (Yaish et al., 2015). Aditionally, in plants, auxin signalling and polar movement play an important role in root reorganization and adaptation mechanisms in response to salinity (Iglesias et al., 2011; Wang et al., 2009). Depending of many circumstances, extra production of IAA has the potential to promote the growth of the plant in interaction with the bacterium. Unfortunately, the chlorophyll content and total biomass failed to recover to the 200 mM NaCl condition, has been mentionated the saline stress lead the destruction of the chloroplast structure and stimulated the instabilty of pigment protein complexes (Sing and Dubey, 1995). It has been reported that chlorophyll content decreases in salt susceptible plants such as tomato (Lapina and Popov, 1970). The other parameter that failed to recover under salinity conditions was dry weight, the reduction in plant dry weight could be associated with reduced rate of leaf production and roots, hence leading to reduced photosynthesis, as well as unbalanced in nutrient intake due to lack of roots (Puvanitha et al., 2017).

We concluded that the modification in the membrane CL composition although important for the adaptation to salinity, is not essential for keep PGP activities and for growth under this conditions (0, 100 and 200 mM NaCl), however it plays a crucial role in *P. fluorescens* UM270 by promoting growth in tomato plants.

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Table 1. Bacterial strains and plasmids use in this study

Strain or plasmid	Relevant characteristic (s)	Reference
Strains		
Pseudomonas fluorescens UM270		
UM270 derivatives		
ClsA-	UM270 carrying a deletion in *clsA*	This work
ClsB-	UM270 carrying a deletion in *clsB*	This work

E. coli		
DH5α	*recA1* _80 *lacZ*_M15; cloning strain	Hanahan, D. 1983
S17-1	*thi pro recA hsdR hsdM*_ RP4Tc::Mu Km::Tn*7*; Tpr Smr Spcr	Simon, R., et al 1983
Plasmids		
pUC19	Cloning vector, carbenicillin resistant	Yanisch-Perron, C., et at 1985
pk18mob*sacB*	Suicide vector, kanamycin resistant	Scha¨fer, A., et al 1994
pUCD04	1 kb fragment upstream of *clsA*, cloned as EcoR1/BamHI fragment inpUC19	This work
pUCD05	1 kb fragment downstream of *clsA* cloned as BamHI/Xba1 fragment in pUC19	This work
pUCD06	1 kb upstream and 1 kb downstream sequences flanking *clsA* cloned into pUC19	This work
pK18D01	Suicide vector for construction of mutant of *ClsA*-	This work
pUCD07	1 kb fragment upstream of *clsB*, cloned as EcoR1/BamHI fragment in pUC19	This work
pUCD08	1 kb fragment downstream of *clsB*, cloned as BamHI/Xba1 fragment in pUC19	This work
pUCD09	1 kb upstream and 1 kb downstream sequences flanking *clsB* cloned into pUC19	This work
pK18D02	Suicide vector for construction of mutant of *ClsB*-	This work

Table 2. Effect of salinity stress on the incorporation of [^{14}C] acetate into phospholipids of P. fluorescens UM270 wild - type.

PL (%)	Growth conditions		
	0mM NaCl	100mM NaCl	200mM NaCl
PE	74.3 ± 5.7	69.8 ± 1.4	75.57 ± 2.8
PG	18.4 ± 4.0	22.8 ± 1.4	15.15 ± 0.8
PC	4.7 ± 0.7	4.4 ± 0.6	1.6 ± 0.7 *
CL	2.5 ± 0.7	2.8 ± 0.6	7.5 ± 0.5 *

PL: phospolipids, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PC: phosphatidylcholine and CL: cardiolipin. Values represent means ± SE of three independent experiments. * indicate that the means differ significantly with respect to the control value (0 mM) according to Duncan's multiple range test ($p < 0.05$).

Table 3. Effect of salinity stress on the incorporation of (^{14}C) acetate into phospolipids of UM270 wild-type and mutant strains.

PL (%)	UM270 Wild-type	Δ*clsA*	Δ*clsB*
PE	75.57 ± 2.8	74.9 ± 3.4	74.3 ± 3.2
PG	15.15 ± 0.8	19.5 ± 0.9	19.2 ± 0.8
PC	1.69 ± 0.7 *	2.4 ± 0.4	3 ± 0.3
CL	7.57 ± 0.5 *	3.2 ± 0.2	3.5 ± 0.3

PL: phospolipids, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, CL: cardiolipin, NI: no identified. Values represent means ± SE of three independent experiments. * indicate

that the means differ significantly with respect to the control value (0 mM) according to Duncan's multiple range test ($p < 0.05$).

Table 4. Summary of plant growth promoting traits showed by *P. fluorescens* UM270 WT and mutant strain in saline conditions.

Strain	Saline Condition NaCl (mM)	IAA secreted (µg/ml)	Siderophore production Halo zone diameter/colony diameter (mm)	Biofilm Absorbance at 570nm (DO)
P. fluorescens UM270 WT	0	47.97 ± 3.6 [a]	14.37 ± 0.31[c]	0.038 ± 0.0003 [b]
	100	30.06 ± 2.6 [b]	14.62 ± 0.42 [c]	0.051 ± 0.0003 [ab]
	200	29.32 ± 2.5 [bc]	14.5 ± 0.28 [c]	0.053 ± 0.004 [b]
P. fluorescens UM270 Δ*clsA*	0	20.45 ± 1.4 [c]	18.87 ± 0.68 [b]	0.054 ± 0.002 [ab]
	100	8.07 ± 1.9 [d]	19.12 ± 1.81 [b]	0.064 ± 0.01 [b]
	200	2.78 ± 1.4 [d]	22.62 ± 1.99 [ab]	0.053 ± 0.004 [ab]
P. fluorescens UM270 Δ*clsB*	0	25.20 ± 1.5 [c]	21.75 ± 1.12 [ab]	0.060 ± 0.003 [ab]
	100	8.57 ± 0.61 [d]	19.75 ± 1.73 [ab]	0.044 ± 0.002 [ab]
	200	2.63 ± 0.9 [d]	23.37 ± 1.28 [a]	0.054 ± 0.002 [ab]

All data are mean values of three independent experiments, error bars indicate standard error. Different letters indicate that the means differ significantly according to Duncan's multiple range test ($p < 0.05$). The statistical analysis was performed by PGP activity considering the three salinity conditions.

Table 4. Tomato plants (*Lycopersicum esculentum cv* Saladette) growth promotion by inoculation of *Pseudomonas fluorescens* UM270 and mutants strains in normal conditions and saline-stress.

Variables	Saline condition (mM NaCl)	Control	*P. fluorescens UM270* WT	*P. fluorescens UM270* *ΔclsA*	*P. fluorescens UM270* *ΔclsB*
Root length (cm)	0	14.22 ± 0.8 ^{bc}	17.5 ± 0.8 ^{a}	16.1 ± 0.9 ^{ab}	18.1 ± 1.4 ^{a}
	100	10.6 ± 0.3 ^{de}	14.72 ± 0.7 ^{bc}	15.93 ± 0.8 ^{ab}	14.14 ± 0.4 ^{bc}
	200	6.4 ± 0.4 ^{f}	14.75 ± 1.25 ^{bc}	9.18 ± 0.1 ^{bc}	5.96 ± 0.2 ^{f}
Shoot length (cm)	0	10.33 ± 0.2 ^{de}	15.75 ± 0.8 ^{a}	11.3 ± 0.6 ^{bc}	13.8 ± 0.4 ^{b}
	100	9.93 ± 0.5 ^{e}	11.83 ± 0.4 ^{cd}	10.25 ± 0.6 ^{e}	11.14 ± 0.4 ^{de}
	200	6.77 ± 0.3 ^{fg}	10.31 ± 0.3 ^{e}	7.81 ± 0.2 ^{f}	7.9 ± 0.3 ^{f}
Chorophyll content (arbitrary units)	0	4.12 ± 0.5 ^{bcde}	9.23 ± 1.2 ^{a}	5.69 ± 0.5 ^{bcd}	6.17 ± 0.7 ^{bc}
	100	2.35 ± 0.4 ^{e}	5.61 ±0.8 ^{bcd}	3.39 ± 0.7 ^{de}	4.14 ± 0.3 ^{bcde}
	200	1.97 ± 0.1 ^{e}	3.30 ± 0.2 ^{de}	3.89 ± 0.6 ^{cde}	1.92 ± 0.04 ^{e}

	0	0.116 ± 0.01 ^{e}	0.233 ± 0.009 ^{a}	0.153 ± 0.01 ^{c}	0.215 ± 0.01 ^{ab}
Plant dry weight (gr)	100	0.067 ± 0.007 ^{gh}	0.126 ± 0.009 ^{ef}	0.090 ± 0.001 ^{efg}	0.090 ± 0.009 ^{efg}
	200	0.049 ± 0.004 ^{h}	0.117 ± 0.008 ^{de}	0.058 ± 0.003 ^{h}	0.048 ± 0.003 ^{h}

Values shown are the mean of twelve independent replicates with ± standard errors values. Different letters indicate that the means differ significantly according to Duncan's multiple range test ($p < 0.05$). The statistical analysis was performed by variable considering the three salinity conditions.

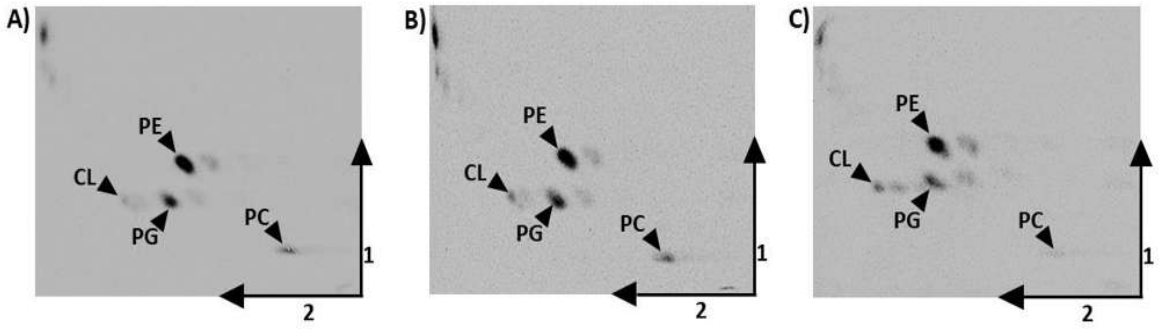


Fig. 1. Membrane lipids of *Pseudomonas fluorescens* UM270 growth in LB medium supplemented (or not) with different salt concentrations: 0 mM (A), 100 mM (B) and 200 mM of NaCl (C).

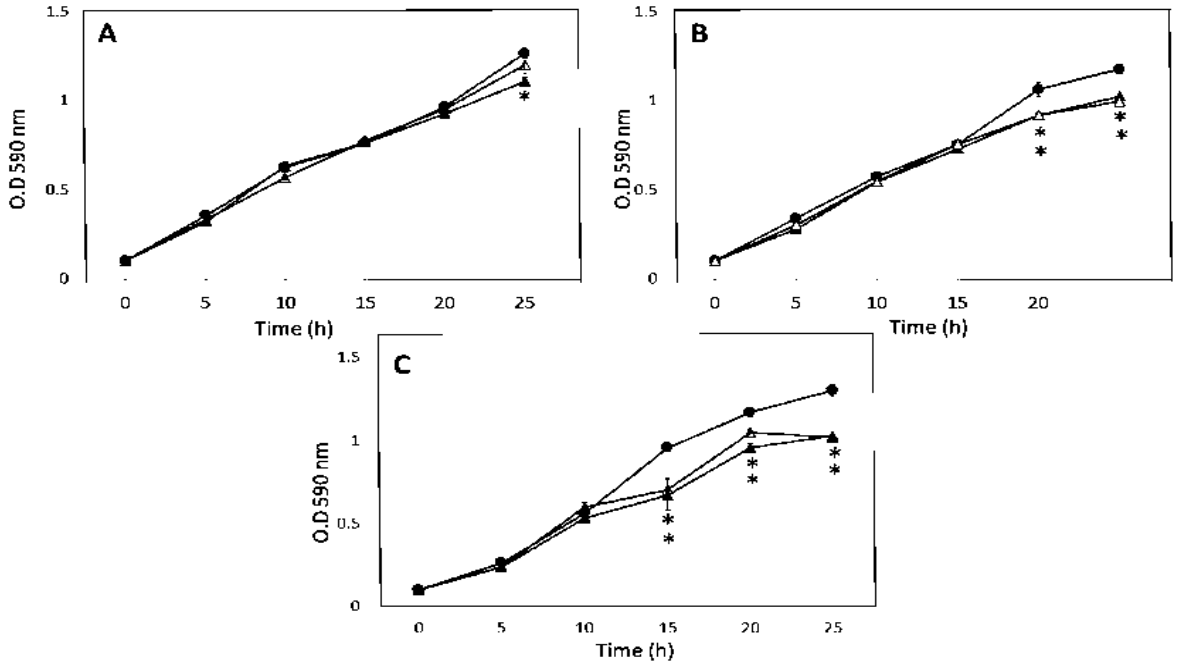


Fig 2. Growth of *P. fluorescens* mutants lacking cls is affected under saline conditions. *P. fluorescens* wild-type UM270 and mutants were grown in LB medium adjusted to 0 mM NaCl (A), 100 mM NaCl (B), or 200 mM NaCl (C). The results of a typical experiment is shown. UM270 – closed circles, *clsA*- closed triangles, clsB- open triangles.

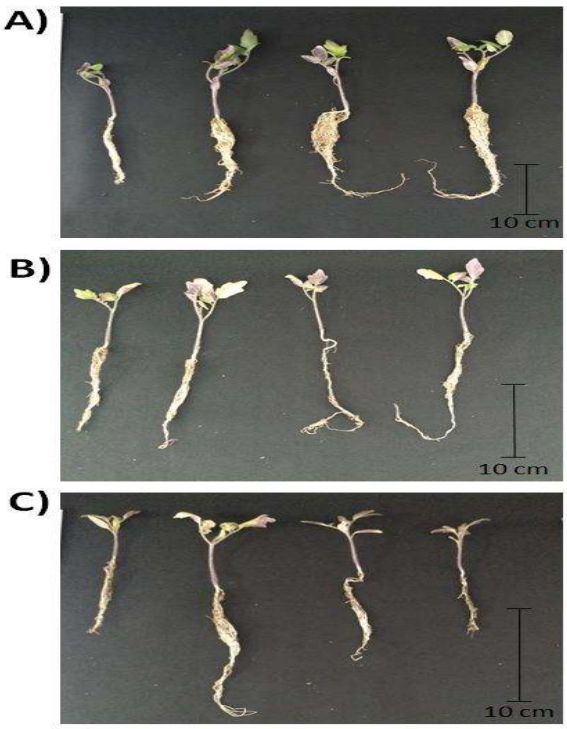
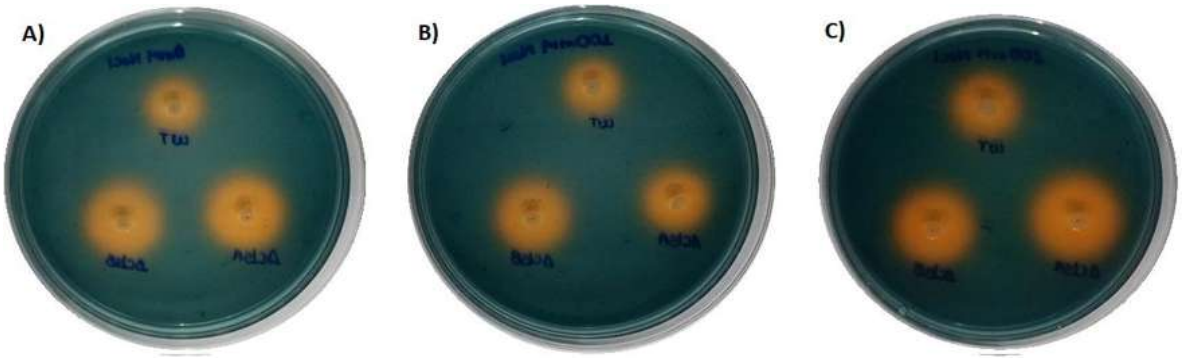


Fig. 3. Composite picture of tomato (*L. esculentum* cv Saladette) plants inoculated with the indicated bacterial strains. Plants irrigated with water (A), plants irrigated with 100 mM NaCl (B), plants irrigated with 200 mM NaCl (B). See the Section "Materials and Methods" for details of the experiment.



Supplementary fig. 1. The effect of NaCl on siderophore production by *P. fluorescens* UM270 (WT, *ΔclsA* and *ΔclsB*) in CAS agar. 0 mM (A), 100 mM (B) and 200 mM of NaCl (C).

13. DISCUSIÓN

La salinidad es uno tipo de estrés abiótico que causa pérdidas importantes en la agricultura actual (Numan et al., 2018). Por lo que resulta de gran importancia la búsqueda de alternativas que permitan el establecimiento de las plantas de interés agrícola ante este tipo de condiciones.

En el capítulo 1, los resultados demuestran que las cepas de *Bacillus*, *Bacillus* sp. CR71 y *Bacillus* sp. E25 de manera individual y en consorcio fueron capaces de conferir tolerancia a plantas de jitomate sujetas a estrés salino, particularmente a la concentración 100 mM NaCl, las plantas inoculadas mostraron un mayor crecimiento del brote, raíz, contenido de clorofila y peso total en comparación con las plantas no inoculadas. Sin embargo, a la concentración 200 mM la interacción de las cepas con las plantas no logró reestablecer el estatus de las plantas crecidas en condiciones sin sal. Además, en ambas condiciones el consorcio formado entre ambas cepas no generó efectos aditivos en el crecimiento, que era algo que se había observado en condiciones sin estrés (Rojas-Solis et al., 2018). La pérdida en la capacidad promotora de las cepas de *Bacillus* en condiciones salinas pudiera estar sujeta a la producción de los COV´s responsables de la promoción de crecimiento como por ejemplo la acetoína y el 2, 3-butanediol (Rojas-Solis et al., 2018), ya que el perfil de estos se pudo ver alterada al colocar a las cepas en las condiciones salinas (Cappellari y Banchio, 2019).

Adicionalmente, al analizar algunos de los mecanismos de promoción de crecimiento vegetal directos e indirectos (producción de sideróforos, proteasas, AIA biofilm e inhibición de fitopatógenos), encontramos que con excepción de la producción de biofilm y AIA para la cepa CR71, el resto de los rasgos presentaron una reducción significativa dependientes de la concentración de sal en el medio sin llegar a eliminarse por completo, factor importante que pudiera estar afectando directamente el no inducir tolerancia a los cultivos de jitomate sometidos a estrés y que correlacionan con algunos otros trabajos (Xu et al., 2014; Ali et al., 2014). Al evaluar la capacidad antagónica de ambas cepas hacia los fitopatógenos *Botrytis cinerea* y *Fusarium oxysporum* las condiciones de sal afectaron mayormente la

capacidad de los COV´s de inhibir el crecimiento de ambos patógenos, probablemente al reducir la cantidad de compuestos azufrados como el DMDS (Rojas-Solis et al., 2018). Además, la disminución en la producción de sideróforos pudo ser otro factor que afectará la capacidad antagónica de ambas cepas (Santos-Villalobos et al., 2012).

Al evaluar los componentes de membrana encontramos que las condiciones de salinidad para *Bacillus* sp. E25 incrementaron los niveles de PE, fosfolípido que proporciona la presión lateral en las membranas, manteniendo la posición de aminoácidos y proteínas (Dowhan et al., 2008), además de los ácidos grasos saturados palmítico (16:0) y esteárico (18:0). Este cambio en el grado de saturación de los ácidos grasos aumenta la rigidez en la membrana para contrarrestar la fluidez y permeabilidad causada por las condiciones salinas (Chibib et al., 2005). Por otro lado, la cepa CR71 sometida a la concentración 200 mM NaCl, incrementó de manera significativa un fosfolípido desconocido, así como el ácido palmitoleíco (16:1 Δ 9) y el ácido 15-metilhexadecanoico (17:0i), resultado que se asemeja al mostrado por Paulicci y colaboradores en 2015, y que permitió que la bacteria *Ochrobactrum intermedium* L115 pudiera promover el crecimiento de plantas de cacahuate.

Debido a que los componentes de membrana de las bacterias regulan procesos como metabolismo, virulencia y respuesta al estrés salino (Kunh et al., 2015), en el capítulo 2 analizamos cambios en los componentes de membrana en *Bacillus toyonensis* COPE52 en condiciones de salinidad y si dichas modificaciones permitían mantener las actividades de promoción de crecimiento en esta bacteria.

Encontramos, que las condiciones de salinidad incrementan la proporción de un lípido desconocido y disminuyeron la síntesis de PE, sin embargo, hacen falta análisis más precisos para determinar el fosfolípido incrementado y de esa manera poder asignar alguna función de protección para la bacteria. Por otro lado, ambas concentraciones de sal aumentaron la cantidad de ácidos grasos ramificados 13-metiltetradecanoico (15:0i) y 15-metilhexadecanoico (17:0i), con lo cual se favoreció un aumento de la fluidez y permeabilidad en la membrana en condiciones salinas

(Murínova y Decová, 2014), por lo que resulta importante que la bacteria presente otros mecanismos que le permitan adaptarse a esta condición.

La habilidad del género *Bacillus* para antagonizar fitopatógenos, así como para promover el crecimiento de las plantas ha sido ampliamente documentada (Orozco-Mosqueda et al., 2020). Sin embargo, hay pocos reportes sobre los efectos beneficiosos que ejercen las cepas de *B. toyonensis* en plantas (Lopes et al., 2017; Rocha et al., 2017; Contreras-Perez et al., 2019). En este trabajo, mostramos que la cepa COPE52 logró inhibir el crecimiento de los hongos fitopatógenos *B. cinerea* y *F. oxysporum*, a través de compuestos difusibles y COVs, esta cepa tiene la capacidad de producir compuestos azufrados como DMDS que pudiera ser el responsable de dicha inhibición (Contreras-Perez et al., 2019), también, Lopes y colaboradores demostraron mediante un análisis *in silico* que *B. toyonensis* BAC315 contiene genes para la síntesis de compuestos antimicrobianos, péptidos no ribosomales y quitinasas, sin embargo, esto no se ha evaluado de manera experimental.

Por otro lado, la inoculación de plantas de jitomate con la cepa COPE52 incrementó la longitud del tallo y raíz, contendido de clorofila y peso seco total en condiciones sin estrés, lo que refuerza la idea de que esta cepa es un potencial bioestimulante ya que Contreras-Perez y colaboradores (2019), reportaron que la inoculación de COPE52 en plantas de arándano estimuló su crecimiento en invernadero. Bajo condiciones salinas, únicamente a la condición 100 mM NaCl las plantas inoculadas con COPE52 presentaron parámetros similares a las plantas crecidas sin estrés, sin embargo, a la condición 200 mM la inoculación de COPE52 no logró reestablecer el estatus de las plantas crecidas sin estrés, esto debido a que los cultivos de jitomate se consideran plantas glicófitas y la condición máxima de sal analizada ocasiona daños graves en su fisiología (Bui, 2013; Orcutt y Nilsen, 2000), el hecho de que COPE52 lograra restaurar los parámetros de crecimiento en la condición de salinidad más baja (100 mM), puede ser atribuida a la producción de COVs como DMDS, acetoína y 2, 3-butanediol, además de que en esta misma concentración salina *B. toyonensis* COPE52 mantuvo la capacidad de producir AIA e

interesantemente incrementó los niveles de biofilm, la sobreproducción de AIA tiene un efecto directo en el crecimiento y desarrollo de la planta, incluyendo el desarrollo de la raíz (Yensen, 2008). Además, el compuesto DMDS tiene la capacidad de modular el sistema radicular de plantas de Arabidopsis, a través de la vía de señalización auxínica (Tyasi et al., 2019).

En cuanto a la producción de biofilm, este se ha propuesto como una estrategia adoptada por las bacterias para su biocontrol y supervivencia en la rizósfera de las plantas, además de ser un mecanismo altamente conservado dentro del género *Bacillus* bajo condiciones de estrés (Kasim et al., 2016; Bais et al., 2004).

Finalmente, en el capítulo 3 evaluamos mutantes de *Pseudomonas fluorescens* UM270 deficientes en el fosfolípido cardiolipina (CL) y su interacción con plantas de jitomate crecidas en condiciones salinas.

Las cepas mutantes Δ*clsA* y Δ*clsB* presentaron una reducción en la síntesis de cardiolipina del 58 % y 53 % respectivamente comparados con la cepa silvestre (WT), afectando su capacidad para crecer a las concentraciones 100 y 200 mM NaCl. Se ha reportado que células de *Escherichia coli* en respuesta a la salinidad acumulan CL en los polos que favorece la transcripción de osmoprotectores, los cuales al sensar un incremento en la osmolaridad generan como respuesta una mayor síntesis de osmolítos en el citoplasma, que mantiene el crecimiento celular sin alteraciones, probablemente esta sea la razón por la cual las cepas mutantes Δ*clsA* y Δ*clsB* presentan un ligero retraso al ser crecidas en medio con sal (Romantov et al., 2007).

Al evaluar mecanismos de promoción de crecimiento vegetal en la cepa silvestre, así como en las mutantes encontramos que estos rasgos se modifican de manera diferencial en las distintas cepas, las cepas mutantes presentaron una reducción en la producción de AIA en comparación con la cepa silvestre, caso contrario al evaluar la producción de sideróforos en donde las mutantes presentaron un incremento de dicha actividad, una posible explicación de los resultados observados es una disminución en la expresión del factor de transcripción de fase estacionaria (RpoS),

debido a que ambos mecanismos se contraponen a lo establecido por Saleh y Glick (2001), en donde reportaron a las cepas *Enterobacter cloacae* CAL2 y *Pseudomonas putida* UW4 que fueron transformadas genéticamente con el gen de *rpoS* de *P. fluorescens*, las cepas mutantes sobreexpresaron este gen, sintetizando mayor cantidad de AIA y menor de producción de sideróforos en comparación de las bacterias no transformadas, este mismo comportamiento fue reportado años más tarde por Sun y colaboradores (2009), en una cepa mutante de *Burkholderia phytofirmans* PsJN deficiente en la actividad ACC desaminasa, ésta presentó una mayor producción de AIA y menor cantidad de sideróforos, por lo que al analizar los niveles de expresión de RpoS encontraron una sobreexpresión de este factor. En nuestro caso al disminuir la cantidad de AIA e incrementar la producción de sideróforos sugerimos que ambos mecanismos se encuentran regulados por este factor trancripcional.

Por otro lado, de forma interesante encontramos que las cepas mutantes incrementaron la producción de biofilm en condiciones sin estrés, resultado que contrasta con lo reportado por Lin y colaboradores en 2015, y en donde al evaluar la producción de biofilm en cepas de *Rhodobacter sphaeroides* deficientes en la síntesis de CL encontraron una disminución de esta actividad, atribuida a una deformación en las células mutantes.

En ensayos de invernadero, la inoculación de plantas de jitomate con las cepas mutantes redujo la capacidad de promover el crecimiento en comparación con la cepa silvestre en condiciones normales y con estrés salino, que podría explicarse directamente por la producción de AIA que presenta cada una de las cepas, este tipo de fitohormona juega un papel importante en los mecanismos de reorganización y adaptación de la raíz en respuesta a la salinidad con lo que mejora su capacidad de adaptación a este factor de estrés (Iglesias et al., 2011). Por lo tanto, la cepa WT al producir mayor cantidad de esta fitohormona presenta un mayor potencial para promover el crecimiento, demostrando así que la síntesis de CL en *P. fluorescens* UM270 es importante para promover el crecimiento de plantas de jitomate crecidas en condiciones normales y con estrés salino.

En conclusión, las cepas de *Bacillus* y *Pseudomonas* empleadas en este estudio mostraron diferente grado de promoción de crecimiento y de protección a la salinidad al ser inoculadas en plantas de jitomate, resultado de la modulación de las actividades de promoción de crecimiento vegetal presentada por cada cepa (Figura 3).

14. CONCLUSIÓN GENERAL

Las bacterias *Bacillus* sp. CR71, *Bacillus* sp. E25, *Bacillus toyonensis* COPE52 y *P. fluorescens* UM270 poseen mecanismos halotolerantes (modificación de componentes de membrana) que en interacción con plantas de jitomate les confiere tolerancia a condiciones de salinidad.

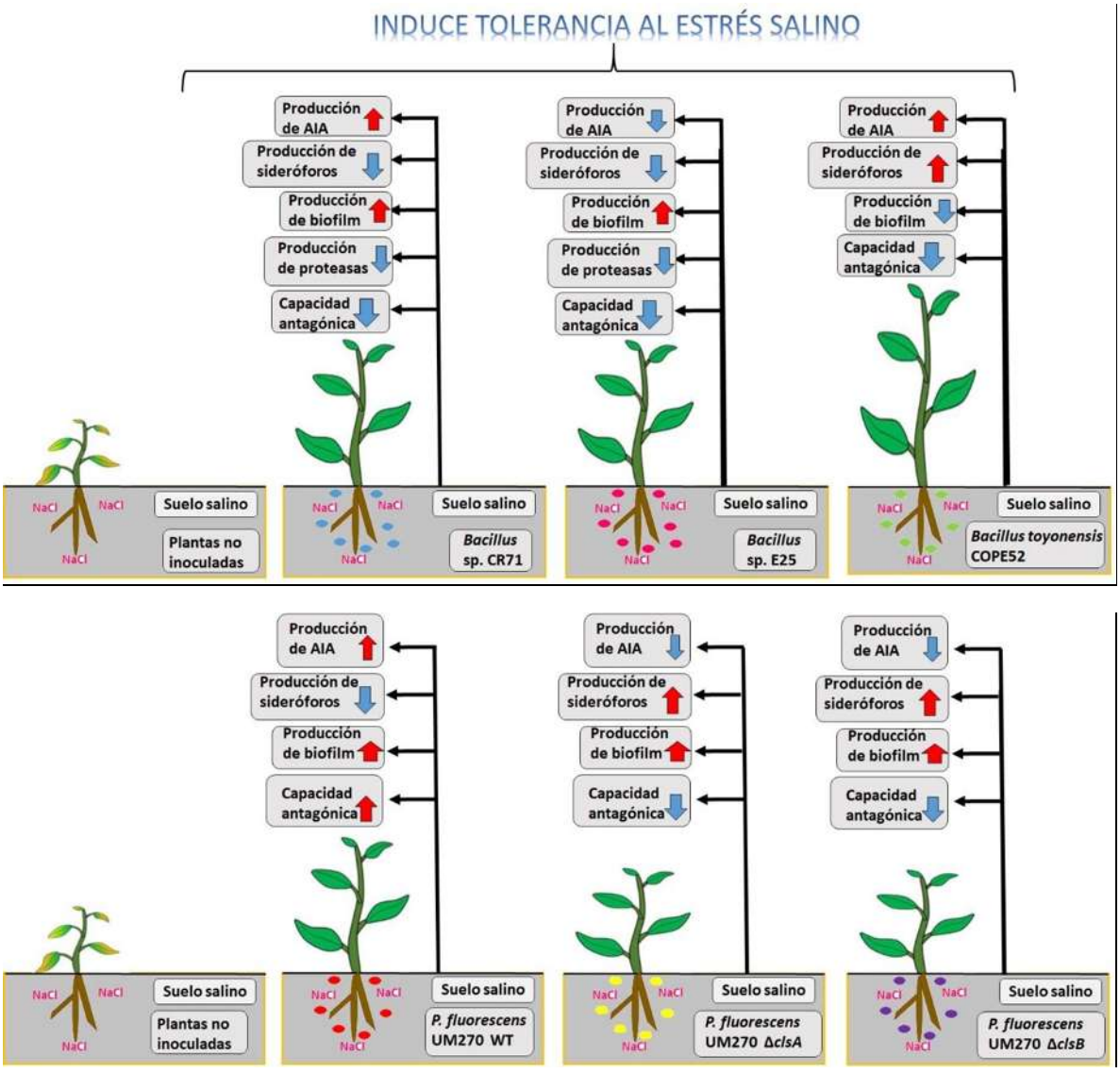


Figura 3. La modulación de las actividades de promoción de crecimiento vegetal ejercidas por las diferentes PGPR´s, confieren tolerancia a plantas de jitomate crecidas en estrés salino.

15. PERSPECTIVAS

- Identificar de forma precisa el fosfolípido de membrana que se incrementó en condiciones de salinidad en *B. toyonesis* COPE52 y *Bacillus* sp. CR71.
- Analizar si la interacción de las PGPR´s con las plantas de jitomate estimulan mecanismos de halotolerancia en las plantas sometidas a estrés salino.
- Crear una doble mutante en *P. fluorescens* UM270 deficiente en cardiolipina y analizar su capacidad para promover el crecimiento de plantas de jitomate en condiciones de salinidad.
- Evaluar otros mecanismos de halotolerancia en las PGPR´s que pudieran estar protegiendo las capacidades de promoción de crecimiento vegetal ejercidos por las bacterias.
- Analizar los niveles de expresión del factor de transcripción de fase estacionaria (RpoS), en las cepas mutantes deficientes en CL, el cual se sugiere modula la producción de sideróforos y AIA.

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17. ANEXOS: PUBLICACIONES ADICIONALES

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Pseudomonas stutzeri E25 and *Stenotrophomonas maltophilia* CR71 endophytes produce antifungal volatile organic compounds and exhibit additive plant growth-promoting effects

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ABSTRACT

Endophytic bacteria are part of the plant microbiome, which can promote the growth of plants and act as biocontrol agents against potential phytopathogens through various mechanisms, including the production of volatile compounds. In this work, we isolated and characterized two new bacterial endophytes, strains E25 and CR71, that exhibited antifungal activity and plant growth promotion. Analysis of the complete 16S ribosomal gene sequences of the strains showed high species-level identity (99%) with *Pseudomonas stutzeri* (E25) and *Stenotrophomonas maltophilia* (CR71). In in vitro assays, both strains showed excellent antagonistic action against *Botrytis cinerea* by emission of volatile organic compounds (VOCs), but not through diffusible compounds. Interestingly, the volatile cocktails emitted by E25 and CR71 were quite similar, highlighting the production of sulphur-containing compounds such as the antimicrobial volatile dimethyl disulphide (DMDS). Analysis of the pure DMDS compound showed mycelial inhibitory activity against the fungal strain. In a greenhouse experiment, inoculation of strains promoted the shoot and root length, chlorophyll content, and total fresh weight of tomato plants (*Lycopersicon esculentum* cv Saladette). Interestingly, when strains were co-inoculated, a better plant growth-promoting effect was observed. In conclusion, the co-inoculation of novel endophytic strains reported herein represents an excellent option to promote growth and achieve the biocontrol of *B. cinerea* through the production of potent volatiles such as DMDS.

1. Introduction

One of the main causes of losses in agricultural crops is the presence of phytopathogenic fungi. These pathogens may be crop-specific or mostly generalists such as the fungus *Botrytis cinerea*, which is responsible for grey mould disease and attacks more than 200 species of plants (Nambeesan et al., 2012; Williamson et al., 2007). In many countries, and especially in developing countries, the first option to control grey mould disease in various crops is the use of agrochemicals; however, the overuse of these compounds has been widely documented to cause various toxic effects on the environment (Adesemoye and Kloepper, 2009; Rosslenbroich and Stuebler, 2000). Therefore, it is important to search for new eco-friendly strategies to control *B. cinerea*.

The use of bioinoculants represents an excellent option to control phytopathogens while promoting plant growth and production, since, to our knowledge, negative effects on the environment, or on human or animal health have not been documented (Santoyo et al., 2012). Moreover, beneficial effects of certain bioinoculants have been reported, which have helped to diminish the employment of agrochemicals (Adesemoye and Kloepper, 2009). For example, several studies have shown the beneficial effects of engineering the plant microbiome to improve biocontrol and/or exhibit plant growth-promoting effects. As part of the beneficial plant microbiome, we here focus on the potential exploitation of bacterial endophytes for eco-friendly pest control (Sturz et al., 2000). Bacterial endophytes inhabit the interior of plant tissues without causing visible harm, and can be isolated from surface-disinfected tissues (Hardoim et al., 2008; Santoyo et al., 2016). The ability of diverse bacterial endophytes to promote plant growth occurs as a consequence of either direct or indirect mechanisms. Direct promotion of plant growth occurs when a bacterium either facilitates the acquisition of essential nutrients or modulates the level of hormones of a plant. Indirect promotion of plant growth occurs when the phytopathogenic activity is inhibited, which therefore decreases the damage to plants (Glick, 2014; Santoyo et al., 2012).

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Some reports have shown that bacteria can produce either antifungal diffusible or volatile compounds (Chaurasia et al., 2005; Hernández-León et al., 2015; Kanchiswamy et al., 2015). For example, the plant growth-promoting rhizobacteria (PGPR) *Arthrobacter agilis* UMCV2 produces volatiles with antagonistic action toward the fungal phytopathogen *B. cinerea* and the oomycete *Phytophthora cinnamomi*. Interestingly, a single compound from the volatile cocktail, namely dimethylhexadecylamine (DMHDA), was found to be responsible for inhibiting mycelial growth (Velázquez-Becerra et al., 2013). In addition, volatiles produced by *A. agilis* UMCV2 have shown the ability to promote growth in *Arabidopsis thaliana* and *Medicago truncatula* plants (Orozco-Mosqueda et al., 2013).

In another study, Huang et al. (2012) reported that dimethyl disulphide (DMDS), a volatile compound produced by the *Bacillus cereus* C1L strain, played a role as an elicitor of induced systemic resistance (ISR) in tobacco and corn plants. This compound elicited a protective response of the plant against *B. cinerea* and *Cochliobolus heterostrophus* under greenhouse conditions. However, DMDS only showed a weak effect on mycelial growth inhibition.

We previously demonstrated that *Pseudomonas fluorescens* strain UM270 is able to produce diffusible and volatile compounds against diverse fungal phytopathogens (Hernández-León et al., 2015). Interestingly, the strain UM270 produced a potent antimicrobial blend of volatiles, including DMDS and DMHDA, which have been reported as antifungals or elicitors of ISR. However, these compounds showed different levels of antagonism against the fungi: for some phytopathogens the mycelial growth inhibition reached up to 100%, whereas for others the inhibitory action was almost non-existent (Huang et al., 2012; Velázquez-Becerra et al., 2013).

Thus, in the present study, we characterized the volatile blends of two new bacterial endophytes with antifungal and plant growth-promoting activity. Both strains produced similar volatiles, including DMDS, which in a pure form was highly antagonistic against a pathogenic *B. cinerea* strain isolated from unhealthy strawberry plants. In addition, co-inoculation of both endophytic strains in tomato plants in a greenhouse experiment showed additive plant growth-promoting effects.

2. Materials and methods

2.1. Isolation of cultured endophytic bacteria

Plants of the tomatillo *Physalis ixocarpa* were collected from an agricultural field close to Morelia, Michoacán, México. The shoots and roots were washed with water to remove soil particles. Endophytic bacteria were collected from several tissues as described by Contreras et al. (2016). In brief, the roots were immersed in 70% ethanol for 30 s, washed with fresh sodium hypochlorite solution (2.5% available Cl^-) for 5 min, rinsed with 70% ethanol for 30 s, and finally washed five times with sterile distilled water. To further confirm the success of the sterilization process, aliquots of the sterile distilled water used in the final rinse were cultured on plates containing nutrient agar (NA) medium. The plates were examined for bacterial growth after incubation at 30 °C for 5 days. Uncontaminated roots, as detected by a culture-dependent sterility test, were used for the isolation of endophytic bacteria.

A collection of approximately 1000 strains was generated. The strains of *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 were selected during preliminary analysis in the search of strains with antifungal and plant growth-promoting activities. The strains E25 and CR71 were grown at 30 °C for 24 h on NA plates and routinely maintained at 4 °C.

2.2. Phytopathogenic fungus

The fungus *B. cinerea* was used as the pathogenic strain, and inoculated in potato dextrose agar (PDA) at 30 °C for 3–5 days in the dark and kept at 4 °C. The same *B. cinerea* strains have been previously employed and reported in other works (Hernández-León et al., 2015; Martínez-Absalón et al., 2014).

2.3. Molecular characterization and phylogenetic analysis of CR71 and E25

Genomic DNA was isolated from the endophytic bacteria E25 and CR71, and the 16S ribosomal DNA subunit (rDNA) was amplified using polymerase chain reaction (PCR) with the universal bacterial primers fD1: 5´-CAGAGTTTGATCCTGGCTCAG-3´ and rD1: 5´-AAGGAGGTGATCCAGCC-3´ under previously reported PCR conditions (Hernández-León et al., 2015). PCR amplifications were performed using the TC-142 Thermocycler Techne thermal cycler (Keison Products, Chelmsford, UK) in Go Taq Master Mix tubes (Promega, Madison, WI, USA). The PCR product was further purified and the 16S rDNA regions of the bacterial isolates were sequenced. The ribosomal sequences were obtained and compared to the GenBank database using the Nucleotide Basic Local Alignment Search Tool (BLAST) program. The alignment of multiple sequences was generated with Clustal W (www.ebi.uk/Tools/clustalw2), and phylogenetic analysis of the 16S rRNA gene sequences was performed using the MEGA 4.0 program (Tamura et al., 2007). To obtain the confidence value for the set of aligned sequences, a bootstrap analysis of 1000 replicates was performed. The phylogenetic tree was constructed using the maximum parsimony algorithm. The GenBank accession numbers of the ribosomal sequences of *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 are MG000977 and MF992168, respectively.

2.4. In vitro evaluation of the antagonistic effects of diffusible and volatile compounds

The antagonism of compounds produced by the endophytic bacteria against *B. cinerea* was evaluated in bioassays performed in Petri dishes as previously reported (Santoyo et al., 2010). In brief, the bacterial isolates were simultaneously striated with the pathogenic fungus in Petri dishes containing PDA. The bacteria were streaked in the cross-shaped dishes and a 4-mm portion of the mycelium was deposited in the centre of each of the formed quadrants on the plates. The Petri dishes were incubated in the dark at 30 °C and mycelial diameter growth was measured at day 6.

To evaluate the antifungal effect of the volatile organic compounds (VOCs) emitted by isolated bacterial endophyte strains, a bacterial inoculum of each strain (1×10^6 CFU) was simultaneously deposited on one side of the Petri dish, and the 4-mm plug of the *B. cinerea* mycelium was inoculated in another portion of the plate. The cultures were incubated in the dark at 30 °C and the growth of the mycelial diameter was measured at day 6.

2.5. Preparation and antifungal bioassay with DMDS

DMDS (Sigma, St. Louis, MO, USA) was prepared as reported by Huang et al. (2012). Thus, DMDS was dissolved in ethanol to make a stock solution of 1.0 M DMDS. The DMDS stock solution was subsequently dispersed in Milli-Q water to make solutions of different concentrations, ranging from 0.1 to 1.0 µM, with an ethanol content of 10 mL/L immediately before application to Petri dishes.

The effect of DMDS on the mycelial growth of *B. cinerea* was investigated as a diffusible or volatile compound in normal or divided Petri dishes. When Petri dishes with separate compartments were used, a 4-mm-diameter portion of the mycelium was inoculated on one side of the divided dish containing PDA medium. Subsequently, the DMDS solution was added to the other side of the dish at different concentrations (0.1, 1, 10, 100, and 1000 µM). The Petri dishes were sealed with parafilm to minimize loss or volatilization of DMDS and incubated at 30 °C. After incubation for 5 days, the diameter of the fungal

mycelium was measured and the degree of inhibition was calculated. The experiment was repeated three times.

When DMDS was evaluated as a diffusible compound, it was directly poured onto the PDA agar medium in normal Petri dishes at different concentrations (0.1, 1, 10, 100, and 1000 μM). Similarly, the dishes were sealed with parafilm and incubated at 30 °C for 5 days, and the mycelial diameter of the fungus was measured to calculate the degree of inhibition.

2.6. Analysis of VOCs emitted by endophyte strains

VOCs produced by strains E25 and CR71 were analysed by solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) on PDMS/DVB fibres (Supelco, Inc., Bellafonte, PA, USA) following a previously reported protocol (Hernández-León et al., 2015), except that the GC-MS system 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. The source pressure was 7 Pa, filament voltage was 70 eV, and the scan rate was 1.9 scan/s. The VOCs 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 bacterial endophytic strain.

2.7. Evaluation of plant growth promotion by endophytic strains in greenhouse conditions

Greenhouse pot experiments with tomato plants (*Lycopersicon esculentum* cv Saladette) were performed in sterile peat moss with a perlite substrate. The experimental design included four treatments: control (24 plants without inoculants), E25 (24 plants with E25 strain inoculant), CR71 (24 plants with CR71 inoculant), and E25 + CR71 (24 plants with both E25 and CR71 inoculants), with a total of 96 experimental units. Tomato seeds were germinated, and after one week, seedlings of the same size were selected and transplanted into pots (one plant was left in each pot). Bacterial inoculants were applied every week after pot transplantation, either individually (E25 and CR71) or combined (E25 + CR71) according to the experimental design, which also included treatments without endophyte inoculations. Bacterial inoculants were adjusted at A600 = 1.0. Throughout the experiment, the plants were irrigated every third day with deionized water. The effect of adding each of the bacterial inoculants on the root length, aerial parts, fresh weight, and chlorophyll concentration was evaluated after 5 weeks of plant growth. The chlorophyll concentration was measured in at least three leafs from each plant as previously reported (Orozco-Mosqueda et al., 2013).

2.8. Statistical analysis

The results were analysed using *Statistica* 8.0 software, and analysis of variance and Duncan's test for mean comparison was used for multiple comparisons ($p < 0.05$).

3. Results

3.1. Molecular characterization of isolates CR71 and E25

The complete molecular sequences of the 16S rDNA gene of isolate CR71 showed high species identity with *S. maltophilia* based on sequences from the NCBI database, whereas those of E25 showed high identity with *P. stutzeri*. These results were confirmed in the phylogenetic analysis, in which the isolates CR71 and E25 were grouped with other species of *S. maltophilia* and *P. stutzeri*, respectively (Fig. 1A and B).

3.2. Antagonism against B. cinerea by P. stutzeri E25 and S. maltophilia CR71

The antagonism towards the phytopathogen *B. cinerea* was evaluated based on the action of diffusible compounds (direct contact) or VOCs (inoculation in divided Petri dishes) produced by the strains *P. stutzeri* E25 and *S. maltophilia* CR71. Both endophytic bacteria showed significant inhibitory effects on *B. cinerea* mycelial diameter growth during the split-Petri dish bioassays (Fig. 2A) and with direct contact co-inoculation (Fig. 2B). In terms of percentages, the VOCs produced by E25 and CR71 reduced the mycelial diameter of the fungus by over 40% and 52%, respectively (Fig. 2A). In the direct co-inoculation assays, *S. maltophilia* CR71 exerted the greatest inhibitory effect against the fungus with a percent inhibition of 24%, whereas *P. stutzeri* E25 reduced the fungal growth by 12% (Fig. 2B).

To further determine whether the antagonistic effect of strains E25 and CR71 on *B. cinerea* was also caused by the production of diffusible compounds, another experiment was performed in which the supernatant of each strain was purified after overnight growth culture. The supernatant was then directly poured onto the Petri dishes at different concentrations and the phytopathogen was inoculated and incubated for 6 days to observe its growth (see Fig. 1 in Ref [Data in Brief]). The results confirmed no inhibition of mycelial growth by the supernatant of both strains, indicating that the observed antagonism towards *B. cinerea* is mainly due to the emission of volatile compounds.

3.3. VOCs analysis

After confirming that the volatile compounds produced by the strains E25 and CR71 were the cause of the inhibitory effect, we proceeded to characterize the mixture of volatiles produced by the bacteria by SPME-GC-MS. The mixture of volatiles for E25 and CR71 showed some differences, with only 11 compounds identified in both strains, and 7 and 16 unique compounds identified for E25 and CR71, respectively (Table 1). Volatile compounds that were detected in NA media without inoculation of the bacterial strains were eliminated from the analysis. The main VOCs produced by the endophyte strains included aldehydes, alcohols, aromatic compounds, esters, ethers, and sulphur compounds. In the profile of VOCs emitted by endophytic bacteria, four sulphur compounds were found: S-methylthiobutyrate, isobutyl isothiocyanate, 2-methylthioethanol, and DMDS. These results suggest that the inhibitory effects detected in the divided Petri dish antagonism assays may be due to several sulphur compound candidates such as DMDS, which has been previously associated with antimicrobial activities (Huang et al., 2012).

3.4. Inhibition of B. cinerea by the volatile compound DMDS

DMDS has been reported to function as an antimicrobial and plant growth-promoting compound (Huang et al., 2012; Hernández-León et al., 2015; Meldau et al., 2013). Since DMDS was one of the main volatiles detected in the mixture of compounds produced by both endophytic strains E25 and CR71, we further evaluated its antifungal role either as a volatile in divided Petri dishes or when poured directly onto the PDA plate where *B. cinerea* was inoculated. In both experiments, DMDS showed a significant inhibitory effect on *B. cinerea* mycelial growth (Fig. 3, panels A and B). However, DMDS was more toxic when it was poured onto the media and was in direct contact with the pathogen, with inhibition observed even at the low concentration of 0.1 μM DMDS (Fig. 3A). DMDS as a volatile had an inhibitory effect at 10 μM (Fig. 3B).

In addition to the strong direct inhibitory effect of mycelial growth observed for DMDS, either as a volatile or as a diffusible compound, its direct plant growth-promoting effect was evaluated; however, no such

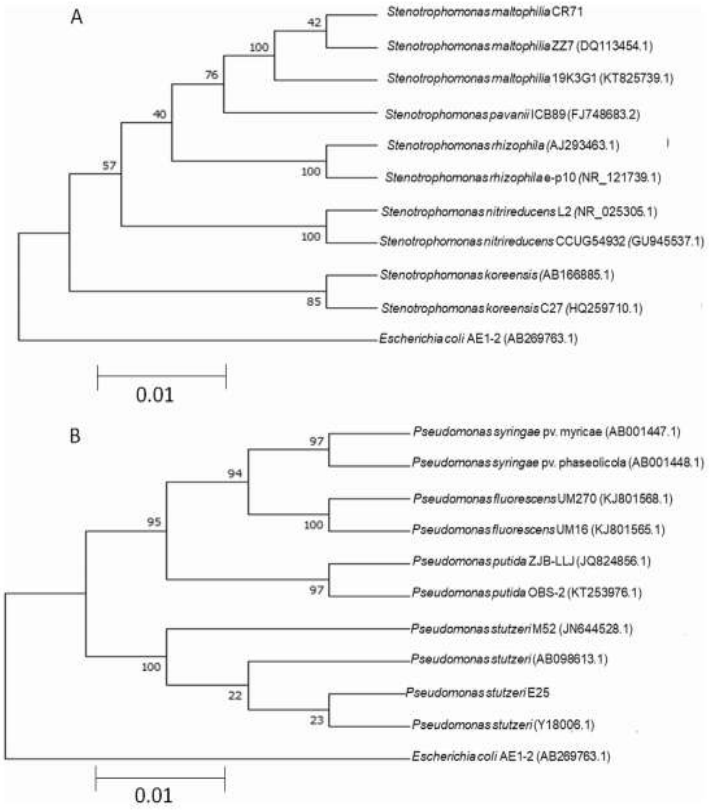

Fig. 1. Molecular characterization of strains CR71 and E25. (A) Phylogenetic analysis of (A) CR71 and (B) E25 based on the complete sequence of the 16S ribosomal gene. Phylogenetic trees were constructed based on the neighbour-joining method (Tamura et al., 2007). Other algorithms showed similar topologies. Bootstrap analysis of 1000 replicates was performed and expressed as percentages.

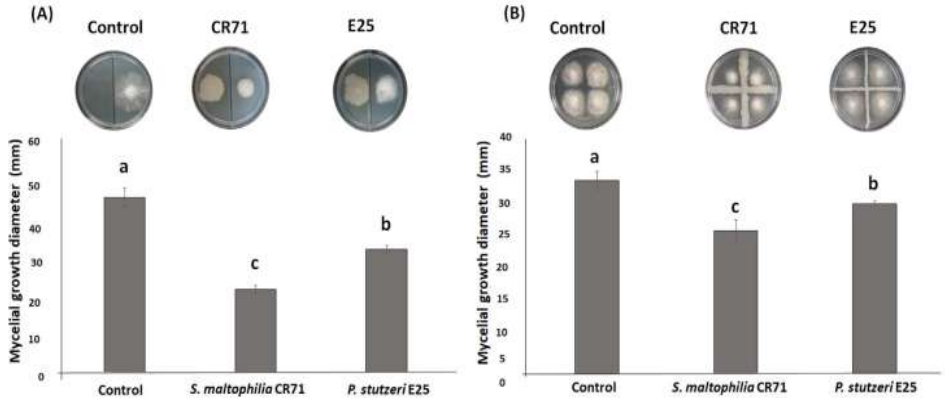

Fig. 2. Antifungal effect of the co-inoculation of endophyte strains *P. stutzeri* E25 and *S. maltophilia* CR71 with the phytopathogen *B. cinerea*. (A) Effect of the volatile organic compounds emitted by CR71 and E25 in divided Petri dishes. (B) Direct effect of the diffusible compounds produced by E25 and CR71 on the mycelial growth of *B. cinerea*. Both experiments were performed independently three times. The respective graphs of each experiment are shown in the lower panels of (A) and (B), where the bars represent the values of the mean ± SE. Letters indicate that the means differ significantly according to Duncan's multiple range test ($p < 0.05$).

beneficial effect or significant differences on plant growth were observed when DMDS was applied to tomato plants in vitro (data not shown).

3.5. Additive plant growth-promotion effect by co-inoculation of CR71 and E25

The strains *P. stutzeri* E25 and *S. maltophilia* CR71 were isolated and selected from a collection of bacterial endophytes according to their ability to restrict the growth of the grey mould phytopathogen *B. cinerea*. However, a desirable characteristic of the endophytic strains would be to have a direct effect on plant growth promotion. Hence, we carried out greenhouse experiments by testing the influence of individual strains or their co-inoculation on the growth of tomato plants. Fig. 4 shows that inoculation of *S. maltophilia* CR71 had a promoting effect on the root length and total plant fresh weight, while *P. stutzeri* E25 increased the root and shoot lengths, as well as resulted in a better plant fresh weight compared to uninoculated plants (control).

Table 1
Analysis of volatile organic compounds produced by E25 and CR71 detected by GC/MS analysis. Grey shadowing indicates sulphur-containing volatiles.

Volatile Compounds	Rt (min)	E25 (%)	CR71 (%)
Acetone	1,4	10,71	n.d
2-Butanone	1,95	2,32	2.24*
Isopropyl alcohol	2,29	0,74	n.d.
Ethyl propionate	2,65	1,14	3.17*
Ethyl isobutyrate	2,76	0,82	6.14*
3-Methyl-2-pentanone	3,77	6,86	n.d.
Trichloromethane	3,96	38,85	n.d.
Ethyl-2-methylbutanoate	4,72	n.d.	3,49
Ethyl isovalerate	5,24	n.d.	1,95
Dimethyl disulphide	5,35	2,11	2.65*
3-Methylbutanenitrile	7,49	12,93	n.d.
S-Methyl thio butyrate	7,56	n.d.	5,91
1-Butanol	8,91	n.d.	0,93
1,3-Diazine	11,3	3,24	n.d.
Ethyl tiglate	12,26	1,92	4.94*
Methyl pyrazine	13,63	1,18	n.d.
Acetoin	14,47	n.d.	8,11
Isobutyl isothiocyanate	15,83	10,47	25.86*
Acetic acid	22,32	n.d.	5,4
Ethyl-3-hydroxybutanoate	24,74	0,48	6.24*
2-(Methylthio)ethanol	25,11	2,1	2.74*
Propionic acid	25,83	n.d.	1,16
2-Methylpropanoic acid	26,96	n.d.	3,72
Phenyloxirane	28,42	2,43	2.14*
Butanoic acid	29,33	n.d.	1,37
3-Methylbutanoic acid	30,87	n.d.	2,32
Methyl salicylate	34,34	n.d.	0,29
2-Butenoic acid	34,49	n.d.	6,07
Acetamide	34,93	1,24	0.31*
Benzyl alcohol	38,27	0,45	1.15*

* Asterisk indicates the VOCs emitted by both strains.

Interestingly, the co-inoculation of E25 plus CR71 exhibited an additive effect of plant growth promotion with respect to both root length and total fresh weight. The inoculant containing both strains also significantly improved the chlorophyll concentration of tomato plants. This result suggests that an inoculant containing both strains, E25 plus CR71, would have superior plant growth-promoting effects.

4. Discussion

B. cinerea affects more than 200 plant species, including important crop plants, causing grey mould disease worldwide with severe consequences for fruit production resulting in huge economic losses (Nambeesan et al., 2012). We have previously reported efforts to isolate, characterize, and select the best antagonistic bacteria for the control of phytopathogens like *B. cinerea* (Hernández-León et al., 2015; Martínez-Absalón et al., 2014). In the present work, we characterized the antagonistic activity of two new endophyte isolates against the phytopathogen *B. cinerea*, which were identified as *P. stutzeri* and *S. maltophilia* at the species level. The genus *Pseudomonas* is well known for having a large arsenal of antifungal and plant growth-promoting metabolites, some of which are volatile compounds (Haas and Défago, 2005; Hernández-León et al., 2015; Yan et al., 2017). For example, Hunziker et al. (2015) recently reported that *Pseudomonas* strains associated with potato plants produced volatiles with high potential for inhibition of *Phytophthora infestans*, an important oomycete phytopathogen. A volatile profile analysis revealed that 1-undecene was a compound produced by *Pseudomonas* strains and responsible for *P. infestans* growth inhibition. Supplying the pure compound 1-undecene to *P. infestans* significantly reduced mycelial growth, germination, sporangium formation, and zoospore release in a dose-dependent manner.

In this sense, the genus *Stenotrophomonas* has also been reported as a biological control and plant growth-promoting agent, owing to its production of a large number of antifungal metabolites and enzymes (Cernava et al., 2015; Elhalag et al., 2015), such as *Stenotrophomonas rhizophila*, a plant-associated bacterium reported to possess antagonistic activity against phytopathogenic fungi like *Verticillum dahliae*, *Rhizoctonia solani*, *Sclerotinia sclerotium*, and *Candida albicans* (Wolf et al., 2002). Interestingly, another *S. rhizophila* strain, DSM14405^T, was reported as a plant growth-promoting agent under stress conditions (by excretion of glucosylglycerol as a protecting mechanism) (Alavi et al., 2013).

In this work, we analysed the volatile profile production of *P. stutzeri* and *S. maltophilia*, which showed some differences in volatile emissions but also some common volatiles such as DMDS. Recent studies have shown that diverse plant growth-promoting rhizobacteria produce volatile S-containing compounds, including DMDS, which has been associated with the suppression of plant fungal diseases by acting as an elicitor of ISR in tobacco and corn (Huang et al., 2012). However, the authors only reported weak direct inhibitory activity of the pure DMDS compound against *B. cinerea* and *Cochliobolus heterostrophus*. In contrast to this previous result, we found potent inhibitory activity of pure DMDS against our *B. cinerea* strain, when analysed either as a diffusible or volatile compound. These results might appear to be conflicting; however, the diversity of phytopathogen strains with different origins could present different responses and resistance mechanisms, some exhibiting a greater capacity to survive exposure to inhibitory compounds. This situation highlights the importance of isolating endemic antagonists from a given region to solve local pest problems.

The ability to promote growth in L. *esculentum* plants by strains E25 and CR71 was also analysed under greenhouse conditions. Both strains

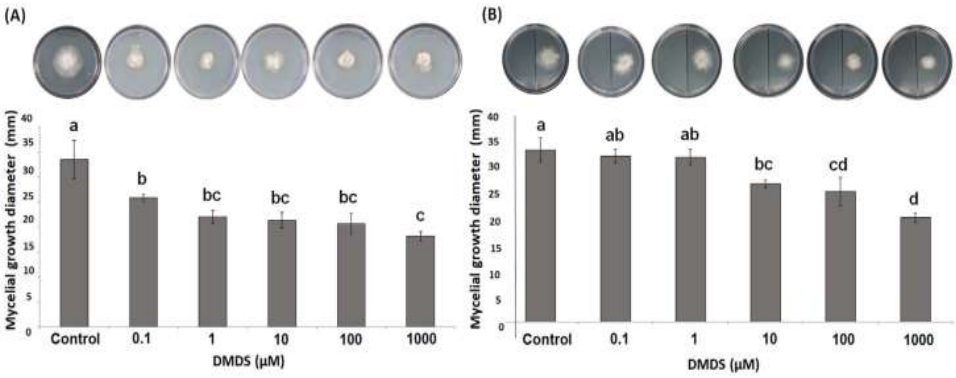

Fig. 3. Antifungal effect of the co-inoculation of the compound DMDS with the phytopathogen *B. cinerea*. (A) Direct effect of DMDS on the mycelial growth of *B. cinerea*. (B) Effect of DMDS on split Petri dishes. Both experiments were performed independently three times. The respective graphs of each experiment are shown in the lower panels (A) and (B), where the bars represent the values of the mean ± SE. Letters indicate that the means differ significantly according to Duncan's multiple range test ($p < 0.05$).

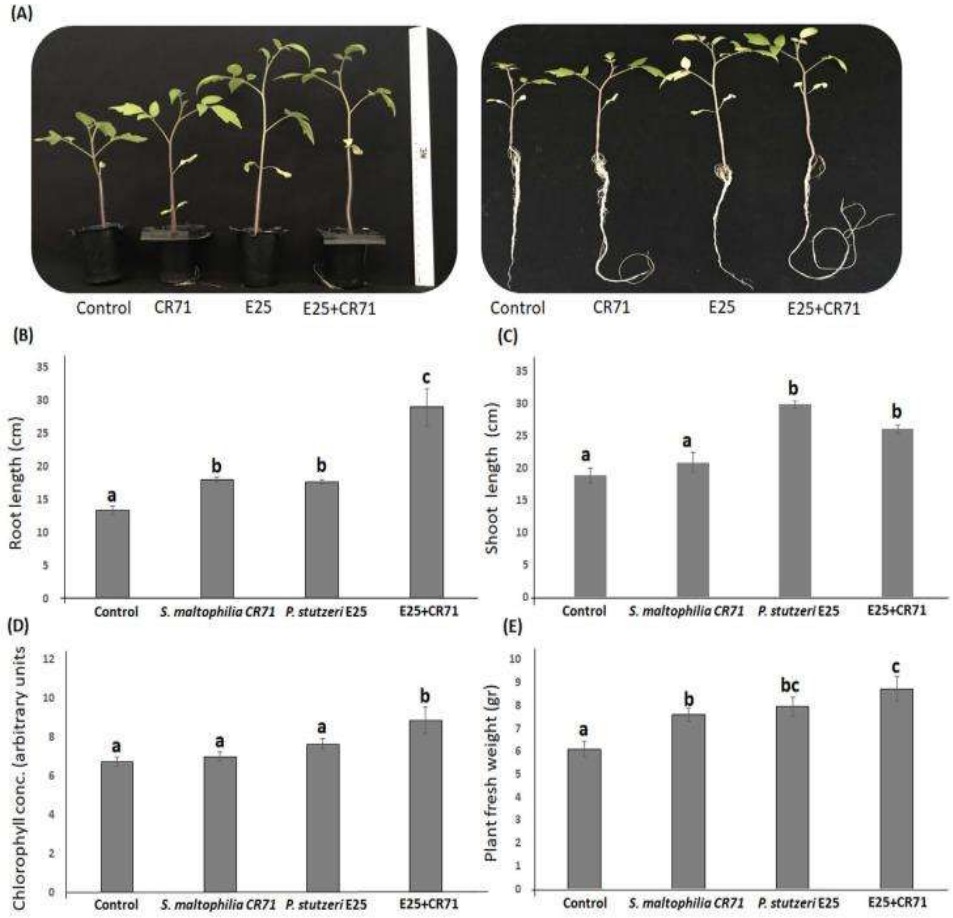

Fig. 4. Effect of inoculation with strains *P. stutzeri* E25 and *S. maltophilia* CR71 individually and in a consortium on L. *esculentum* plants grown under greenhouse conditions. Representative plants of the experiment are shown in Panel (A). Graphics show the root length (B), shoot length (C), chlorophyll concentration (D), and total fresh weight (E). The bars represent the values of the mean ± SE. Letters indicate that the means differ significantly according to Duncan's multiple range test ($p < 0.05$).

were independently able to improve some parameters analysed in tomato plants, such as root and shoot length, as well as total plant fresh weight. Acetoin (3-hydroxybutanone) was included within the blend of volatile compounds produced by *S. maltophilia* CR71. This volatile has been widely reported as a promoting compound of *A. thaliana* seedlings, along with the volatile 2,3-butanediol emitted by *Bacillus subtilis* and *Bacillus amyloliquefaciens* (Ryu et al., 2003). As previously mentioned, the profile of volatile compounds also included DMDS. Meldau et al. (2013) reported that DMDS produced by a *Bacillus* strain induced the growth of *Nicotiana attenuata* wild tobacco plants. The authors demonstrated that DMDS contributes to sulphur (S) nutrition in *N. attenuata* and concluded that DMDS by B55 production is a plant growth-promotion mechanism that likely enhances the availability of reduced S, which is particularly beneficial for wild-type plants growing in S-deficient soils. Since we did not analyse the S-deficient growing condition in our experiment, this might be a possible explanation for the lack of observing any direct promoting activity of DMDS applied to tomato plants.

One other interesting result found in this work was the additive positive effects exerted by the co-inoculation of strains E25 and CR71. Similar effects have been observed by Timm et al. (2016) with a mixture of *Pseudomonas* and *Burkholderia* strains treated to *Populus* plants, which improved root biomass and photosynthetic capacity compared to individual inoculation (or no inoculation). Interestingly, transcriptomic analysis of the plant response revealed that several genes involved in the synthesis of thiamine, sulphate, and lipids were modulated by the mixed inoculum. The impact of co-inoculation of the strains on the metabolic profile of the leaf was also different from the individual inoculation effect, demonstrating a better plant response with the mixed inoculum.

Similarly, Rojas-Solis et al. (2016) recently reported an additive or better beneficial effect achieved by co-inoculating a mixture of PGPR strains. They found that a certain *Bacillus thuringiensis-Pseudomonas fluorescens* consortium had a beneficial interaction on Mexican husk tomato seedlings, but not all of the possible strain combinations were analysed.

In conclusion, this work describes the characterization of two new strains, *P. stutzeri* E25 and *S. maltophilia* CR71, which exhibit excellent abilities to antagonize the grey mould phytopathogen *B. cinerea* by emitting VOCs but not diffusible compounds, including DMDS. Finally, the two new endophyte strains reported herein may be best applied as a consortium to act as effective biocontrol agents against pathogens while simultaneously promoting plant growth.

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Data Article

Data on the effect of *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 culture supernatants on the mycelial growth of *Botrytis cinerea*

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ABSTRACT

Plant growth-promoting bacterial endophytes (PGPBEs) produce volatile and diffusible compounds that inhibit phytopathogens (Santoyo et al., 2016) [1]. A recent work by Rojas-Solis and colleagues [2] demonstrated the antifungal effect of volatile organic compounds exerted by the *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 endophytes, highlighting the production of sulfur-containing compounds such as the antimicrobial volatile dimethyl disulfide (DMDS). The data presented in this article include the effect of two culture supernatants from the same strains, E25 and CR71, on the mycelial growth of the gray mold phytopathogen *Botrytis cinerea*. These data may help to further evaluate the specific compounds produced by endophyte isolates E25 and CR71 with antifungal activity. This article is submitted as a companion paper to Rojas-Solís et al. (2018) [2].

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Specifications Table

Subject area	Biology
More specific subject area	Biological Control
Type of data	Graph, figure
How data was acquired	Plate bioassays
Data format	Raw data statistically analyzed
Experimental factors	The data concern filtered overnight bacterial cultures.
Experimental features	The experimental design included potential antifungal activity of bacterial culture supernatants.
Data source location	Morelia, México
Data accessibility	Data is within this article

Value of the data

- The data show the effect of culture supernatants of *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 on the mycelial growth of the gray mold phytopathogen *Botrytis cinerea.*
- The data highlight the weak antifungal effect of diffusible compounds produced by bacterial endophytes *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71.
- The data are useful to further explore the compounds responsible for antifungal action.

1. Data

PGPBEs can produce either antifungal diffusible or volatile compounds [1, 2].

These data show the direct effect of *P. stutzeri* E25 and *S. maltophilia* CR71 culture supernatants on the mycelial growth of the phytopathogen *B. cinerea* using culture assays on potato dextrose agar (PDA) plates (Table 1). Values are presented as the means ± standard errors of three replicates from repeated experiments. Different letters in each column indicate significant ($p < 0.05$) differences according to the least significant difference test.

Table 1
Antifungal activities of *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 culture supernatants on mycelial growth of *Botrytis cinerea.*

Treatment	Mycelial growth area (cm^2)	Mycelial growth inhibition (mm)
Control	45.96 ± 8.86 ab	Not detected
E25 (0.1X)	41.69 ± 11.05 ab	7.28 ± 0.47 a
E25 (0.5X)	44.47 ± 5.10 ab	7.83 ± 0.52 a
E25 (1X)	32.47 ± 3.08 a	7.3 ± 0.25 a
CR71 (0.1X)	50.42 ± 8.86 b	7.0 ± 0.59 a
CR71 (0.5X)	30.07 ± 3.48 ab	6.75 ± 0.39 a
CR71 (1X)	47.79 ± 7.95 ab	6.95 ± 0.44 a

2. Experimental design, materials and methods

The culture supernatants of isolates *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 were tested for antifungal activities against *B. cinerea* through plate bioassays. Overnight cultures were grown (O.D. 1.2) on liquid nutrient media and filter-sterilized using 0.2-μm membranes. Then, 1 mL of media at 1×, 0.5× and 0.01× concentrations were inoculated via flooding onto PDA plates and allowed to dry in a laminar flow cabinet. A 4-mm mycelial plug (from a freshly pre-grown culture of *B. cinerea*) was deposited in the center of each plate and incubated in darkness at 30 °C. Mycelial growth inhibition (mm) and mycelial growth area (cm^2) were measured at day 6. The mycelial growth area (cm^2) was determined using ImageJ software.

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Transparency document. Supporting information

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Bacterias endófitas de plantas y su posible repercusión en la salud humana

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Resumen: Las bacterias constituyen al grupo más representativo de los microorganismos unicelulares, y se encuentran presentes de forma natural en nuestro ambiente, incluyendo los alimentos. El siguiente artículo se enfoca en las bacterias patógenas para el hombre que viven dentro de las plantas y que para éstas no representan mayor riesgo (bacterias endófitas), que son capaces de trasladarse a los alimentos que consumimos día con día, a través de diversas fuentes, como son: el uso de estiércol como fertilizante, el riego de cultivos con agua contaminada, contacto con heces fecales, así como su propagación a través de insectos, plagas y hongos. La mayoría de las bacterias endófitas que llegan a nuestros alimentos y que ocasionan enfermedades están representadas por las bacterias entéricas (microorganismos que habitan generalmente el intestino de animales y personas), entre los que destacan los géneros Salmonella, Listeria y Escherichia, sin embargo, no son los únicos y la sintomatología que causan es muy diversa. Son necesarias diversas condiciones para que una bacteria endófita pueda representar un riesgo serio para la salud humana.

Palabras clave: bacterias endófitas, alimentos, salud humana.

Introducción

Los alimentos que consumimos, incluyendo los vegetales, son reservorios comunes de patógenos potenciales que pueden causar diversas infecciones en los humanos, por lo que constituyen uno de los riesgos de salud pública más generalizados. Para combatir las enfermedades causadas por la ingesta de alimentos, una de las recomendaciones es lavar y desinfectar bien las frutas y verduras. Sin embargo, la desinfección se realiza únicamente en la superficie de los vegetales, pero ¿sabías que aun así existen microorganismos patógenos que viven dentro de los tejidos de las plantas?, y que estos no son eliminados por la desinfección superficial. Pues es verdad, dentro de las plantas viven una infinidad de microorganismos, incluyendo bacterias de las especies más conocidas como patógenos oportunistas, incluyendo *Salmonella enterica*, *Listeria monocytogenes, Vibrio cholerae*, *Pseudomonas aeruginosa o Staphylococcus aureus,* los cuales potencialmente pueden causar diversas enfermedades en las personas.

Estas bacterias que viven dentro de las plantas, incluyendo los vegetales y frutas que consumimos, se les conocen como organismos endófitos. Una característica de ellos es que no causan enfermedades o daño a las plantas donde viven, aun cuando se encuentran habitando en los diferentes tejidos, como las raíces, tallos, hojas, flores y frutos (Akhtyamova, 2013). Pero ¿qué sucede si consumimos estos vegetales que contienen endófitos patógenos de humanos? ¿son realmente una amenaza para nuestra salud? A continuación revisaremos algunos datos interesantes que nos harán evaluar si de verdad es un riesgo consumir estos alimentos.

En su mayoría las bacterias que pueden causar enfermedades en humanos son consideradas como oportunistas (organismos que no suponen un problema en la salud del hombre hasta que el sistema

Tabla 1. **Bacterias patógenas oportunistas de humanos presentes en frutos y vegetales de consumo por el hombre.**

Especie bacteriana	Enfermedades que causan al hombre	Frutos y vegetales hospederos	Referencia
Pseudomonas aeruginosa	Endocarditis, neumonía, infecciones en vías urinarias, infecciones gastrointestinales y meningitis	Jitomate, zanahoria	Mahajan-Miklos et al., 1999
Serratia spp.	Conjuntivitis, queratitis, infecciones respiratorias, meningitis y endocarditis	Pepino	Kurz, 2003
Staphylococcus aureus	Infección de la piel, neumonía, septicemia	Zanahoria, cebolla y jitomate	Prithiviraj, 2005
Salmonella spp. Escherichia coli 0157:H7	Gastroenteritis y salmonelosis	Jitomate, mango, espinaca, naranjas, lechuga	Deering, 2011
Staphylococcus epidermidis	Infecciones sanguíneas	Cebolla	Nithya y Badu 2017
Stenotrophomonas maltophilia	Asociado con conjuntivitis, queratitis, escleritis, celulitis y endoftalmitis	Zanahoria, cebolla y jitomate	Islam et al., 2016
Enterobacter aerogenes	Infección del tracto urinario, endocarditis, artritis séptica, e infección de la piel	Zanahoria	Regli y Pages 2015
Enterobacter hormaechei	Se asocia con infección en el torrente sanguíneo	Zanahoria	Overbeek et al., 2014
Pseudomonas stutzeri	Neumonía, meningitis y septicemia neonatal.	Jitomate, pepino y cebolla	Miron et al., 2007
Pseudomonas stutzeri	Neumonía, meningitis y septicemia neonatal.	Jitomate, pepino y cebolla	Miron et al., 2007

inmunológico falla) y estas deben cumplir con ciertos criterios para caer dentro de esta categoría, como son: ser cultivables, antagonizar a otros organismos, ser altamente competitivos, presentar versatilidad en su nutrición, tener capacidad de formar biofilm (biopelículas formadas por microorganismos compuestas de exopolisacáridos), además de mostrar resistencia contra diversos antibióticos y toxinas (Berg et al., 2014), aunque estos patógenos usualmente no causan enfermedades en personas sanas, si representan un gran riesgo en personas inmunocomprometidas (condición en la cual la capacidad de un organismo para combatir infecciones se encuentra reducida) y esto podría resultar alarmante si consideramos que el número de individuos inmunocomprometidos se eleva continuamente en todo el mundo, de acuerdo con el Sistema de Vigilancia de Infecciones de los Estados Unidos de América, en 2002 el número de infecciones causadas a pacientes inmunocomprometidos por bacterias oportunistas fue de 1.7 millones (Klevens et al., 2007).

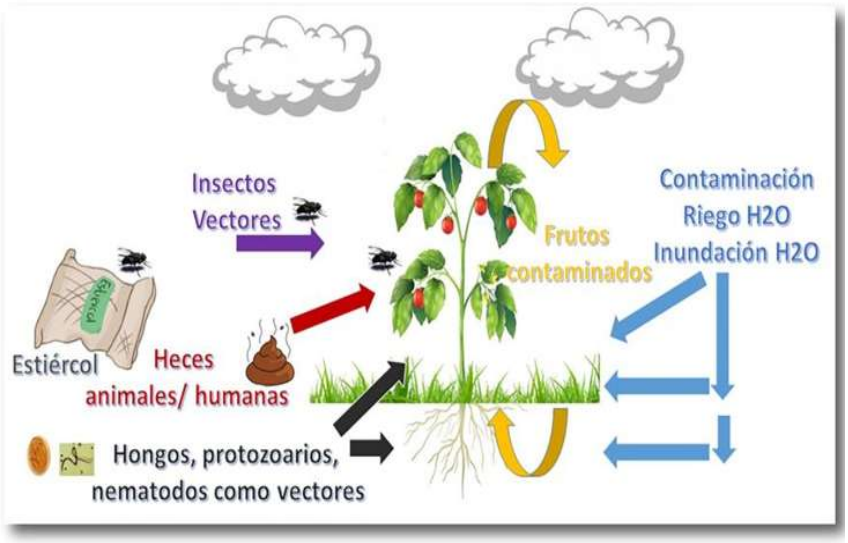

Figura 1. Factores que contribuyen a la contaminación de frutos y vegetales con bacterias patógenas de humanos (Modificado de Brandl, 2006).

En la tabla 1 se enlistan algunas de las bacterias patógenas oportunistas de humanos que pueden hospedar algunas plantas de consumo por el hombre.

Aunque como mencionamos la mayoría de las bacterias que afectan la salud del hombre son en su mayoría oportunistas, existen algunas excepciones tal es el caso de *Bacillus anthracis* que es un patógeno obligado causante del ántrax y que puede colonizar cultivos de cebolla y jitomate (Ganz et al., 2014).

Dentro de las bacterias oportunistas, el grupo más representativo es el correspondiente a bacterias entéricas (microorganismos que habitan generalmente el intestino de animales y personas) entre las que causan mayor repercusión en la salud del hombre podemos destacar a *Salmonella enterica* que es capaz de colonizar cultivos de alfalfa, jitomate y cebada e infectar un gran número de alimentos, responsable de causar Salmonelosis una enfermedad de preocupación mundial que se manifiesta con la aparición de fiebre, diarrea, cólicos abdominales, dolor de cabeza, náuseas y vómito y que en países como Estados Unidos de América y México es la enfermedad más común transmitida por alimentos (García et al., 2014).

Otro ejemplo lo tenemos en *Listeria monocytogenes* que ha sido vinculada a enfermedades potencialmente graves transmitidas por los alimentos como meningitis cuyos síntomas son la aparición de fiebre súbita, dolor de cabeza, rigidez en el cuello, náuseas y vomito o complicaciones como septicemia que es una infección en la sangre resulta potencialmente mortal (Farber y Peterkin, 1991), esta bacteria es capaz de colonizar inicialmente la rizósfera (porción del suelo que se encuentra influenciada por la raíz) de plantas de cebada para posteriormente colonizar los tejidos internos, ocupando apoplastos (espacios extracelulares por el que fluyen agua y otras moléculas) y la corteza interna de la planta (Kutter et al., 2005), hay evidencia además de que la invasión de las raíces por bacterias patógenas de humanos podría conducir a la propagación sistémica y contaminación de semillas y frutos (Guo et al., 2001).

Muchos de los patógenos potencialmente oportunistas presentan una etapa de vida endofítica y algunas de éstos tienen la capacidad de promover el crecimiento de plantas como consecuencia de mecanismos directos e indirectos; la promoción directa se presenta cuando la bacteria facilita la adquisición de nutrientes esenciales o modula el nivel de fitohormonas (moléculas producidas dentro de la célula vegetal que regulan diversos procesos en las plantas), mientras que la promoción indirecta engloba a las bacterias que disminuyen el daño a las plantas después de la infección de un patógeno (Santoyo et al., 2016).

Un ejemplo de esto lo encontramos en *Stenotrophomonas maltophilia* CR71 y *Pseudomonas stutzeri* E25 dos patógenos oportunistas de humanos, que son capaces de promover el crecimiento de plantas de jitomate al ser inoculadas en la raíz además de inhibir el crecimiento de *Botrytis cinerea* un hongo patógeno de este cultivo (Rojas et al. 2018).

Esta dualidad que presentan las bacterias a ser potenciales patógenos en un organismo (humano) y por el contrario promover el crecimiento de otro (plantas) resulta de más interesante.

¿Pero cómo es que llegan estos patógenos de humanos a las plantas?

Se pueden destacar las siguientes formas en que las bacterias patógenas colonizan las plantas (Figura 1):

- El estiércol es comúnmente aplicado a los campos de cultivo empleándolo como fertilizante, además de las heces humanas y animales lo que les permite a las bacterias patógenas sobrevivir por periodos prolongados de tiempo pudiendo colonizar las plantas.

- Riegos de cultivos y aplicaciones de pesticidas con agua contaminada, que llegan a los cultivos favoreciendo su contaminación.

- Transmisión de bacterias patógenas a los cultivos por medio de insectos, hongos, protozoarios y nematodos como vectores actuando como un factor de contaminación antes de la cosecha (Brandl, 2006).

Pese a este panorama no debemos alarmarnos por la presencia de bacterias patógenas en nuestros alimentos, debido a que siempre han estado presentes, incluso la comida enlatada según la FDA (Administración de Alimentos y Medicamentos) presenta partes de insectos, áfidos y huevecillos y tienen valores máximos permisibles de acuerdo a las diversas normas establecidas, y lo mismo ocurre con las bacterias patógenas que mientras no rebasen un umbral (cantidad de unidades formadoras de colonias), no representan mayor daño su consumo, incluso para pacientes inmunocomprometidos, aunque por otra parte siempre es bueno tomar todas las medidas de higiene al momento de ingerir nuestros alimentos.

Conclusiones y perspectivas

La aparición de brotes de enfermedades transmitidas por alimentos asociados con frutas y verduras ha recibido gran interés entre las agencias de salud pública, provocando una nueva ola de investigaciones en cuanto a la seguridad alimentaria y su relación con la contaminación microbiana de frutos y vegetales.

Para disminuir estos brotes es necesaria una mejora en las prácticas agrícolas y de consumo para disminuir el potencial de contaminación del producto, poniendo especial atención en las plantas, frutos y vegetales que son de mayor consumo por el hombre.

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