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**“Estudio de los mecanismos de
bioestimulación por bacterias del maíz en
Arabidopsis thaliana”**

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RESUMEN

Las comunidades bacterianas son esenciales en el microbioma vegetal, las cuales residen en la rizosfera, una región del suelo altamente influenciada por exudados radicales a través de una señalización química compleja. Una parte del carbono fijado durante la fotosíntesis es transformado y secretado por la raíz en forma de compuestos orgánicos que actúan como atrayentes químicos, ayudando al establecimiento de relaciones simbióticas. Las Rizobacterias Promotoras del Crecimiento Vegetal (PGPR; *Plant Growth Promoting Rhizobacteria*) producen metabolitos que estimulan el crecimiento y mejoran las respuestas de inmunidad en las especies vegetales.

Aunque el potencial de los microorganismos como bio-inoculantes (formulaciones de microorganismos vivos con propiedades benéficas de fitoestimulación) es altamente promisorio, existen escasos conocimientos sobre los mecanismos que regulan sus interacciones con las plantas. En este trabajo se identificaron molecular y fenotípicamente dos rizobacterias aisladas de la rizosfera del cultivo de maíz en un suelo alcalino, del predio denominado “el Lometón” en el municipio de Tarimbaro, Michoacán, México. La secuencia del ADN que codifica para el ARN ribosomal 16S, posicionó taxonómicamente a dichos aislados como *Micrococcus luteus* y *Bacillus* sp. LC390B. Ambas bacterias presentaron respuestas promotoras sobresalientes en la biomasa y el desarrollo de *Arabidopsis* en ensayos *in vitro* por contacto directo, aunque su influencia sobre la morfología de la raíz y sus atributos son distintas. *M. luteus* detiene el crecimiento de la raíz primaria incrementando la respuesta auxínica, y la inducción de la ramificación de raíces laterales y la mayor producción de pelos radicales, mientras que *Bacillus* incrementó el peso fresco del follaje y de la raíz, así como el contenido de clorofila a través de los fitocromos PHYA y PHYB. Estos resultados contribuyen con conocimiento fundamental en las interacciones planta microorganismo de cada grupo para su implementación como herramientas biotecnológicas y su aplicación en sistemas de cultivos agrícolas y hortícolas.

Palabras clave: Rizobacterias, fitocromos, auxinas, arquitectura vegetal, fitoestimulación.

ABSTRACT

Bacterial communities are integral to the plant microbiome and reside in the rhizosphere, a soil region influenced by root exudation. Part of the fixed carbon during photosynthesis is transported to the root and secreted in the form of organic compounds that act as chemical attractants, and help establishing symbiotic relationships. Plant Growth Promoting Rhizobacteria (PGPR; *Plant Growth Promoting Rhizobacteria*) produce metabolites that stimulate growth and enhance immune responses in vegetables.

Although many efforts have been done applying microbes as bioinoculants in many crops, there is limited knowledge about the mechanisms that regulate plant growth promotion under bacterial symbiosis. In this regard, we analyzed and identified molecularly and phenotypically the biostimulation properties of two rhizobacteria isolated from alkaline soil from the rhizosphere of maize crops, typified molecularly as *Micrococcus luteus* and *Bacillus* sp. LC390B. Both bacteria showed outstanding growth promoting responses in Arabidopsis seedlings by direct co-cultivation *in vitro* assays. However, the morphological characteristics of roots exposed to each bacteria varied. *M. luteus* halted root growth by a mechanism involving auxin signaling that drives root branching and root hair formation, whereas *Bacillus* sp. LC390B increased foliage and root fresh weight and chlorophyll content through the phytochromes PHYA and PHYB. These results contribute with basic knowledge on plant-microorganism interactions that highlight the probiotic properties of each group for their implementation as biotechnological tools and their application in agricultural and horticultural crop systems.

Keywords: Rhizobacteria, phytochromes, auxin, plant architecture, phytostimulation.

1. INTRODUCCIÓN

El crecimiento, desarrollo y defensa en las plantas, están modulados por la homeostasis de un conjunto de fitohormonas, que regulan procesos específicos o de manera conjunta en una red compleja de interacciones a partir de la integración de señales ambientales (Murphy, 2015). El tallo y las ramas son estructuras de soporte para las hojas, órganos encargados de realizar la fotosíntesis, así como las flores con funciones en la reproducción (Reinhardt y Kuhlemeier, 2002). El sistema radical, por otra parte, es un sistema dinámico que está especializado en la captura de agua y nutrientes, exploración del suelo y el establecimiento de interacciones con microorganismos (Birt *et al.* 2022). Los fotosintatos provenientes del follaje se transportan hacia la raíz y pueden secretarse hacia la rizosfera promoviendo eventos de simbiosis con la microbiota residente. Dicha comunicación impacta en el crecimiento e inmunidad vegetal a través de compuestos con actividad biológica, que son moléculas de señalización (Backer *et al.* 2018).

Las rizobacterias promotoras del crecimiento vegetal o PGPR (*Plant Growth Promoting Rhizobacteria*), por sus siglas en inglés, producen reguladores del crecimiento, como auxinas y citocininas, modulando así la formación de raíces laterales y pelos radicales (Teale *et al.* 2006; Méndez-Gómez *et al.* 2021; García-Cárdenas *et al.* 2022). El conocimiento generado a partir de la investigación básica por ensayos *in vitro* con *Arabidopsis*, nos ha permitido avanzar en el entendimiento de las interacciones planta-bacteria, no obstante, para poder aplicar este conocimiento en especies de interés agronómico en campo, es importante distinguir las propiedades de cada aislado bacteriano, entre los que se incluyen la solubilización de nutrientes, la fijación de nitrógeno, la resistencia a patógenos, la tolerancia a estrés abiótico por pH alcalino y ácido, por la salinidad, altas temperaturas, estrés hídrico o causado por sequías. Al respecto, la diversidad taxonómica de las PGPR es amplia, en el grupo de las Gram negativas, encontramos los géneros *Azospirillum*, *Rhizobium*, *Pseudomonas*, *Azotobacter*, y *Paenibacillus* (Swarnalakshmi *et al.* 2020; Francis *et al.* 2010), en tanto que en las Gram positivas destacamos a las Actinobacterias y al género *Bacillus* por su ubicuidad y prevalencia en los ecosistemas. Por ejemplo, *B. subtilis*, *B. megaterium*,

B. cereus, *B. amyloliquefaciens* y *B. licheniformis* se han reportado como bacterias probióticas en vegetales (Akinrinlola *et al.* 2018). Entre las actinobacterias, la familia *Micrococcaceae* incluye representantes con propiedades sobresalientes, como la producción de auxinas y otros reguladores. *Micrococcus luteus* y *Kocuria* sp, son cepas halotolerantes, solubilizadoras de fosfato, productoras de auxinas y promueven el crecimiento vegetal (Dastager *et al.* 2010; Li *et al.* 2020).

La comunicación planta-bacteria implica la producción de exudados radicales, que depende de la maquinaria fotosintética. Sin embargo, poco se sabe sobre la regulación de los programas de crecimiento en simbiosis benéficas que involucren fotorreceptores de las plantas. En este estudio, se identificaron las rizobacterias *Micrococcus luteus* LS570 y *Bacillus* sp LC390B, que incrementaron el peso fresco y la ramificación de la raíz a través de diferentes mecanismos. Estos atributos son importantes para considerarlas como probióticos y aplicarlas eventualmente en la agricultura.

2. ANTECEDENTES

2.1. *Arabidopsis thaliana*

La implementación de *Arabidopsis thaliana* en la investigación como organismo modelo, ha contribuido a generar conocimiento en múltiples áreas de las ciencias biológicas. Taxonómicamente, es una angiosperma de la familia Brassicaceae, nativas del norte de África y Eurasia. Este grupo también incluye especies de gran importancia agronómica como el nabo (*Brassica rapa*), el colinabo (*Brassica napus*), la coliflor (*Brassica oleracea*), y la mostaza (*Brassica nigra*). *Arabidopsis* destaca en la investigación científica como organismo modelo por tener un genoma pequeño (115 Mb) distribuido en 5 cromosomas, un ciclo de vida corto, de aproximadamente de 6-8 semanas, produce una gran cantidad de semillas, es fácil de cultivar y se han generado colecciones amplias de mutantes y plantas transgénicas para estudiar todos los procesos de crecimiento, desarrollo, nutrición, metabolismo, respuestas hacia estímulos y múltiples procesos fisiológicos (Szymanski, 2013).

Bajo condiciones adecuadas, las pequeñas semillas de *Arabidopsis* son altamente viables en comparación con otras especies y germinan dentro de un periodo de 2 días. Cuando se establece la planta sobre un sustrato, durante el crecimiento vegetativo, las hojas ovaladas forman una roseta compacta de la que emerge el tallo principal que da soporte a las flores, frutos y semillas. La configuración del sistema aéreo y radical, permite el estudio de interacciones con microorganismos que influyen las respuestas de crecimiento, nutrición, desarrollo y defensa frente a diversos estímulos bióticos y abióticos.

2.2. Organización tisular en *Arabidopsis*

La organización y estructura celular determinan las funciones de los distintos órganos. En los organismos pluricelulares, las células están en constante recambio, en las plantas, particularmente, es un proceso constante en el que las células muertas se reemplazan por nuevos linajes para mantener la tasa de crecimiento y diferenciación (Wendrich y Weijers, 2013). En este proceso intervienen células

iniciales que producen dos células hijas y se encuentran en compartimentos específicos, llamados meristemos.

En los meristemos, se encuentran células pluripotentes mitóticamente activas, localizadas en las regiones apicales en el sistema aéreo y en la raíz, las cuales regulan la transición de la división a la elongación y diferenciación celular (Fig. 1) (Greb y Lohmann, 2016). El meristemo apical radical (RAM; *Root Apical Meristem*) y el meristemo apical del follaje (SAM; *Shoot Apical Meristem*), por sus siglas en inglés, producen continuamente células que originan los tejidos de la raíz y del sistema aéreo, respectivamente (Chang *et al.* 2020). En plántulas de *Arabidopsis* de 7 días después de la germinación (7 d.d.g), el grupo de células iniciales en el RAM rodea a cuatro células que conforman el centro quiescente (QC), que se forman durante el desarrollo embrionario, raramente se dividen, por lo que su función principal es mantener la identidad de las células iniciales y prevenir e inhibir su diferenciación. Estos programas están coordinados genéticamente por factores de transcripción, fitohormonas y moléculas de señalización (Pacifici *et al.* 2015; Rovere *et al.* 2016).

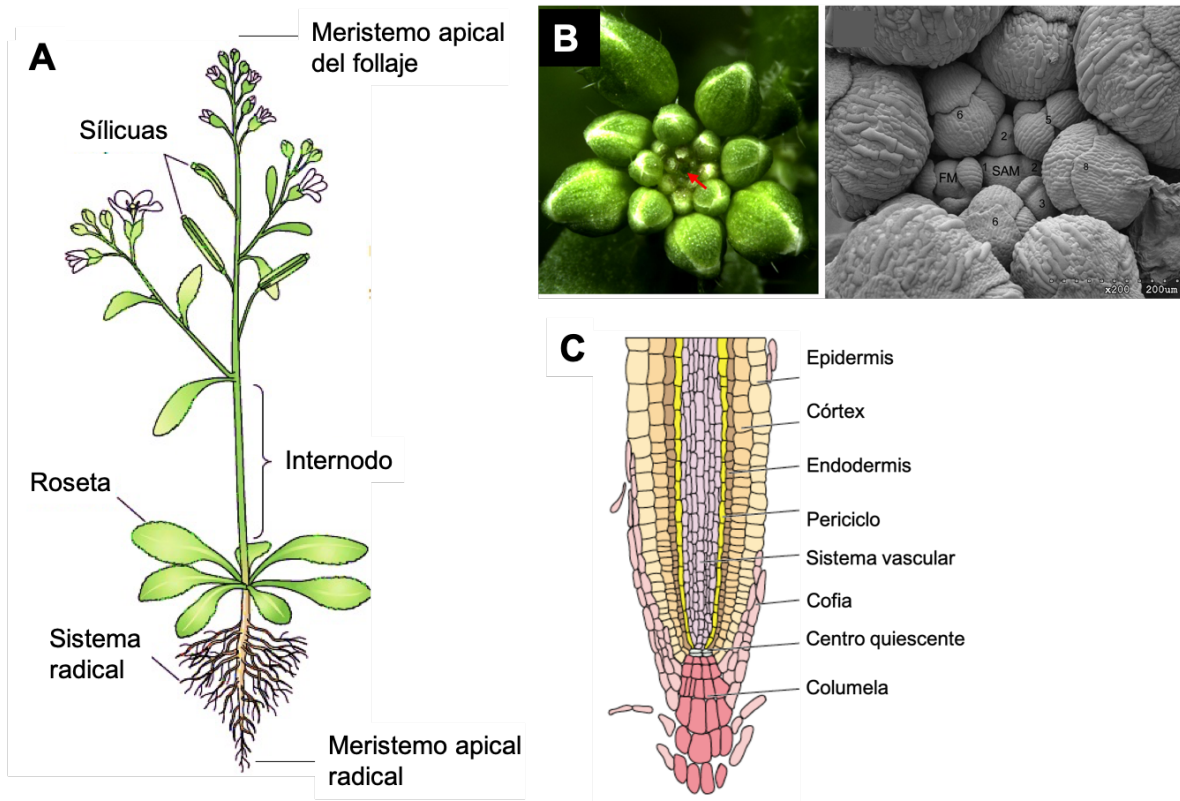


Figura 1. Representación del meristemo apical del follaje y raíz en *Arabidopsis*. A. Estructura completa de *Arabidopsis*. B. Meristemo apical del follaje (SAM) en el centro, mostrando los meristemos florales. En la micrografía tomada con microscopía electrónica de barrido, los números representan el estadio de desarrollo de los meristemos florales (FM). C. Estructura del meristemo apical radical (RAM). Adaptado de Chang *et al.* (2020) y Taiz y Zeiger (2002).

La disposición y organización espacial de los órganos vegetales a lo largo del tallo se conoce como filotaxia y comprende la arquitectura vegetal, la cual está en constante cambio debido a las fluctuaciones ambientales, factores bióticos y abióticos, así como señales intrínsecas y extrínsecas (Reinhardt y Kuhlemeier, 2002; Hodge *et al.* 2009). Como organismos sésiles, las plantas perciben y responden rápidamente a los estímulos modificando sus programas de crecimiento y desarrollo, de manera que pueden cambiar su arquitectura como una estrategia

adaptativa ante la adversidad. Esta propiedad se define como plasticidad fenotípica y contribuye a optimizar la adquisición de recursos (Satbhai *et al.* 2015).

2.2.1. Estructura de la raíz primaria

La raíz de *Arabidopsis* tiene una configuración en forma de cilindro con varias capas, de la más externa al interior se encuentran la epidermis, la corteza, la endodermis, el periciclo y el cilindro vascular, constituido por xilema y floema. Las plantas dicotiledóneas presentan una raíz principal o primaria de origen embrionario y raíces laterales post-embrionarias que se forman específicamente de las células del periciclo, localizadas en la capa adyacente al cilindro vascular (Tian *et al.* 2014). Adicionalmente, se forman pelos radicales en las células epidérmicas denominadas tricoblastos. Las raíces laterales y los pelos radicales contribuyen al incremento del área de superficie, aumentando la capacidad de exploración del sustrato, y la captación de agua y nutrientes.

En una vista longitudinal, en el meristemo apical radical se distinguen cuatro zonas de crecimiento, i) la zona meristemática, ii) la zona de transición, iii) la zona de elongación y iv) la zona de diferenciación (Petricka y Benfey, 2008). En la zona meristemática, ocurre una alta tasa de proliferación celular, los análisis moleculares de plántulas transgénicas que expresan el gen reportero *uidA* fusionado a la ciclina B1 (*CYCB1:uidA*), demostraron que la zona meristemática ocupa una región de unos 200 μm sobre la columela. En la interfase entre la zona meristemática y la de elongación se denomina meristemo basal, en la que las células se preparan para una rápida elongación. El tamaño de esta zona de la raíz comprende los 200 a 520 μm desde la columela. Enseguida se localiza la zona de elongación, la cual se identifica por la rápida expansión longitudinal de las células que excede la amplitud, abarcando aproximadamente de 520 a 850 μm (Verbelen *et al.* 2006).

2.2.2. Origen de las raíces laterales

En la zona de diferenciación, las células adquieren sus funciones distintivas, aquí es posible visualizar el sistema vascular, los pelos radicales y las raíces laterales. Las raíces laterales determinan la capacidad exploratoria y con ello el potencial de absorción de agua y nutrientes. Su origen es a partir de las células del periciclo, proceso que ocurre en cinco fases, i) la formación del sitio de pre-iniciación, que se lleva a cabo en la zona meristemática y de elongación, ii) la iniciación de las células fundadoras de las raíces laterales, donde ocurre una migración nuclear en estas células y las células individuales o en pares del periciclo se dividen anticlinalmente (división celular que se lleva a cabo en el ángulo perpendicular al plano de división) y de forma asimétrica, adyacentes a las células fundadoras, que resulta de la formación de dos células hijas que continúan dividiéndose hasta formar un primordio (De Smet, 2011). En esta etapa, se acumulan las auxinas localmente y se inducen genes de señalización como *AUX/IAA14* o *SOLITARY-ROOT (SLR)* y *AUXIN RESPONSE FACTORS (ARF7/ARF19)*. La mutación con ganancia de función *slr1*, resulta en el bloqueo de las primeras divisiones celulares en el periciclo, por lo que la degradación de los represores transcripcionales es esencial para las divisiones asimétricas que ocurren durante la formación del primordio. iii) Formación de una estructura en forma de domo llamada primordio, iv) la salida de la raíz primaria, que da lugar cuando el primordio formado atraviesa las capas celulares externas de la raíz principal, la endodermis, el córtex y la epidermis y, v) la activación del meristemo de la nueva raíz lateral, adquiere un meristemo totalmente funcional, similar al de la raíz parental, con un centro quiescente y células madre que lo rodean (Fig. 2; Péret *et al.* 2009; Du y Scheres, 2018).

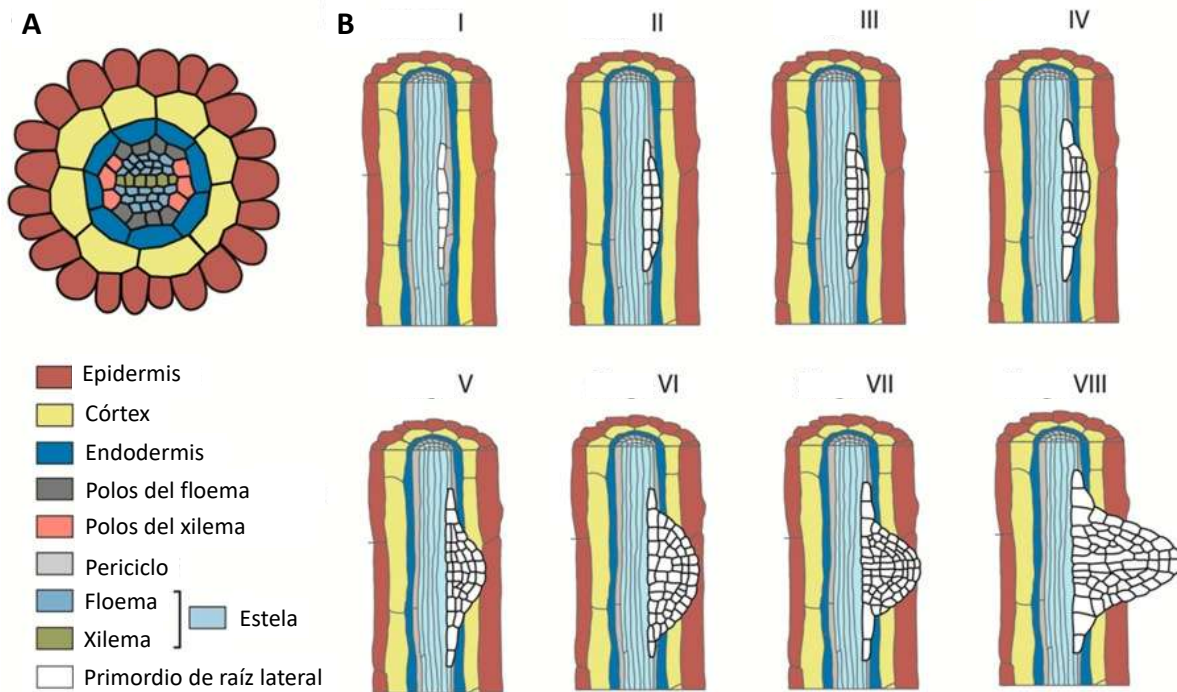


Figura 2. Iniciación y emergencia de las raíces laterales. A. Vista transversal de la estructura de la raíz primaria. B. Número de las etapas del desarrollo y emergencia de las raíces laterales. Modificado de Péret *et al.* (2009).

2.2.3. Las auxinas en la formación de raíces laterales

Los estudios farmacológicos con inhibidores del transporte de auxinas proveen evidencias que sustentan la función de estas fitohormonas en el desarrollo de las raíces laterales. Los tratamientos con el inhibidor *N*-1-ácido naftilftalámico (NPA), evitan que las células del periciclo pasen de la fase G1 a S del ciclo celular, por la activación del inhibidor de ciclinas dependientes de cinasas (CDK), KRP2, y las células no entran en mitosis. Por lo contrario, la aplicación de auxinas promueve la ramificación de la raíz (Teale *et al.* 2005).

Las auxinas dirigen la especificación de las células fundadoras del periciclo, que al dividirse forman un primordio. Dichas células adquieren características diferenciales, poseen un núcleo más grande, citoplasma denso y vacuolas pequeñas (Casimiro *et al.* 2003; Dubrovsky *et al.*, 2008). Huang y col. (2019), describieron detalladamente la relación entre las auxinas y la vía de señalización de

las MAP cinasas (MAPK) en la formación de raíces laterales mediante ensayos moleculares con protoplastos de *Arabidopsis*, en donde se encontró que las auxinas activan a las MAPK5 y 6 a través de cinasas transmembranales (TMK), que en conjunto con auxinas regulan la división celular en distintas etapas del desarrollo de las raíces (Huang *et al.* 2019).

El incremento en los niveles endógenos de auxinas aumenta el tamaño vacuolar y la presión de turgencia, consecuentemente, el tamaño de las células aumenta. En este contexto, las auxinas polimerizan la pectina y activan ATPasas de la membrana plasmática que provocan la acidificación del apoplasto, se activan enzimas que degradan la pared celular ejerciendo una presión turgente que expande la célula. Así mismo, se estimula la expresión de numerosos genes que codifican para los componentes de la pared celular en el tejido nuevo que se forma durante el desarrollo de los primordios (Majda y Robert, 2018).

La rizotaxia, que conlleva a la orientación de las raíces laterales sobre la raíz principal, también es modulada por auxinas. En cada curvatura que presenta la raíz durante su crecimiento, se forma una raíz lateral en una disposición de derecha-izquierda alternada. Los modelos obtenidos a partir de análisis moleculares y anatómicos explican la relevancia del transporte de influjo mediado por AUXIN RESISTANT1/LIKE-AUX1 (AUX1/LAX), lo que provoca una acumulación de auxinas en las células del periciclo que se requieren para la formación de un nuevo órgano (De Smet *et al.* 2007; Laskowski *et al.* 2008).

La regulación del desarrollo de las raíces laterales no es un evento único de las auxinas, sino que su interacción con otras fitohormonas contribuye a coordinar este proceso de organogénesis actuando de manera conjunta o antagónica. Las citocininas, moléculas derivadas de la adenina, inhiben la iniciación de las raíces laterales a causa de la interrupción del flujo auxínico y el nivel de las proteínas transportadoras. En relación con este resultado, en las mutantes afectadas en la vía de señalización de citocininas se forman más raíces laterales (Su *et al.* 2011; Kieber y Schaller, 2014; Jing y Strader, 2019).

El etileno es una fitohormona de naturaleza química gaseosa, que se difunde libremente en las células, se deriva de la *L*-metionina y su precursor es el ácido 1-

aminociclopropano-1-carboxílico (ACC). En las plantas, está directamente relacionado con la activación de la respuesta inmune, el desarrollo de las hojas, la maduración de los frutos, la senescencia y la respuesta a estrés biótico y abiótico (Iqbal *et al.* 2017). En el meristemo apical de la raíz, inhibe la proliferación celular y este efecto se revierte en las mutantes insensibles a etileno *ETHYLENE INSENSITIVE ein2* y *ein3*. El etileno promueve la biosíntesis auxínica, inhibiendo la elongación celular y el crecimiento de la raíz (Street *et al.* 2015). En la formación de raíces laterales, el etileno modifica los transportadores PIN3 y PIN7 del cilindro vascular y afecta la acumulación de auxinas mediada por AUX/LAX, que se requiere en las células del periciclo para la iniciación de los primordios (Zemlyanskaya *et al.* 2018).

2.2.4. Biosíntesis de auxinas

Las auxinas se producen en hojas jóvenes, cotiledones, y meristemos (Ljung *et al.* 2005). La principal vía de biosíntesis inicia a partir del aminoácido triptófano, siguiendo cuatro posibles rutas: la vía del ácido indol-3-piruvato (IPA), la del indol-3-acetamida (IAM), la de la indol-3-acetaldoxima (IAN) y la que involucra a la triptamina (TAM) (Fig. 3) (Hiroyuki, 2016).

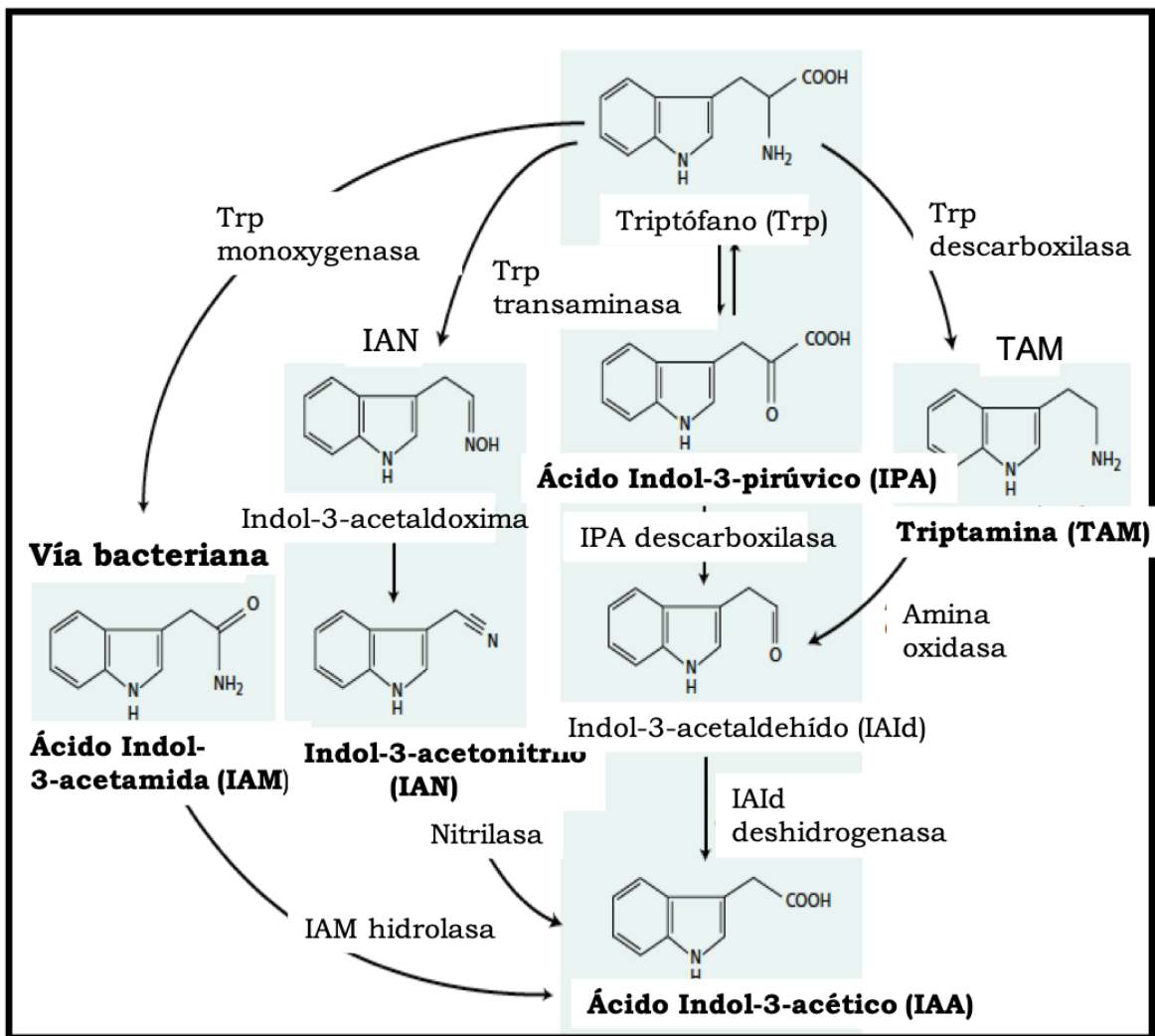


Figura 3. Rutas de biosíntesis auxínica dependiente del triptófano y sus principales intermediarios.

La vía IPA es la más conservada en las plantas e involucra dos reacciones, una desaminación y posteriormente una descarboxilación para formar indol-3-acetaldehído (IAId), el cual mediante la acción de una deshidrogenasa es oxidado a IAA. En la conversión a auxinas participan las enzimas aminotransferasas de triptófano (TAA) y YUCCA (YUC), de la familia de las flavin monooxigenasas. Los genes *YUC* están conservados en las plantas, en el genoma de *Arabidopsis* existen 11 genes con diversas funciones en el desarrollo.

La mutación en un gen *yuc* no compromete el crecimiento, sin embargo, al afectar más de un gen en combinaciones dobles, triples y cuádruples conlleva a fenotipos

con múltiples alteraciones, mientras que la sobreexpresión de estos genes causa defectos en el desarrollo vegetal (Chen *et al.*, 2014; Munguía-Rodríguez, 2020; (Zhao, 2014).

Durante la conversión de triptamina (TAM) a AIA ocurre una descarboxilación seguida de una desaminación, contrario a la vía IPA, en tanto que en la ruta de la indol-3-acetamida, el primer paso en la conversión de triptófano a IAM es catalizado por la enzima triptófano-2-monooxigenasa y posteriormente se convierte a auxinas por la hidrolasa de indol-3-acetamida, codificada por el gen *AMI1*. Esta vía fue identificada por medio de la caracterización de *Agrobacterium rhizogenes*, el cual produce un incremento notable en la ramificación de la raíz y la formación de pelos radicales (Mano y Nemoto, 2012).

Los citocromos de la familia P450 CYP79B2 y CYP79B3 convierten el triptófano en IAOx en una reacción de oxidación, posteriormente, IAOx actúa como sustrato para la formación de indol-3-acetonitrilo. Finalmente, una nitrilasa convierte IAN en AIA. Estos citocromos fueron identificados en un estudio molecular de mutantes sobreproductoras de auxinas, denominadas *superroot* (*sur1* y *sur2*). Ambos participan en la vía de biosíntesis de glucosinolatos (Brumos *et al.* 2013; Zhao, 2014).

Existe evidencia sobre una vía independiente del triptófano para la producción de auxinas a través de una sintasa de indol (INS) localizada en el citosol. La mutante (*ins-1*) afecta severamente el patrón de división celular y produce letalidad del embrión en un ensayo con la doble mutante *ins1trp3-1*, en la que se afecta la biosíntesis de auxinas de manera dependiente e independiente del triptófano (Wang *et al.* 2015). La diversidad de rutas dependientes e independientes del triptófano, así como de los intermediarios que modulan la biosíntesis de auxinas resaltan la importancia en las funciones que desempeñan dichas fitohormonas en el crecimiento y desarrollo vegetal.

2.2.5. Transporte y señalización de auxinas

Las auxinas se transportan mediante proteínas de influjo y eflujo localizadas en la membrana plasmática de las células, formando gradientes locales desde sus sitios de síntesis (Petrášek y Friml, 2009). El modelo quimiosmótico elaborado por Rubery y Sheldrake (1974), permitió explicar el flujo y la direccionalidad auxínica dependiente de su naturaleza química. Debido a que es ligeramente ácida ($pK_a=4.85$), una parte se encuentra protonada en el apoplasto (pH 5.5), lo que facilita su entrada en la célula a través de la membrana plasmática, mientras que en el citoplasma el pH es alcalino (pH 7) favoreciendo la forma aniónica (IAA^-) y no pueden atravesar la membrana celular, de manera que se necesitan los transportadores de eflujo para su distribución (Krupinski y Jönsson, 2010; Geisler, 2021).

Los acarreadores de influjo se identificaron mediante la mutante agravitrópica, *auxin resistant1* (*aux1*) en *Arabidopsis* y codifica para una familia de proteínas transmembranales tipo permeasa implicados en la toma de auxinas que promueve el movimiento del floema hacia el ápice de la raíz (Taiz y Zeiger, 2002). Por otra parte, el eflujo de auxinas está mediado por ocho transportadores de la familia PIN. Cada miembro presenta regiones trans-membranales de distinta composición y función, (PIN 1-4 y 7) contienen de 10 a 12 regiones trans-membranales separados por una región hidrofílica. En el retículo endoplasmático se encuentran PIN5, 6 y 8 cuyas funciones son internalizar a las auxinas para su conjugación.

La señalización de las auxinas ocurre a través de las proteínas TRANSPORT INHIBITOR RESPONSE1 (TIR1) y AUXIN SIGNALING F-BOX PROTEINS (AFB), ésta última incluye 6 miembros en *Arabidopsis*. La regulación de la transcripción de genes de respuesta a auxinas está sujeta a la degradación de represores transcripcionales AUX/IAA por ubiquitinación en el proteosoma 26S, a través del complejo SKP1-Cul1 F-Box SCF^{TIR1/AFB}, al que se unen los receptores una vez que los niveles de auxinas se incrementan. Consecuentemente, los activadores de transcripción de la familia AUXIN RESPONSE FACTOR (ARF) forman multímeros que se unen directamente a los elementos de respuesta a auxinas (*AuxRES*) en los promotores para iniciar la transcripción (Fig. 4). Los genes que se transcriben a

partir de esta vía incluyen a *GRETCHEN HAGEN 3 (GH3)*, *AuxIAA*, y *SMALL AUXIN UP-REGULATED RNA (SAUR)* (Wang y Estelle, 2014; Powers y Strader, 2020).

Cuando los niveles de auxinas son bajos, ocurre una inhibición de la transcripción de genes a distintos niveles. La regulación por el complejo TOPLESS (TPL) recluta desacetilasas de histonas del nucleosoma (HDACs), provocando la compactación del ADN y la represión transcripcional. La interacción del receptor TIR1 con óxido nítrico promueve la S-nitrosilación, mejorando la degradación de los represores de transcripción por el complejo SCF, el cual constituye un nivel de regulación post-traducciona (Wang y Estelle, 2014; Powers y Strader, 2020).

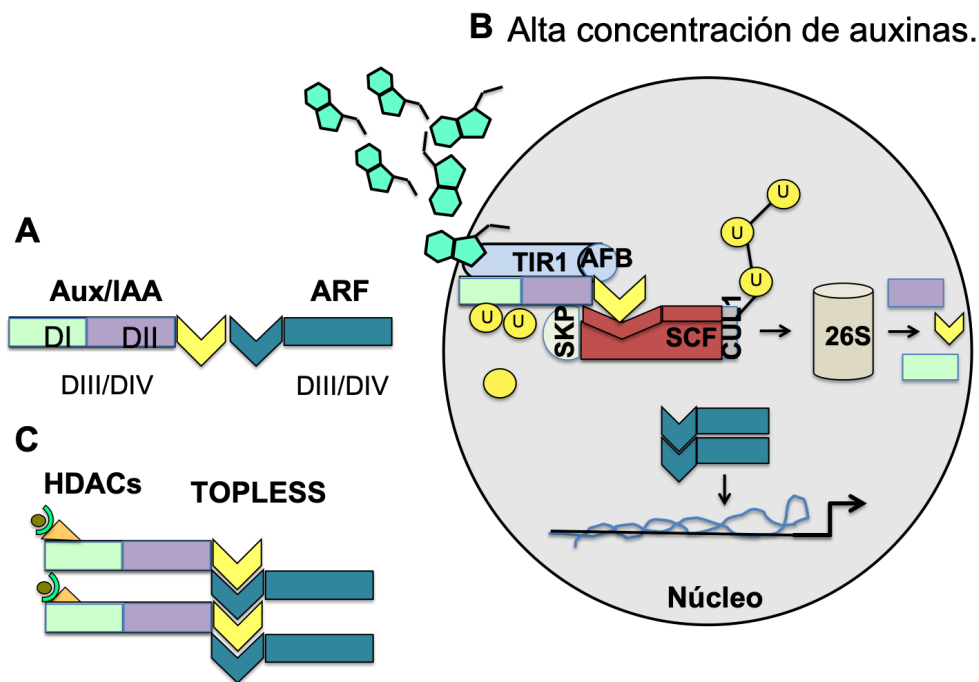


Figura 4. Vía de señalización auxínica. A. Estructura de las proteínas represoras de la transcripción (Aux/IAA) y factores de transcripción (ARF) con sus dominios de interacción DIII/DIV. B. Activación de la vía de señalización en altas concentraciones de auxinas, en la que se degradan los represores transcripcionales en el proteosoma 26S, permitiendo la dimerización de ARFs para la transcripción de genes de respuesta a auxinas. C. Complejo multimérico formado entre las proteínas Aux/IAA, ARF y TOPLESS, que interactúa con desacetilasas de histonas (HDACs) reprimiendo así la transcripción de genes relacionados a auxinas.

El genoma de *Arabidopsis* codifica para 29 proteínas de AUX/IAA y 23 de la familia ARF. En la vía auxínica, Aux/IAA forma un complejo multimérico con ARFs para reprimir la transcripción, interactuando a través del dominio DIII/IV. De las 23 proteínas ARF, únicamente ARF5, 6, 7, 8 y 19 se identificaron y caracterizaron como activadores transcripcionales. De los procesos más importantes que regulan está la formación de raíces laterales a través de las proteínas *LATERAL ORGAN BOUNDARIES-DOMAIN16/ASYMMETRIC LEAVES2-LIKE18* (LBD29/ASL16), ya que como se ha demostrado, la pérdida de función en la doble mutante *arf7/arf19* exhibe un fenotipo severamente afectado en la formación de estas estructuras (Okushima *et al.* 2007). Por otra parte, *SOLITARY ROOT* o *IAA14* (SLR/IAA14) es uno de los genes que se expresa en la raíz y en el periciclo, cuyo producto proteínico interactúa con ARF7 y ARF19. La mutación con ganancia de función en *slr1* bloquea la división celular en la fase de transición de G1-S para la iniciación de las raíces laterales, exhibiendo un fenotipo carente de estas estructuras y una raíz primaria agravitrópica (Goh *et al.* 2012).

Diversos genes interactúan con los distintos módulos entre Aux/IAA y proteínas ARF en la regulación de las etapas para el desarrollo de raíces laterales. Mediante estudios transcriptómicos se logró identificar al factor de transcripción GATA23, que se expresa específicamente en las células del periciclo y determina la identidad de las células fundadoras (De Rybel *et al.* 2010; Vangheluwe y Beeckman, 2021). De igual forma, la proteína nuclear ABERRANT LATERAL ROOT FORMATION 4 (ALF4) es necesaria para el mantenimiento de las células del periciclo en mitosis, cuya interacción ocurre con una subunidad del complejo SCF que controla la degradación de proteínas Aux/IAA (Bagchi *et al.* 2018).

2.2.6. Las auxinas como moléculas de señalización en la rizosfera

En la rizosfera, los microorganismos establecen una comunicación dinámica con las plantas a través del intercambio de diversas señales químicas. Las auxinas son biomoléculas móviles que cumplen funciones esenciales en las plantas, y que son

producidas por las bacterias y los hongos (Nadarajah y Rahman, 2021). *Agrobacterium tumefaciens* y *Pseudomonas savastanoi* producen ácido indol-3-acético, causando un desbalance en la división celular que causa la formación de tumores y agallas en el huésped, sin embargo, también microorganismos considerados benéficos las producen, impactando positivamente en el crecimiento, desarrollo e inmunidad vegetal (Ouzari *et al.* 2008; Spaepen y Vanderleyden, 2011). Esta dualidad de las auxinas en las interacciones con organismos benéficos o patógenos es interesante, y depende de diversos factores, como el tipo de microorganismo que la produce, cómo ésta es percibida por el huésped, así como el tipo de señalización que desencadena (Spaepen y Vanderleyden, 2011).

Las rutas de biosíntesis de auxinas en los microorganismos son similares a las de plantas. En experimentos de cultivo de bacterias *in vitro*, al suplementar triptófano se incrementa la producción de ácido indol-3-acético (Apine y Jadhav, 2011; Oliveira *et al.* 2021). Estudios bioquímicos y genéticos demuestran que los patógenos utilizan la vía de IAM, mientras que los benéficos utilizan la ruta IPA, sin embargo, varias vías están presentes y activas tanto en microorganismos benéficos como patógenos. También pueden producir AIA por la vía TAM descarboxilando el triptófano a triptamina y su conversión a indol-3-acetaldehído (IAAld) por una amina oxidasa. Por otra parte, la vía triptófano oxidasa (TSO) convierte el triptófano directamente a IAAld y éste es después oxidado en IAA (Keswani *et al.* 2020).

El gen *ipdC* que codifica para una indol-3-piruvato descarboxilasa es esencial en la síntesis de auxinas por microorganismos, como *Azospirillum brasilense* Sp245 en los que su mutación disminuye la producción de auxinas (Spaepen *et al.* 2007; Spaepen *et al.* 2014). Diversos estudios demuestran que las auxinas son moléculas de señalización microbiana. El ácido indol-3-acético regula la expresión de genes de virulencia (*vir*) en patógenos como *Agrobacterium tumefaciens*. En la levadura *Saccharomyces cerevisiae* la aplicación de auxinas provoca un crecimiento invasivo dependiente de la concentración, donde los procesos de filamentación y adhesión son modulados por *FLO11*. Los genes que están involucrados en vías metabólicas también son sobre regulados por AIA, así como la formación de biofilm (Prusty *et al.* 2004). Entre las especies del género *Rhizobium* spp, la nodulación está

relacionada con la producción de auxinas (Herrbach *et al.* 2016). Otros reportes denotan el rol de protección de las auxinas frente a condiciones de estrés biótico. En arroz ante estrés por patógenos, ocurrieron cambios en los perfiles de expresión de más de 300 genes relacionados con auxinas incluyendo *GH3*, *Aux/IAA*, *SAUR* y *ARF* (Ghanashyam y Jain, 2009). El tratamiento con auxinas, así como una sobreproducción de estas moléculas en plántulas que sobreexpresan el gen *YUCCA1* resulta de un incremento en la susceptibilidad a la infección (Kunkel y Harper, 2018).

En las bacterias, las auxinas modulan una amplia diversidad de procesos, entre los que están la biosíntesis de antibióticos (Matilla *et al.* 2018), la expresión de genes de virulencia y moléculas efectoras (Djami-Tchatcou *et al.* 2020), también confieren protección contra estrés oxidativo provocado por la producción de especies reactivas de oxígeno (Tzipilevich *et al.* 2021). Por ello, las auxinas son, además de señales que coordinan eventos de crecimiento y desarrollo vegetal, verdaderas moléculas de señalización microbiana, debido a que pueden regular la expresión de distintos genes y su interacción con las plantas.

2.3. Composición del microbioma vegetal

El microbioma vegetal incluye los hongos, las bacterias, arqueas, protistas, nemátodos y oomycetes que establecen relaciones con las plantas. Los tipos de interacción pueden ser benéficos, patogénicos o neutrales. En las asociaciones benéficas, se promueve el crecimiento e inmunidad vegetal, destacando las asociaciones micorrízicas (hongos-raíces), los hongos del género *Trichoderma* y diversas especies bacterianas (Bulgarelli *et al.* 2013).

Los microorganismos habitan distintos compartimentos o tejidos vegetales, la filosfera, comprende el sistema aéreo y aquí habitan epífitos (residen en la superficie de los tejidos) o filosféricos (se encuentran en la superficie de los tejidos aéreos de la planta, principalmente en las hojas). La endosfera abarca el interior de los tejidos y los microbios que pueden internalizarse son los endófitos. En la rizosfera, por otra

parte, residen microorganismos rizosféricos, circundantes al sistema radical (Rossman *et al.* 2017).

La rizosfera es una zona que alberga una gran diversidad y abundancia de microorganismos, a diferencia de los compartimentos anteriores, en el que los exudados liberados por las raíces atraen y potencian el crecimiento de poblaciones microbianas (Alawiye y Babalola, 2019; Ling *et al.* 2022). Actualmente, los análisis tradicionales de aislamiento, establecimiento de cultivos puros y determinación taxonómica se complementan con el uso de técnicas metagenómicas (genoma), metabolómicas (metabolitos), herramientas bioinformáticas y metatranscriptómicas (expresión genética capturando el ARN mensajero total) que proporcionan información sobre la composición, estructura y función de comunidades bacterianas que interactúan con plantas bajo distintas condiciones (Escobar-Zepeda *et al.* 2015; Nwachukwu y Babalola, 2022).

En *Arabidopsis thaliana*, se identificó un grupo de microorganismos endófitos y rizosféricos que constituyen el microbioma central de la planta. Los grupos de microorganismos dominantes fueron Actinobacterias y Proteobacterias de las familias Pseudomonadaceae, Rhizobiaceae, Metylobacteriaceae y Moraxellaceae (Lundberg *et al.* 2012). En las raíces de jitomate (*Solanum lycopersicum*), predominaron los géneros *Bacillus* y *Pseudomonas* (Dong *et al.* 2019).

El suelo es un ambiente complejo, por lo tanto, presenta numerosos factores que pueden modificar e influenciar la abundancia y diversidad microbiana, como son el pH, la salinidad, la disponibilidad de nutrientes, la temperatura, el tipo de cultivo, humedad, entre otros. En cultivos agrícolas de maíz y soya, la diversidad y abundancia de las poblaciones microbianas se vieron afectadas conforme incrementó la profundidad del suelo. A 15 cm se observó la mayor abundancia y disminuyó drásticamente entre los 90 y 180 cm de profundidad. La diversidad taxonómica también fue afectada, sin embargo, además de la cantidad de materia orgánica presente y el tipo de suelo, el tipo de cultivo y la retención de agua fueron variaciones importantes que moldearon la estructura de la microbiota (Hao *et al.* 2020).

Las prácticas agrícolas modernas pueden perturbar la estructura de comunidades microbianas. Brisson y col. (2019) compararon líneas de maíz endogámicas, híbridas y de teosinte crecidas en un suelo desprovisto de nutrientes, en el que sugieren que, durante la domesticación del maíz, hubo modificaciones en el reclutamiento de microorganismos del suelo, encontrando diferencias significativas en la composición, abundancia y diversidad del microbioma en cada variedad genética (Brisson *et al.* 2019). La complejidad del suelo representa un enorme reto al considerar todos los factores que afectan las interacciones planta-microorganismo. La necesidad de contar con bio-inoculantes, altamente efectivos, formulados con microorganismos individuales o en consorcios ha motivado los escrutinios para identificar microorganismos con potencial de incrementar productividad vegetal, así como la resistencia a patógenos, la resiliencia frente a los efectos del cambio climático global y la tolerancia a distintos tipos de estrés ambiental, cuyo impacto a mediano y largo plazo queda por esclarecerse (Trivedi *et al.* 2022).

2.4. Rizobacterias promotoras del crecimiento

De los microorganismos que residen en la rizosfera, las bacterias son las más abundantes, ya que representan alrededor del 95% en biomasa (Glick, 2012). Las rizobacterias promotoras del crecimiento vegetal o PGPR (Plant Growth Promoting Rhizobacteria) por sus siglas en inglés, presentan propiedades interesantes como agentes de biocontrol y mejoran el crecimiento vegetal a través de la capacidad de colonizar la raíz, un crecimiento rápido, sobrevivir bajo estrés, proliferar en una amplia variedad de sustratos, competir con otros microorganismos por nutrientes, y sintetizar metabolitos antimicrobianos contra patógenos (Fig. 5; Souza *et al.* 2015). Un estudio reciente por Ling y col. (2022) sobre las comunidades bacterianas presentes en distintos tipos de rizosferas indica que la diversidad, abundancia y funciones del microbioma varían entre los tipos de ecosistemas evaluados. Los grupos más abundantes en suelos rizosféricos fueron del phylum Bacteroidetes, Proteobacteria y Actinobacteria para la mayoría de las plantas. Firmicutes se

encontró en cultivos de gramíneas, las cianobacterias en las cucurbitáceas y pastos. Los géneros *Rhizobium* y *Mezorhizobium* en las gramíneas, leguminosas, cucurbitáceas y pastos, principalmente. Los géneros de *Burkholderia* y *Variovorax* fueron exclusivos de las leguminosas y *Pedobacter* y *Aeromicrobium* de las gramíneas (Ling *et al.* 2022).

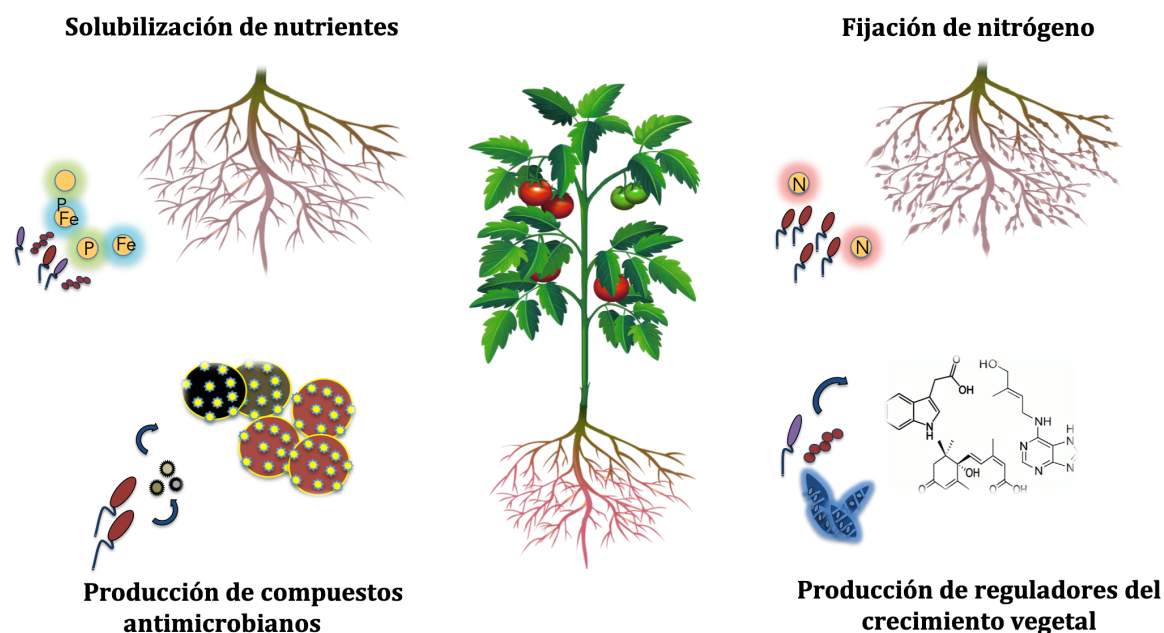


Figura 5. Mecanismos de bio-estimulación por rizobacterias. Entre los mecanismos directos están la solubilización de nutrientes, la fijación de nitrógeno y la producción de reguladores del crecimiento vegetal, mientras que los indirectos comprenden la producción de compuestos antimicrobianos y sideróforos.

2.5. Importancia de los exudados radiculares en las interacciones planta-microorganismo

Los exudados radiculares están constituidos por azúcares (glucosa, fructosa, sacarosa, arabinosa, xilosa, manitol), aminoácidos (glicina, arginina, asparagina, histidina, leucina, citrulina, triptófano, lisina), ácidos grasos (ácido linoleico, palmítico, oleico, linolenico y esteárico), ácidos orgánicos (ácido málico, acético,

benzoico, cítrico, láctico, propiónico, succínico) vitaminas, compuestos fenólicos, mucílago, e inclusive proteínas (Ortiz-Castro *et al.* 2009).

La rizodeposición ocurre principalmente en las puntas de las raíces, a través de mecanismos de transporte activo. Aquellas partículas que no pueden difundirse libremente a través de la membrana plasmática, como los azúcares, aminoácidos y ácidos orgánicos, requieren de acarreadores específicos. Los transportadores de las familias UMAMIT, transportadores catiónicos de aminoácidos (CAT) y GDU para aminoácidos, SWEET para azúcares y ALMT para ácidos orgánicos, actúan como acarreadores trans-membranales con base en el gradiente de concentración de dichas sustancias (Canarini *et al.* 2019; Kawasaki *et al.* 2021). Algunos microorganismos pueden influir sobre la maquinaria de transporte de exudados radicales como parte de su proceso de colonización, por ejemplo, *Trichoderma atroviride* induce la expresión de genes *SWEET* por la emisión de compuestos orgánicos volátiles, lo que conlleva a la exudación de azúcares (Esparza-Reynoso *et al.* 2020).

En la asociación entre bacterias del género *Rhizobium* y leguminosas, la producción de iso-flavonoides por la planta funcionan como quimio-atrayentes, estimulando la colonización por estos microorganismos y la nodulación (Pascale *et al.* 2020). *Bacillus* sp es un género predominante en distintos tipos de suelo. Se ha demostrado que ciertas especies promueven el crecimiento vegetal a través de la colonización y protegen a las plantas frente al ataque por patógenos, en este contexto, la cepa de *Bacillus velezensis* SQR9 presentó mayor afinidad a un grupo de exudados radicales constituidos principalmente por ácidos orgánicos, azúcares y aminoácidos, mientras que el ácido salicílico, decanoato de sodio y el ácido pentadecanoico mostraron propiedades como repelentes (Feng *et al.* 2021). En *Arabidopsis thaliana*, la producción de ácido málico incrementó hasta cuatro veces más la colonización de la rizobacteria benéfica *Bacillus subtilis* FB17 cuando se infectaron zonas del área foliar con el patógeno *Pseudomonas syringae* pv *phaseolicola* (NPS3121) (Rudrappa *et al.* 2008). La respuesta quimiotáctica ejercida por tres ácidos orgánicos (ácido oxálico, ácido málico y ácido fumárico) del plátano mejora la producción de biopelículas de la cepa *Bacillus amyloliquefaciens* NJN-6

(Yuan *et al.* 2015). El conjunto de estos antecedentes denota el potencial y la influencia de moléculas microbianas bio-activas que influyen una comunicación dinámica en la estructuración de asociaciones simbióticas con plantas.

2.6. Señalización por *N*-acil homoserina lactonas

En las bacterias Gram negativas, la comunicación química célula a célula ocurre por pequeñas moléculas difusibles llamadas *N*-acil homoserina lactonas (AHLs), también conocidas como autoinductores. La acumulación de AHLs en el medio sincroniza la expresión genética, y afecta el comportamiento, la motilidad, bioluminiscencia, formación de biofilm, resistencia a fármacos, producción de factores de virulencia, colonización de la raíz, entre otros (Xue *et al.* 2022). En *Pseudomonas aeruginosa*, una bacteria patógena oportunista de animales y plantas, se han descrito dos sistemas principales que incluyen a las sintasas LasI y RhII que producen a los autoinductores 3-oxo-dodecanoil homoserina lactona, y butanoil homoserina lactona, respectivamente, que son reconocidas por los receptores específicos LasR y RhIR (Waters y Bassler, 2005).

Además de actuar como moléculas de señalización entre las bacterias, también se ha reportado que las plantas poseen mecanismos para percibir AHLs, provocando modificaciones en su patrón de crecimiento. La aplicación de distintas AHLs de 8 a 14 carbonos en *Arabidopsis* inhibió el crecimiento de la raíz primaria (Ortiz-Castro *et al.*, 2008), a través del incremento en la producción de especies reactivas de oxígeno (EROs), óxido nítrico, calcio y la actividad de MPK6 (Cao *et al.* 2022).

En *Arabidopsis thaliana*, la interacción directa con la cepa mutante de *Pseudomonas aeruginosa* Δ lasI promovió el crecimiento a través de la producción de ciclodipéptidos (L-pro-L-tyr), L-pro-L-val) y (L-pro-L-phe) con actividad auxínica (Ortiz-Castro *et al.* 2011). La *N*-3-oxo-hexanoil homoserina lactona promovió la elongación de la raíz primaria en *Arabidopsis* incrementando la división celular y la expansión en el meristemo (Zhao *et al.* 2016).

2.7. Sistemas de quorum-sensing en bacterias Gram positivas

Las bacterias Gram positivas utilizan oligopéptidos como moléculas autoinductoras con funciones fisiológicas como la esporulación, la competencia, la producción de factores de virulencia, entre otros procesos, regulando la transcripción de genes

(Wang *et al.* 2020). Las Actinobacterias utilizan γ -butirolactonas como compuestos difusibles, mediadores en la señalización, que al alcanzar una concentración umbral activan un receptor tipo cinasa que fosforila el regulador de respuesta, y a su vez, esta proteína regula la expresión de genes al unirse al promotor y activar la transcripción (Fig. 6). En este contexto, recientemente se reportaron genes homólogos a LuxR en el genoma de *Arthrobacter agilis* implicados en la motilidad bacteriana y la producción del compuesto orgánico volátil, la *N*-dimetilhexadecilamina, con propiedades de bio-estimulación en plantas (Chávez-Moctezuma *et al.* 2022).

Algunos metabolitos secundarios, como el factor PI, identificado como 2,3-diamino-2,3-bis(hidroximetil)-1,4-butanediol, están involucrados en la síntesis de pimaricina. Adicionalmente, los furanos metilenomicinas (MMFs) y los butanóidos de *Streptomyces coelicolor* (SCBs) regulan funciones de percepción de quorum. En la Fig. 6 se ejemplifican los sistemas de señalización representativas de las bacterias (Polkade *et al.* 2016).

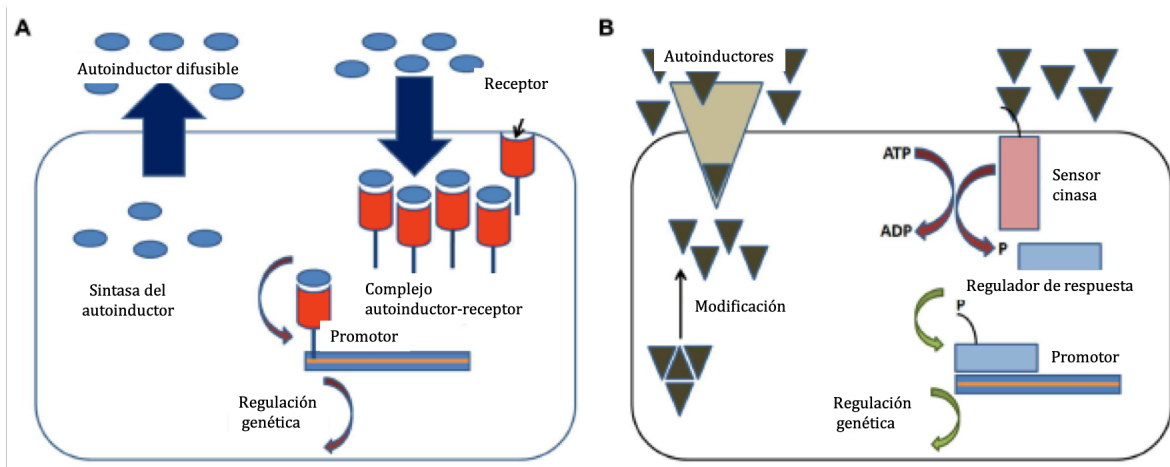


Figura 6. Comparación entre el sistema de quorum-sensing en bacterias Gram negativas y Gram positivas. A. Modelo de la regulación de QS en bacterias Gram negativas. B. Esquema representativo de quorum en bacterias Gram positivas. Adaptado de Polkade *et al.* (2016).

2.8. Fotosíntesis

La fotosíntesis es un conjunto de reacciones por las que las plantas y las bacterias autótrofas convierten la energía luminosa en energía química. Este proceso involucra dos fases, en la primera se llevan a cabo reacciones dependientes de la luz en la membrana del tilacoide, conocida como fase luminosa, en la que se captura energía solar para sintetizar ATP y NADPH mediante reacciones de transferencia de electrones a partir de moléculas de agua. En la segunda fase ocurre la fijación y asimilación de carbono en el estroma del cloroplasto, a partir del ATP y NADPH obtenidos en la fase luminosa, los cuales se utilizan como fuente de energía para reducir el CO₂ y transformarlo en triosas fosfato por la vía del ciclo de Calvin-Benson (Johnson, 2016). En resumen, la fotosíntesis se describe con la siguiente reacción química:



Los fotosintatos derivados de esta reacción son triosas fosfato que siguen dos rutas para su aprovechamiento: i) se almacenan como almidón para utilizarlo como reservas durante la noche y ii) son exportados al citosol para transformarlas en sacarosa mediante diversos intermediarios enzimáticos para transportarla a los órganos y tejidos de demanda por el floema (Everard y Loescher, 2017).

2.8.1. Fase luminosa

La organización funcional de clorofilas y pigmentos accesorios involucrados en la fotosíntesis, forman complejos multiproteicos que catalizan la transferencia de electrones y la fijación del CO₂, estos procesos son mediados por dos fotosistemas localizados en el cloroplasto. Ambos se encuentran unidos mediante el citocromo *b₆f* (Cyt *b₆f*), dos acarreadores de electrones, un transportador de electrones entre el fotosistema II al Cyt *b₆f* y una proteína soluble, la plastocianina, que une al Cyt *b₆f* con el fotosistema I (Gao *et al.* 2018).

Por medio de los complejos de captura de luz, los cuales se forman por moléculas de clorofila a, b y luteína se realizan dos funciones importantes, la absorción de luz y la transferencia de electrones hasta el sitio donde se convierte la energía luminosa en energía química, llamado centro de reacción. Las clorofilas y los pigmentos accesorios funcionan como antenas, capturando la luz y transfiriéndola entre moléculas hasta alcanzar el centro de reacción (Caffarri *et al.* 2014). Cada fotosistema contiene un centro que se complementa funcionalmente para la captura y transferencia de electrones.

En las plantas, el fotosistema II participa primero en la captura de energía luminosa para romper las moléculas de agua a través de un centro de reacción tipo quinona, generando protones y electrones del agua a través de la enzima plastoquinona oxidorreductasa, cuando se excita el citocromo P680 con luz y se producen especies oxidantes. Los productos resultantes son moléculas con gran potencial reductor y energéticas como el NADPH y el ATP, respectivamente.

Este fotosistema posee el mismo contenido de clorofila a y b. Su centro de reacción excita a los electrones del agua por un par de clorofilas a una longitud de onda de P680 y los conduce a través del citocromo *b₆f*. El orden del flujo de electrones ocurre primero hacia la plastoquinona, después al citocromo *b₆f* y a la plastocianina (Lu, 2016).

2.8.2. Fase oscura

El fotosistema II se encuentra acoplado al I. En el fotosistema I, se lleva a cabo la fase oscura o también conocida como ciclo de Calvin. En este sistema, predomina el contenido de clorofila b y el centro de reacción es de tipo hierro. A partir de la plastocianina, P700 transfiere los electrones a la ferredoxina en el estroma, posteriormente, dona el par de electrones a NADP⁺ reduciéndolo a NADPH. Por otra parte, la energía liberada durante el transporte de electrones a través del complejo multiproteico de los fotosistemas, se utiliza para bombear protones desde el estroma hasta el lumen del tilacoide. Este proceso provoca una acumulación en el gradiente de protones que no son permeables en la membrana, debido a ello, la

ATP sintasa forma ATP promoviendo la difusión de protones en favor a un gradiente de concentración, un proceso llamado quimiosmosis (Jagendorf, 2002).

Como productos de la fase luminosa, se obtienen ATP y NADPH los cuales son utilizados para formar moléculas orgánicas a partir del CO_2 por el ciclo de Calvin. La fijación de carbono ocurre en tres fases. En la primera, una molécula de CO_2 reacciona con la enzima ribulosa bifosfato carboxilasa/oxigenasa (RubisCo), como producto se obtiene una molécula de 6 carbonos que inmediatamente se descompone en dos moléculas de fosfoglicerato (PGA). El carbono que constituye al CO_2 se ha fijado en un esqueleto de 3 carbonos a partir de la reacción inicial. Por esta razón, al ciclo de Calvin se le conoce como ruta C_3 . En la segunda fase, se reduce el carbono, las moléculas de PGA son convertidos en gliceraldehído-3-fosfato (G3P) utilizando ATP y NADPH. En promedio, 3 moléculas de CO_2 se fijan con 3 enzimas RubisCo para formar 6 moléculas de G3P y en la última etapa, 5 de las 6 triosas fosfato se utilizan para regenerar 3 enzimas RubisCo y comenzar nuevamente el proceso de fijación de carbono (Jensen, 2000).

Los electrones que se pierden a través de la transferencia del fotosistema II al I, son reemplazados por la oxidación de moléculas de agua para producir oxígeno. Existen organismos que sólo poseen un fotosistema, los cuales no realizan fotosíntesis oxigénica, tanto las algas, como las cianobacterias y las plantas la producen, a diferencia de las bacterias púrpura y sulfurosas, donde no se produce oxígeno.

2.8.3. Metabolismo del carbono

La síntesis de compuestos orgánicos como las proteínas, celulosa, almidón y lípidos producidos por organismos autótrofos derivan de la obtención de triosas fosfato a partir de la reducción del dióxido de carbono. Las triosas fosfato se utilizan para sintetizar biomoléculas, principalmente sacarosa para transportarla a los tejidos y órganos no fotosintéticos y la producción de almidón como fuentes de almacenamiento de reservas energéticas. La síntesis de sacarosa involucra la exportación de dos triosas fosfato al citosol para formar fructosa-1,6-bifosfato, catalizado por una aldolasa. La unión con la UDP-glucosa forma sacarosa 6 fosfato

mediante las enzimas sacarosa fosfato sintasa (Sucrose Phosphate Synthase) (Maloney *et al.* 2015). El grupo fosfato de la sacarosa es removido por fosfatasas sacarosa 6-fosfato exportando la sacarosa a través del floema a los tejidos vegetales demandantes (Fig. 7).

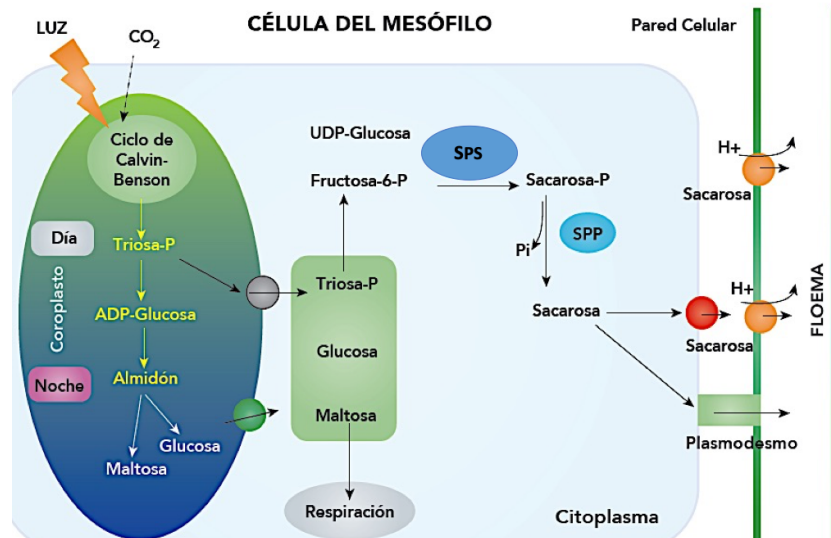


Figura 7. Representación de la fotosíntesis y el metabolismo de las triosas fosfato.
Adaptado de Esparza-Reynoso (2018).

Por otra parte, el gliceraldehído 3-fosfato que no se utiliza para la producción de sacarosa, sigue una ruta distinta para obtener almidón, un polisacárido constituido por monómeros de glucosa unidos por enlaces alfa 1-4 que se produce durante el día para ser degradado por la noche en residuos de maltosa y glucosa. La ADP-glucosa se forma por la enzima AGP al condensar la glucosa 1-fosfato con ATP, produciendo pirofosfato. Para la síntesis del almidón, la ADP-glucosa transfiere el grupo glucosil en el extremo no reductor de la cadena en síntesis, en la posición 4 (Pfister y Zeeman, 2016).

2.9. Fotorreceptores involucrados en los programas de crecimiento y desarrollo vegetal

En las plantas se pueden distinguir dos procesos dependientes de la luz, la fotosíntesis y la regulación de distintos programas de crecimiento y desarrollo por fotorreceptores. En este escenario, la luz se percibe como una señal mediante tres clases distintas de fotorreceptores, los criptocromos, fototropinas y fitocromos. Los criptocromos y fototropinas perciben la luz ultravioleta y azul en el rango de los 320-500 nm, mientras que los fitocromos absorben en longitudes de onda roja y roja lejana con longitudes de onda de 600 y 750 nm.

Los fitocromos son apoproteínas diméricas unidas covalentemente a un cromóforo tetrapirrol lineal tipo bilina (fitocromobilina) posicionado en el residuo 374 del dominio amino terminal, adherido a una cisteína. El cromóforo bilina se encuentra conservado en angiospermas, gimnospermas, helechos, musgos, algas, hongos filamentosos, cianobacterias y bacterias no fotosintéticas. Estos grupos comparten tres dominios conservados en los fitocromos Per/Arndt/Sim (PAS), monofosfato de guanosina cíclica fosfodiesterasa/adenil ciclasa/FhIA (GAF) y dominio específico de fitocromo (PHY), que en conjunto se le denomina núcleo fotosensorial (PCM). En las plantas, la estructura química de los fitocromos presenta cuatro subdominios en el amino terminal; una extensión N-terminal (NTE) únicamente presente en plantas, (PAS), GAF y PHY (Fig. 8). El grupo carboxilo terminal también se subdivide en PAS-related domain (PRD), dos repetidos de PAS, y un dominio relacionado a histidin cinasa (HKRD) (Rockwell *et al.* 2006).

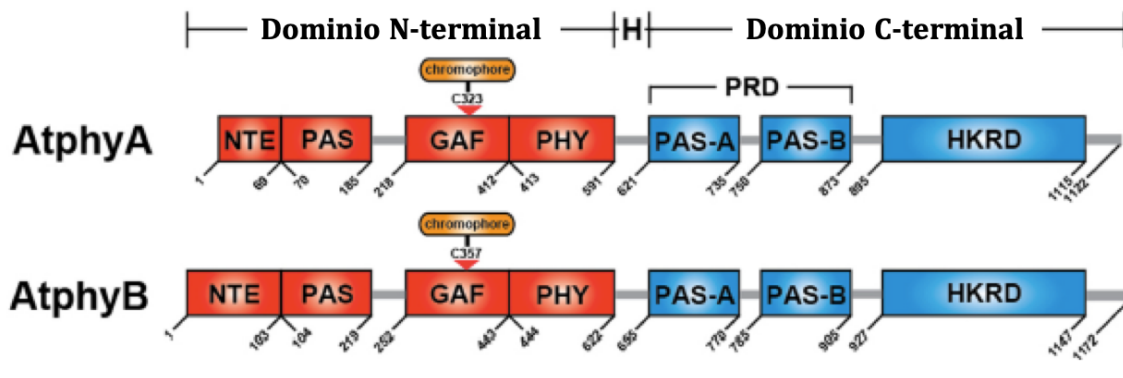


Figura 8. Estructura de las proteínas PHYA y PHYB. Dominios de los fitocromos PHYA y PHYB. NTE (extensión N-terminal), PAS (Proteína de periodo circadiano), HKRD (dominio relacionado a histidin cinasas), PHY (fitocromo). Adaptado de Li *et al.* (2011).

El peso molecular de los fitocromos oscila entre los 120-125 kDa. En *Arabidopsis thaliana* existen 5 tipos, PHYA-PHYE. La propiedad de fotoreversibilidad que presentan, les permite activarse bajo la absorción de fotones en la luz roja (Pfr) y pasar a la forma inactiva (Pr) por fotones de luz roja lejana. El mecanismo de reversibilidad entre estas dos fases requiere una reconfiguración del cromóforo.

La percepción y absorción de la luz por las plantas a través de los fitocromos, les confiere la propiedad de detectar la proximidad de competidores por luz en altas densidades poblacionales y de esta manera, modificar su crecimiento. Cuando el radio de luz roja lejana disminuye, las plantas exhiben una elongación de los tejidos para proyectar sus hojas hacia la luz, evitando la sombra. Si la luz no es percibida con la elongación, se modifican otros programas de crecimiento y desarrollo como son la aceleración de la floración y formación de frutos para garantizar la supervivencia.

Se ha propuesto que los fitocromos actúan como cinasas que perciben la luz y transducen la señal bioquímica migrando al núcleo o se asocian con otros componentes que regulan la expresión de genes. Su sitio de síntesis ocurre en el citosol y pueden translocarse al núcleo para la modulación de la expresión de genes. Hay diferencias entre la localización nuclear de cada fitocromo, PHYA se localiza

exclusivamente en el citosol en oscuridad en la forma inactiva Pr, cuando se expone a la luz se importa al núcleo, no tiene una señal de localización nuclear, por lo que se acopla con dos pequeñas proteínas, FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) y FHY1-LIKE (FHL) que inducen su acumulación para su importación, la cual ocurre rápidamente. A diferencia de PHYB, su acumulación en el núcleo es más lenta y ocurre bajo la continua exposición a luz roja. En el grupo terminal carboxilo posee una señal de localización nuclear para su traslocación (Li *et al.* 2011; Legris *et al.* 2019). Los fitocromos constituyen importantes fotorreceptores con dualidad de funciones en la percepción y transducción de la luz para modificar los programas de crecimiento vegetal.

2.9.1. Funciones de los fitocromos en plantas

Con el análisis de mutantes afectadas en la función de los fitocromos, se ha esclarecido su papel en el ritmo circadiano, la floración, la dormancia, la expansión de la hoja, entre otros procesos (Smith, 2000; Tepperman *et al.* 2004). PHYA es abundante en plántulas crecidas en oscuridad y sus niveles disminuyen drásticamente en la exposición a la luz blanca y roja (Franklin y Whitelam, 2007). La mutante *phyA* exhibe una exacerbada elongación del hipocótilo bajo luz roja lejana, mientras que en luz blanca no hay diferencias respecto a las plantas silvestres. La mutante *phyB* presenta un fenotipo con clorosis y una elongación mayor del hipocótilo respecto a plántulas silvestres. Esta respuesta se exagera en la doble mutante *phyAphyB* bajo luz blanca en plántulas y la elongación de los tallos en plantas adultas (Fig. 9).

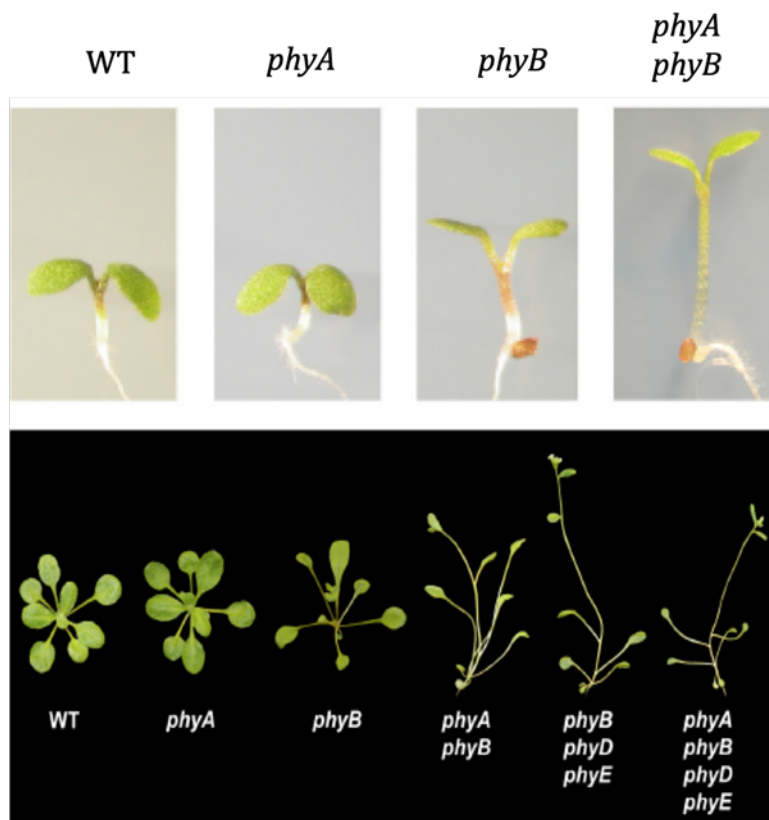


Figura 9. Fenotipos en luz blanca de plantas de *Arabidopsis silvestres* y mutantes en los fitocromos en distintas etapas de desarrollo. A. Efecto de plántulas mutantes sencillas afectadas en *phyA*, *phyB* y la doble mutante en la elongación del hipocótilo a los 4 días después de la germinación. B. Plantas silvestres (WT) y mutantes de 3 semanas crecidas en luz blanca. Modificado de Li *et al.* (2011).

Los fitocromos PHYA y PHYB son los más abundantes en *Arabidopsis*. Varios reportes han documentado su relación con la maquinaria fotosintética y el metabolismo primario, donde la doble mutante *phyAphyB* muestra una acumulación reducida de almidón (Han *et al.* 2017). La mutante *phyB* mostró una disminución drástica en el número de estomas, así como una fotosíntesis reducida (Boccalandro *et al.* 2009).

La exposición a luz ultravioleta y alta radiación pueden ocasionar daños en el sistema fotosintético, provocando una acumulación de especies reactivas de oxígeno. El fotosistema II constituye un sistema de percepción a este tipo de estrés

y se ha correlacionado con los fitocromos en la protección contra luz UV. El mecanismo que conlleva a esta respuesta deriva de distintos reportes de investigación en donde plantas transgénicas que sobre-expresan fitocromos aumentan el contenido y actividad de antioxidantes de bajo peso molecular, la capacidad de difusión y captura del CO₂, pigmentos que absorben luz UV y cambios en la composición de las membranas del cloroplasto. Al pasar a la forma activa de Pr a Pfr se activa la producción de catalasa y peroxidasa y se induce la síntesis de carotenoides y flavonoides (Kreslavski *et al.* 2018).

3. JUSTIFICACIÓN

Las auxinas coordinan eventos de crecimiento y morfogénesis en las plantas y su producción por microorganismos impacta en la fisiología y arquitectura vegetal. Existen grupos diversos de microorganismos con características de bio-estimulación únicas y contrastantes, por lo tanto, es importante esclarecer si sus efectos dependen de auxinas u otras moléculas bioactivas.

4. HIPÓTESIS

Los mecanismos de bio-estimulación vegetal por rizobacterias dependen de mecanismos auxínicos y de su impacto en la fotosíntesis.

5. OBJETIVOS

5.1. Objetivo general

Caracterizar los mecanismos de fitoestimulación por dos rizobacterias aisladas de la rizosfera de maíz con efectos contrastantes sobre la arquitectura radical de *Arabidopsis*.

5.2. Objetivos específicos

- ❖ Caracterizar el efecto de las rizobacterias *M. luteus* LS570 y *Bacillus* sp. LC390B sobre el crecimiento de *Arabidopsis thaliana in vitro*.
- ❖ Identificar los componentes de la vía de señalización por auxinas y elementos que modifican la fotosíntesis en la interacción.
- ❖ Analizar la influencia de interacción con las plantas sobre el crecimiento de la bacteria.

6. RESULTADOS

En esta sección se presentan los principales resultados obtenidos durante el proyecto de investigación de Doctorado relacionados con los mecanismos de bioestimulación del crecimiento vegetal por dos rizobacterias, *Micrococcus luteus* LS570 y *Bacillus* sp LC390B en *Arabidopsis thaliana*.

6.1. Publicaciones con arbitraje estricto en revistas indizadas en el *Journal of Citation Reports*

6.1.1. Capítulo I

1. García-Cárdenas E, Ortiz-Castro R, Ruíz-Herrera LF, Valencia-Cantero E, López-Bucio J. 2021. *Micrococcus luteus* LS570 promotes root branching in *Arabidopsis* via decreasing apical dominance of the primary root and an enhanced auxin response. *Protoplasma* 259(5):1139-1155. doi:10.1007/s00709-021-01724-z

6.1.2. Capítulo II

2. García-Cárdenas E, Ortiz-Castro R, Ruíz-Herrera LF, Valencia-Cantero E, López-Bucio J. 2022. *Bacillus* sp. LC390B from the maize rhizosphere improves plant biomass, root elongation and branching and requires the phytochromes PHYA and PHYB for phytostimulation. *Journal of Plant Growth Regulation*. <https://doi.org/10.1007/s00344-022-10770-7>

6.1.3. Capítulo III

6.2. Artículo de revista de divulgación

1. Carta de aceptación
2. Constancia de artículo de portada
3. García-Cárdenas E y Ruíz-Herrera LF. (2022, noviembre). El potencial de la microbiota vegetal para alimentar al mundo. *Saber más*, (65). https://www.sabermas.umich.mx/revistas/no_65/index.html#p=37.

6.3. Capítulo IV

6.3.1. Capítulo de libro

1. García-Cárdenas E, Muñoz-Parra E, López-Bucio J, Beltrán Peña E. (2018). Las auxinas: hormonas que coordinan el crecimiento y desarrollo de las plantas y su interacción con el ambiente. Beltrán Peña E, López-Bucio J, Martínez-Trujillo M (Eds.), *Fronteras en la Biología: Señalización y comunicación de las plantas* (pp. 17-26). Morevalladolid.

Publicaciones de arbitraje internacional

Capítulo I



Micrococcus luteus LS570 promotes root branching in *Arabidopsis* via decreasing apical dominance of the primary root and an enhanced auxin response

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Abstract

The interaction of plant roots with bacteria is influenced by chemical signaling, where auxins play a critical role. Auxins exert positive or negative influences on the plant traits responsible of root architecture configuration such as root elongation and branching and root hair formation, but how bacteria that modify the plant auxin response promote or repress growth, as well as root structure, remains unknown. Here, we isolated and identified via molecular and electronic microscopy analysis a *Micrococcus luteus* LS570 strain as a plant growth promoter that halts primary root elongation in *Arabidopsis* seedlings and strongly triggers root branching and absorptive potential. The root biomass was exacerbated following root contact with bacterial streaks, and this correlated with inducible expression of auxin-related gene markers *DR5:GUS* and *DR5:GFP*. Cellular and structural analyses of root growth zones indicated that the bacterium inhibits both cell division and elongation within primary root tips, disrupting apical dominance, and as a consequence differentiation programs at the pericycle and epidermis, respectively, triggers the formation of longer and denser lateral roots and root hairs. Using *Arabidopsis* mutants defective on auxin signaling elements, our study uncovers a critical role of the auxin response factors ARF7 and ARF19, and canonical auxin receptors in mediating both the primary root and lateral root response to *M. luteus* LS570. Our report provides very basic information into how actinobacteria interact with plants and direct evidence that the bacterial genus *Micrococcus* influences the cellular and physiological plant programs ultimately responsible of biomass partitioning.

Keywords Rhizobacteria · Root architecture · Biomass distribution · Auxin · Mitosis

Introduction

Plants harbor a very rich and diverse microbiome that helps them survive and adapt to challenging environments (Ortiz-Castro and López-Bucio 2019; Trivedi et al. 2020). The root

is the prime organ engaged in biotic interactions, where the so-called plant growth-promoting rhizobacteria (PGPR) are attracted through chemotaxis driven by carbon-rich exudates and exert benefits to the host that include increased nutrient availability, improved immunity, and modulation of shoot and root architecture (Ortiz-Castro et al. 2009; Backer et al. 2018; Jiménez-Vázquez et al. 2020; Ortiz-Castro et al. 2020).

An important role of bacterially produced auxins or molecules that mimic the action of auxins such as cyclodipeptides, which can recognize auxin receptors in plant-microbe communication, has been evidenced (Spaepen and Vanderleyden 2011, Ortiz-Castro et al. 2011). *Azospirillum brasilense* is well known for its growth-promoting traits in cereals and horticultural species, and the changes elicited in root architecture rely in complex mechanisms that involve both the perception of auxins and cytokinins as well as the lipopolysaccharide signatures (Méndez-Gómez et al. 2021a, b). Diffusible molecules and cell structural components from *Pseudomonas*

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aeruginosa PAO1 and *A. brasilense* sp245 enhance plant metabolism and cell proliferation within root meristems acting as drivers of the target of rapamycin (TOR) signaling pathway (Méndez-Gómez et al. 2020; González-López et al. 2021).

A recent study involving synthetic bacterial communities identified the *Variovorax* genus as dominant bacteria in an ecological context, which harbors an auxin-degradation operon that encodes proteins that not only destroyed the auxin message for cellular communication, but also reversed the inhibitory effects on primary root growth of *Arabidopsis* by other bacteria that naturally produce auxin (Finkel et al. 2020). The interference by certain bacteria on plant hormone levels also involves ethylene, a signaling gas produced by plants under stress and strongly represses root growth (Artner and Benkova 2019; Qin and He 2019). In this regard, bacteria might possess an enzyme termed ACC deaminase, which degrades 1-aminocyclopropane-1-carboxylate (ACC), an ethylene precursor in plants, and in this manner enables growth to proceed under a variety of challenging situations such as salinity and high temperatures (Nascimento et al. 2018; Gupta and Pandey 2019). Manipulation of endogenous hormone levels is integral to the ecological balance that enables a long-lasting symbiosis and plant productivity. However, deep studies aimed at characterizing how auxin production and/or degradation orchestrates all complex traits that determine root architecture and overall plant growth are needed to understand the communication networks that shape plant organogenesis.

Gram-positive bacteria are very prolific, representing up to 25–30% of the soil microbiota, and their biological roles in ecosystems are not negligible. Among this group, the phylum Actinobacteria and their biostimulation mechanisms related to plant growth and development have just started to be defined (Solans et al. 2019). *Micrococcus luteus* LS570 belongs to the Micrococcaceae family, which include aerobic, non-spore-forming cells. In maize (*Zea mays* L.) and cowpea (*Vigna unguiculata*) interactions, these bacteria were able to solubilize phosphate and increase yield (Dastager et al. 2010; Raza and Faisal 2013). In grapevine (*Vitis vinifera* L. cv. Malbec), it reduced heavy metal stress and promoted growth, yield, and production of photosynthetic pigments in consortia with *Pseudomonas* and *Bacillus* isolates (Funes et al. 2018). The *Micrococcus* genus is considered a strong auxin producer that employs both tryptophan-dependent and independent precursors (Ahmad et al. 2020), but currently, no specific root architectural or biomass responses have been correlated with auxin production by this genus.

The phytohormone auxin (IAA), from microbial or plant origin, is critically involved in cell division, elongation, and differentiation depending upon the plant tissues and developmental stages (Vanneste and Friml 2009; Paque and Weijers 2016). Such bio-activity depends on the endogenous gradients modulated by biosynthesis and transport, and ultimately, its

perception by a family of receptors belonging to the TIR1/AFB family (Parry et al. 2009; Prigge et al. 2020).

The role of auxin as a probiotic factor in plant–microbe interactions is complex and not well understood. A few examples of pathogenic bacteria that produce auxin suggest its role in virulence. For instance, disruption of the *ipdC* gene encoding indole-3-pyruvate decarboxylase in *Salmonella* leads to decreased IAA release and reduced pathogenesis and root colonization of *Medicago truncatula* (Cox et al. 2018). Along with stimulation of cellular growth during infection by *Agrobacterium tumefaciens* to induce tumor formation and to boost lateral root production by *A. rhizogenes*, auxin may behave as an effector molecule that antagonizes plant defense responses (Kunkel and Harper 2018). Therefore, little is known into how rhizospheric bacteria that produce auxin affect plant productivity and or disease during the life cycle of the host.

In the model plant *Arabidopsis thaliana*, the postembryonic root is composed of a major, dominant axis that grows through three well-defined zones: meristematic, transition, and differentiation. The root apical meristem (RAM) produces the cells that later on elongate and differentiate to make the functional tissues of the primary root of embryonic origin (Smith and de Smet 2012). Later on, root hairs and lateral roots can be produced de novo from either root epidermal or pericycle tissues, respectively, and these structures along with discrete root movements assist in water and nutrient uptake (Pandey and Bennett 2019). These morphogenic events are under the influence of both endogenous and environmental programs in a cross-talk with plant hormones or bacterial derived quorum-sensing signals, antibiotics, peptides, and/or secondary metabolites and ultimately, root branching structures represent highly valuable niches for root colonization by microbes (Ortiz-Castro and López-Bucio 2019; Jiménez-Vázquez et al. 2020).

In this report, we identified and characterized a plant probiotic bacterium, *Micrococcus luteus* LS570, and tested its growth-promoting attributes in the model plant *Arabidopsis thaliana*. Genetic and molecular evidences suggest a critical role for auxin signaling in root architecture remodeling and development events triggered by the bacterium during interaction with roots. Our work also uncovers the critical role for the ARF7 and ARF19 proteins in mediating root sensing of bacteria.

Materials and methods

Bacterial isolation and electronic microscopy characterization

Micrococcus luteus LS570 is an aerobic strain obtained from maize (*Zea mays* L.) roots in an alkaline soil (pH 8.6) located on the Lometón field, Tarímbaro, Mich. Méx. (19°42'N and 101°8'O). This actinobacterium has an optimal

growth on Luria–Bertani (LB) media, showing small, punctiform, cream, flat, yellow-colored colonies at 28 °C, pH 7 after 24 h of growth. Scanning electron microscopy (SEM) (Quanta FEG 250, FEI Company Quanta TM, USA) was performed cultivating the bacterial strain in LB media. Bacterial growth plugs (8-mm diameter) were cut and fixed with 1 ml of 2.5% glutaraldehyde for 20 h at 4 °C. The stopper samples were dehydrated with ethanol at gradual concentrations (30–90%) and three times at 100% for 10 min. The sample was dried to a critical point using CO₂ (Quorum K850, Quorum Technologies Ltd, USA). Finally, the samples were piece-mounted and gold-plated with a cathodic spray coating (Quorum Q150RS, Quorum Technologies, Ltd, USA) obtaining representative electron micrographs.

Molecular rRNA 16S gene amplification

DNA genomic extraction from *Micrococcus luteus* LS570 was carried out following the methodology described by Tzecz-Interián et al. (2020). The rRNA 16S gene marker was amplified with forward and reverse oligonucleotides (F27: AGAGTTTGATCCTGGCTCAG and R1494: TACGGC TACCTTGTTACGAC). Gene amplification was done following the manufacturer's specifications (Qiagen). Finally, the amplicons were sent to the National Laboratory of Genomics for Biodiversity (LANGEBIO), CINVESTAV, for sequencing. The results were analyzed in the GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), with MEGA 7 software (Kumar et al. 2016) to construct the dendrogram and some type strains were included. The evolutionary history was inferred by the maximum likelihood method and Tamura-Nei model (Tamura and Nei 1993).

Arabidopsis seedlings and treatment conditions

The first procedure in elucidating plant-bacteria interaction mechanism was to use a wide molecular battery of seedlings such as *Arabidopsis thaliana* wild-type ecotype Columbia-0 (Col-0), as well as transgenic lines for auxin response *DR5:uidA* (Ulmasov et al. 1997), and *DR5:GFP* (Ottenschläger et al. 2003), and auxin signaling mutants *tir1* (Ruegger et al. 1998), *tir1/afb2/afb3* (Parry et al. 2009), *slr1* (Fukaki et al. 2002), and *arf7/arf19* (Okushima et al. 2007). The seeds were washed with ethanol 95% (v/v) for 5 min, bleach 20% (v/v) for 7 min, and distilled sterilized water for 1 min, repeating this last step five times before including them into a refrigerator (4 °C) for 48 h to synchronize germination.

Plant-bacteria interaction and growth conditions

After cold treatment, seeds were placed on agar plates containing 0.2 × MS (Murashige and Skoog Basal Salts Mixture; Sigma-Aldrich). Plates were incubated vertically on a growth chamber (Percival Scientific AR95L) with 16 h:8 h light:darkness photoperiod, light intensity of 300 μmol m⁻²s⁻¹, 80% humidity, and 22 °C temperature. Seedlings at 4 days after germination (4 d.a.g), were transferred with their roots placed over a horizontal bacterial streak of *Micrococcus luteus* LS570. The position of the primary root tips was marked behind the dish to quantify the primary root growth after interaction with the bacterial streak. The phenotypes of roots and shoots were analyzed after 8 days of root-bacteria interaction.

To test the influence of the plant on *M. luteus* LS570 growth in vitro, the bacterial streaks were recovered from the media after 5 days in interaction with roots or grown over agar plates with MS 0.2 × media, or over LB solid media as a positive control for comparable times. The bacterial biomass was collected from the media and re-suspended in 1 ml deionized water to quantify optical density at 600 nm. Four dilutions were also performed to determine viable counts per plate and graph them as colony-forming units, spreading 50 μl of each dilution in agar plates containing LB broth, incubating at 28 °C for 24 h until single colonies were observable.

Root system analysis

We performed an analysis from shoot and root architecture after root contact with *Micrococcus luteus* LS570. Primary root and lateral root lengths were measured using a ruler; lateral root number was counted using a stereoscopic microscope (Leica EZ4D). A total of 90 lateral roots were registered per treatment. The lateral root density was determined dividing the lateral root number by the primary root length parameter. For root hair analysis, representative photographs were taken each day during bacterial interaction by stereoscopic microscopy (Leica MZ6) at 0.6 × magnification.

Fresh weight quantification

After 8 days in interaction with *Micrococcus luteus* LS570, the roots and shoots were dissected using a scalpel. The total fresh weight comprised both the shoot and root systems together; shoot and root fresh weights were quantified separately using an analytical scale.

Histochemical detection of GUS activity

Transgenic seedlings expressing the β -glucuronidase reporter gene *uidA* (GUS) were grown and to $0.2 \times$ MS media with or without a bacterial streak. Upon bacterial exposure of roots, plants were incubated overnight with 5-bromo-4-chloro-3-indolyl β -D-glucuronide at 37°C . A clarification protocol was applied in order to remove starch and pigments. Briefly, methanol 20% (v/v), HCl 0.24 N was supplied and the seedlings were incubated at 60°C for 40 min; after removal of the first solution, NaOH 7% was added to cover the material and maintained at room temperature for 30 min, and then washed with ethanol 40, 20, 10% (v/v) for 30 min each, and finally stored with glycerol 50% (v/v) at 4°C . The activity was monitored and analyzed with Nomarski microscopy (Leica ZR5000) in 20 and $40\times$ magnification. Plants were placed on slides covering the samples with glycerol 50% (v/v), and representative images were taken for at least 18 seedlings for each treatment.

Confocal microscopy analysis and fluorescence determination

This procedure let us monitor auxin gradients with transgenic plants expressing *DR5::GFP* construct at 488-nm excitation/505–550-nm emission using an Olympus FV1000 (Olympus Corp., Tokyo, Japan) confocal microscope. For all treatments, roots were placed carefully in a clean glass slide immersing the primary root in a propidium iodide solution for 1 min before observation. Images were taken in 20 and $40\times$ objectives. Fluorescence of the green fluorescent protein was scanned *in vivo*, and images were taken and analyzed using ImageJ software. Fluorescence determinations were done for an area covering 20 cortical cells and from the quiescent center in the root apical meristem. Standardization of the values was applied for controls and treatments, given the value 1 for the controls, whereas treatments were compared to this value, obtaining the relative fluorescence parameter in arbitrary units (A.U.).

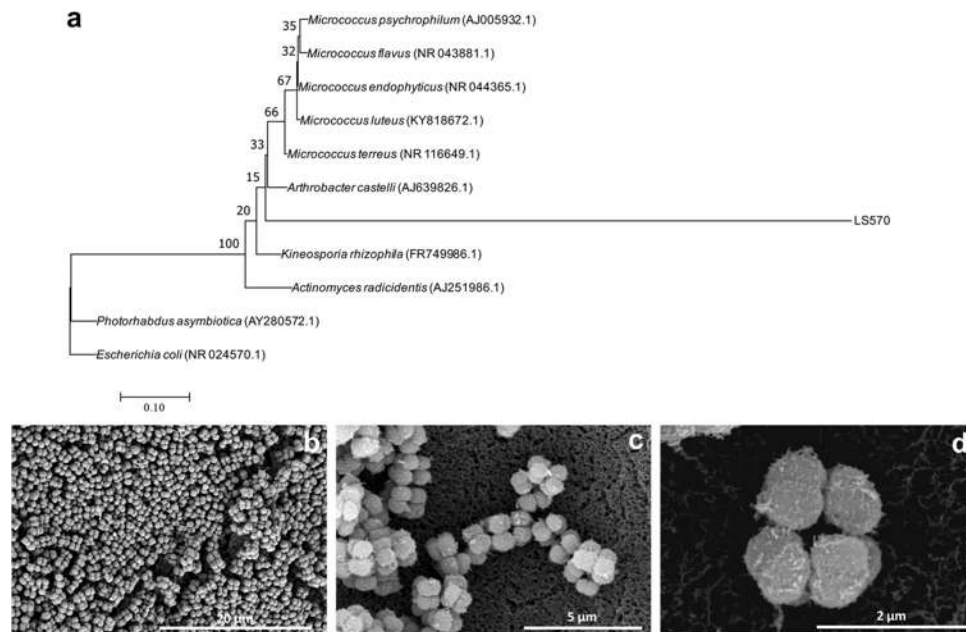


Fig. 1 Identification and characterization of strain LS570. (a) Phylogenetic relationship among strain LS570 with the cluster of the genus *Micrococcus*. (b–d) Representative images of bacterial growth in LB

culture medium. The images were taken with the SEM at different magnifications. Scale bars: 20 μm , 5 μm , and 2 μm , respectively

Fig. 2 Effect of *M. luteus* LS570 in *Arabidopsis* seedlings biomass. ▶ Total fresh weight (a), shoot fresh weight (b), and root fresh weight (c). The results were analyzed with Student's *t* test, when comparing two means, or with an ANOVA test and Tukey's means separation test for multiple comparisons ($p < 0.05$). $n = 18$. Different letters represent statistical significant differences. The experiment was repeated three times with comparable results

Statistical analysis

For each experiment, at least 24 individuals were analyzed, eight of them taken from single Petri plates and three plates comprised the overall sample. Statistical analysis was performed using the STATISTICA program, with a Tukey's post hoc tests, and univariate and factorial ANOVA. Differences were reported with different letters and a significance of ($P < 0.05$).

Results

Isolation and molecular characterization of *Micrococcus luteus* LS570

As part of the search for new bacterial isolates with potential biological activities in plant growth, around 1000 bacterial isolates associated with the rhizosphere of maize from an alkaline soil were evaluated for their ability to promote plant growth using an *Arabidopsis* in vitro system in co-culture with bacterial isolates (Ortiz-Castro et al. 2011). We could identify the bacterial isolate LS570 with strong biological activity, repressing main root elongation and triggering the formation of new root branches that were longer with time.

The identity of the LS570 strain was determined by comparison of 16S rRNA gene sequences against NCBI sequence database according to Blast similarity matches. We identified that the isolate LS570 was grouped into a cluster of bacteria of the genus *Micrococcus*, closely to *M. luteus* (Fig. 1a). The phenotypes of the colony were determined by scanning electron microscopy (SEM), where it can be observed that the round, single cells are around 1 μm in diameter and remain grouped in the form of tetrads without flagella (Fig. 1b–d). This phenotype is characteristic of *Micrococcus luteus* and is consistent with the molecular analysis of the LS570 isolate.

Micrococcus luteus LS570 modulates growth and root system architecture of *Arabidopsis* seedlings

Our first aim was to test *Micrococcus luteus* LS570 as a possible beneficial bacterium and elucidate its potential to affect

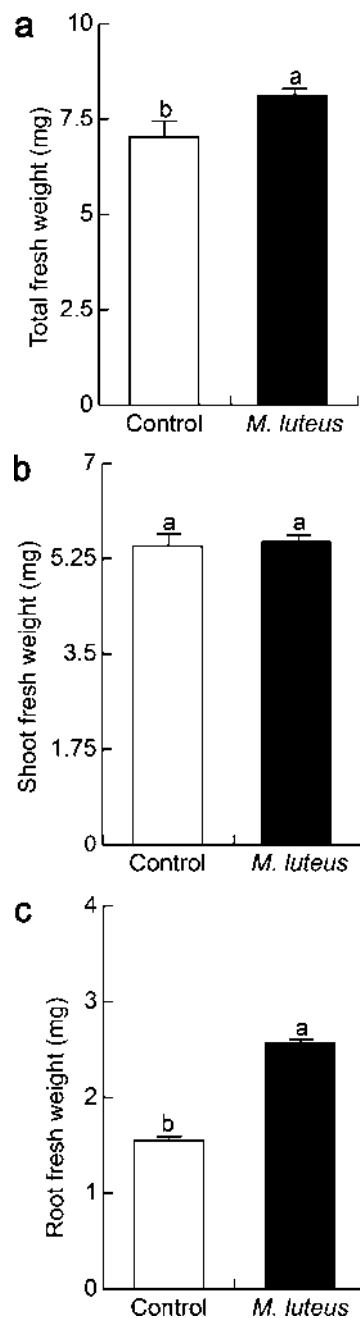
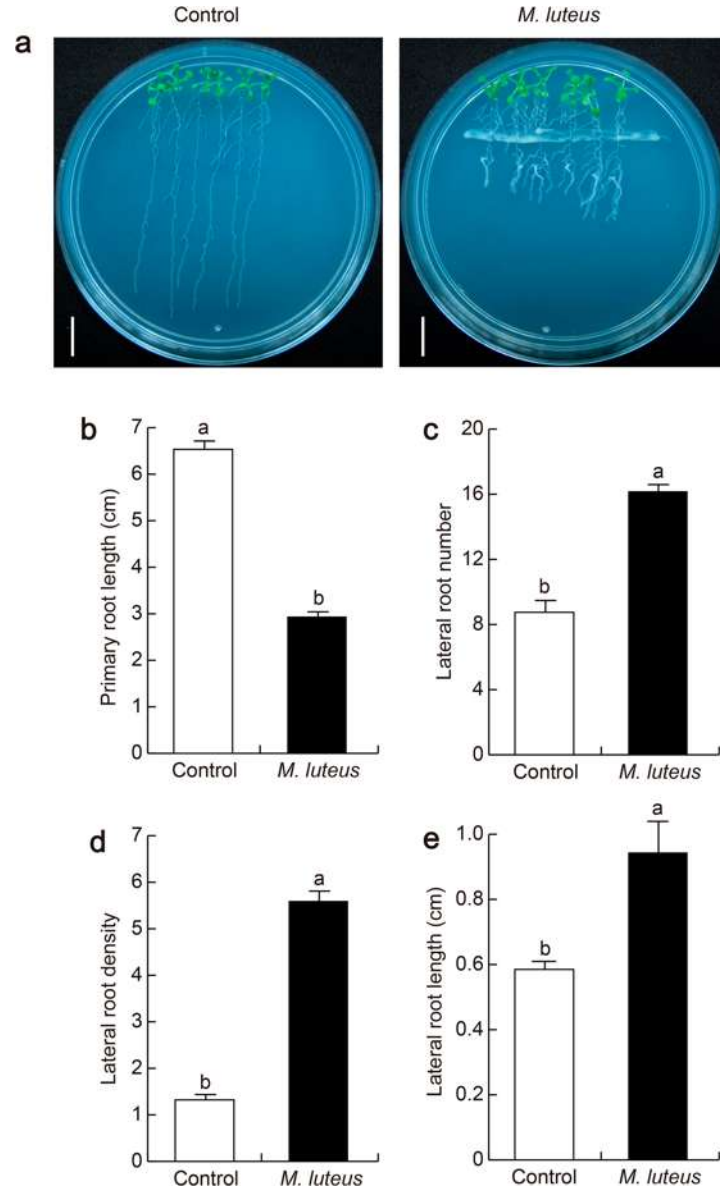


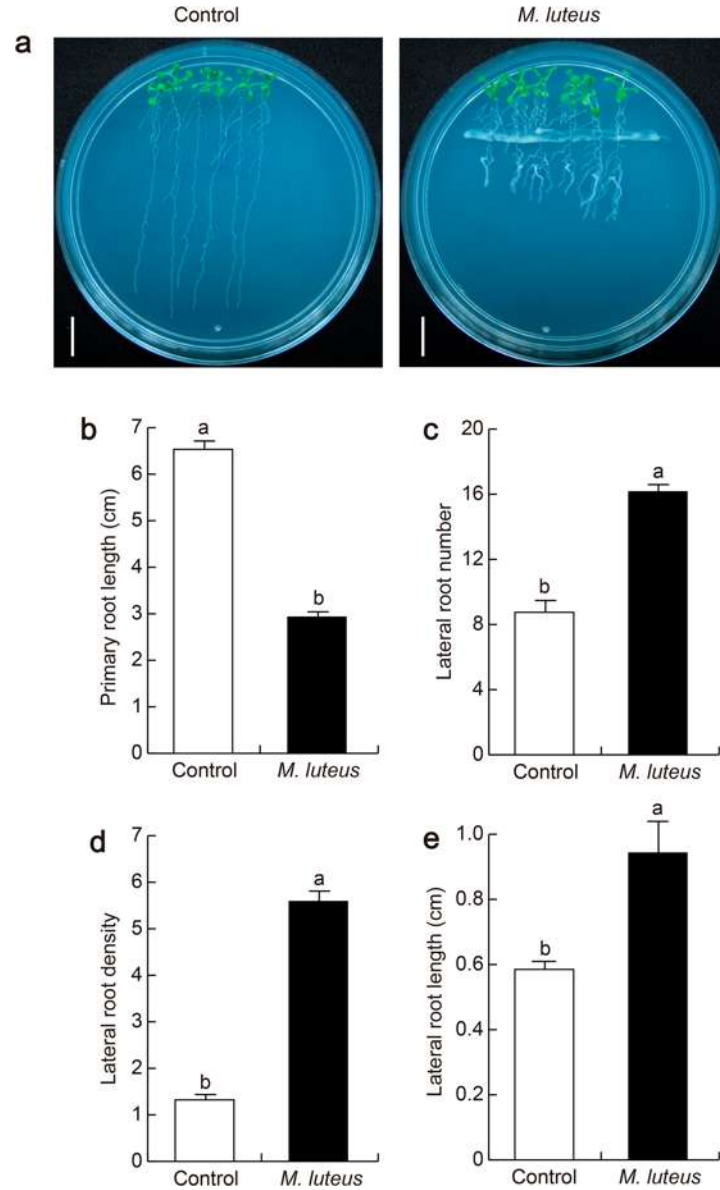
Fig. 3 Effect of *M. luteus* LS570 in overall plant growth and root architecture. *Arabidopsis* seedlings were germinated and grown for 4 days and transferred to axenic 0.2×MS medium with or without a bacterial streak. Representative images of Petri plates 8 days after interaction (a). Primary root length (b), lateral root number (c), lateral root density (d), and lateral root length (e). Scale bar: 1 cm. The results were analyzed with Student's *t* test, when comparing two means, or with an ANOVA test and Tukey's means separation test for multiple comparisons ($p < 0.05$). $n = 18$. Different letters represent statistically significant differences. The experiment was repeated three times with comparable results



growth in vitro of *Arabidopsis thaliana* seedlings via direct contact of the roots with a bacterial streak, which is a fast and very suitable method to rapidly distinguish pathogenic

or beneficial effects. The seedlings were germinated and grown for 4 days in Petri dishes containing agar-solidified 0.2×MS nutrient medium and then transferred over a

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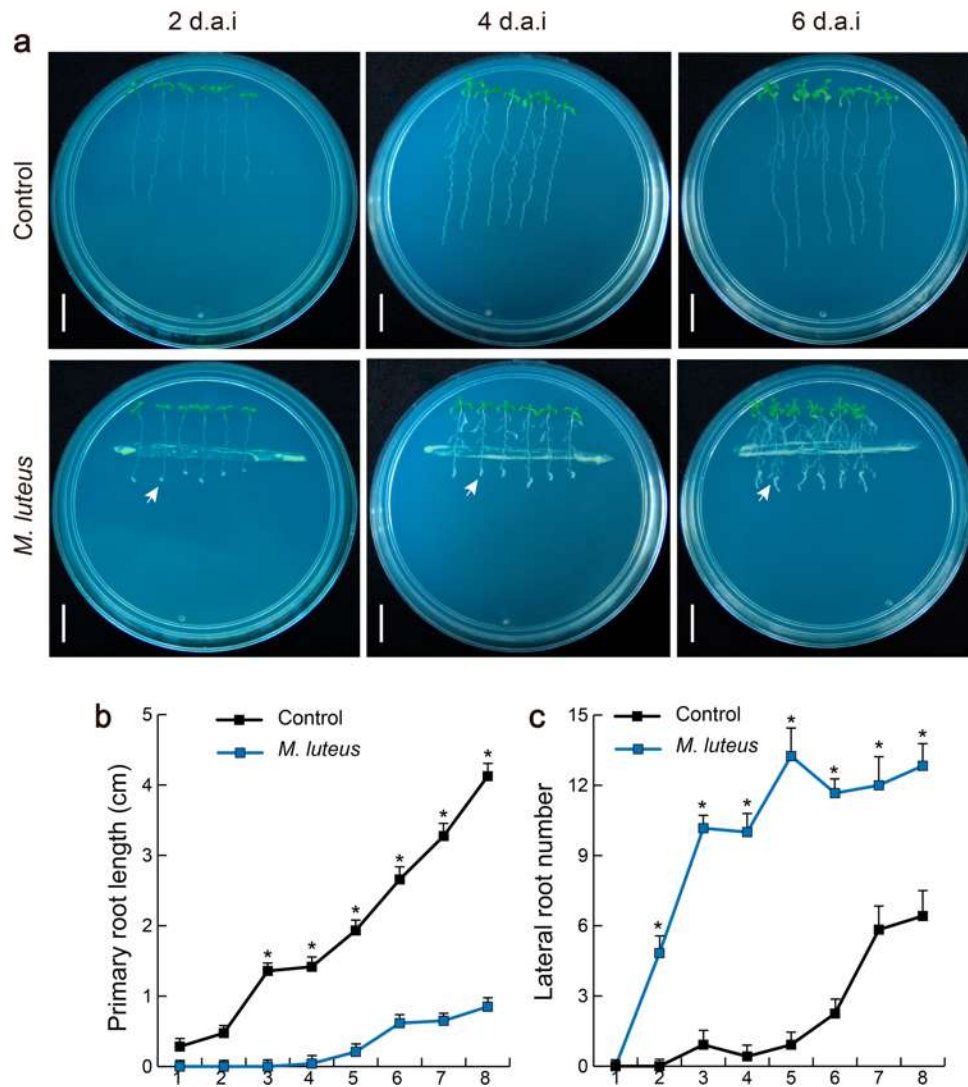


Fig. 4 *M. luteus* LS570 disrupt root apical dominance and trigger root branching. Representative images of Petri dishes at 2, 4, and 6 days after *M. luteus* interaction (a). White arrow shows the position of the primary root tip. Kinetics of primary root growth (b) and lateral root number (c) after root-bacteria contact. Scale bar: 1 cm, $n=18$.

The results were analyzed with Student's *t* test, when comparing two means, or with an ANOVA test and Tukey's means separation test for multiple comparisons ($p<0.05$). Different letters represent statistically significant differences. The experiment was repeated three times with comparable results

consolidated bacterial streak at the surface of plates with the same medium. The growth and development of seedlings were assessed during 8 days after root bacterization. There was a strong growth-promoting effect in roots, but not in shoots, suggesting that the bacterium favors biomass partitioning in roots over shoots (Fig. 2). Noteworthy, the primary root in inoculated seedlings was 55% shorter with a twofold increase in lateral root number, sixfold increase in lateral root density, and their lateral roots were roughly 30% greater than the controls (Fig. 3). Thus, *M. luteus* has a major impact in promoting lateral root formation and elongation.

***Micrococcus luteus* LS570 halts apical dominance of primary roots**

To analyze the correlation between halted primary root growth and promotion of root branching by *M. luteus* LS570, the morphological events in roots that took place during the first 2, 4, and 6 days in interaction were monitored by measuring primary root growth and lateral root number. The non-inoculated seedlings show continuous growth of the primary roots, the foliage develops new leaves, and the first lateral roots are visualized in the primary root axis following four days (Fig. 4a). In roots placed in contact with *M. luteus*, the growth of

the main root axis was maintained, but at much slower rate (Fig. 4a). A kinetic analysis performed every day for 8 days indicated that along with primary root growth repression, the bacterium induced the production of up to four times more lateral roots since the first day onwards (Fig. 4b, c). The new branches being formed were densely covered by long root hairs, which overall increased the root volume and absorptive capacity of the root system (Fig. 5). These data show the correlation between the loss of apical dominance of the primary root and the reconfiguration of root system architecture.

***Micrococcus luteus* LS570 induces the auxin response in roots and shoots**

To know if the changes in root architecture triggered by *M. luteus* in *Arabidopsis* could be explained by an auxinic effect, we next monitored the plant auxin response using *Arabidopsis* transgenic seedlings that express the auxin-inducible construct *DR5::uidA* grown in axenic medium or placed over the bacterial streak. *M. luteus* LS570 clearly promoted GUS histochemical detection in shoots, lateral forming zones of the primary root and the primary root tip, revealed by the blue color in the different plant tissues (Fig. 6a–c). Interestingly, the most

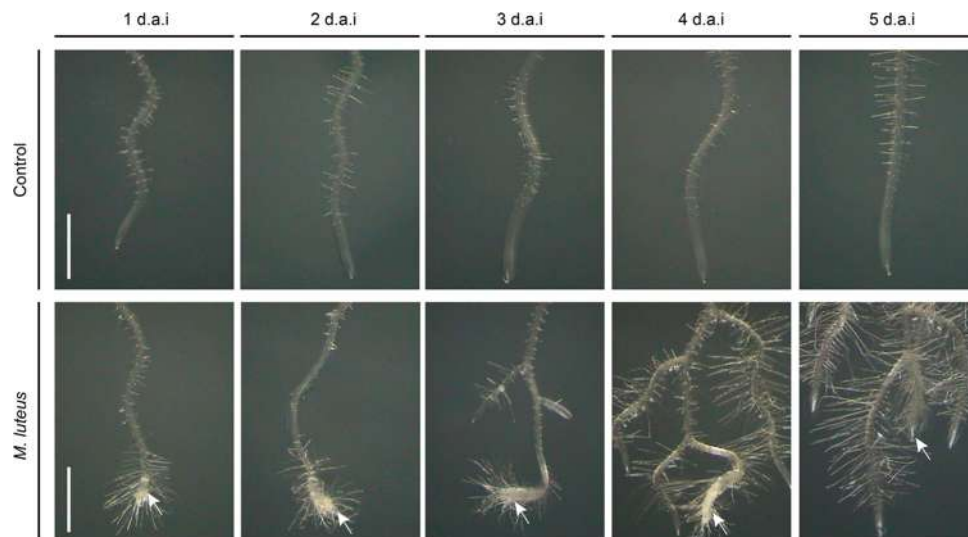


Fig. 5 Changes in root tip structure and induction of root hairs following contact with *M. luteus* LS570 streak. Representative images of *Arabidopsis* primary root tips analyzed with a stereoscopic microscope from 1 to 5 days after transfer over a bacterial streak. Images

of single root tips were selected from six independent images taken from seedlings cultivated in three independent plates, $n=18$. The experiment was repeated three times with comparable results. Scale bar = 1 mm

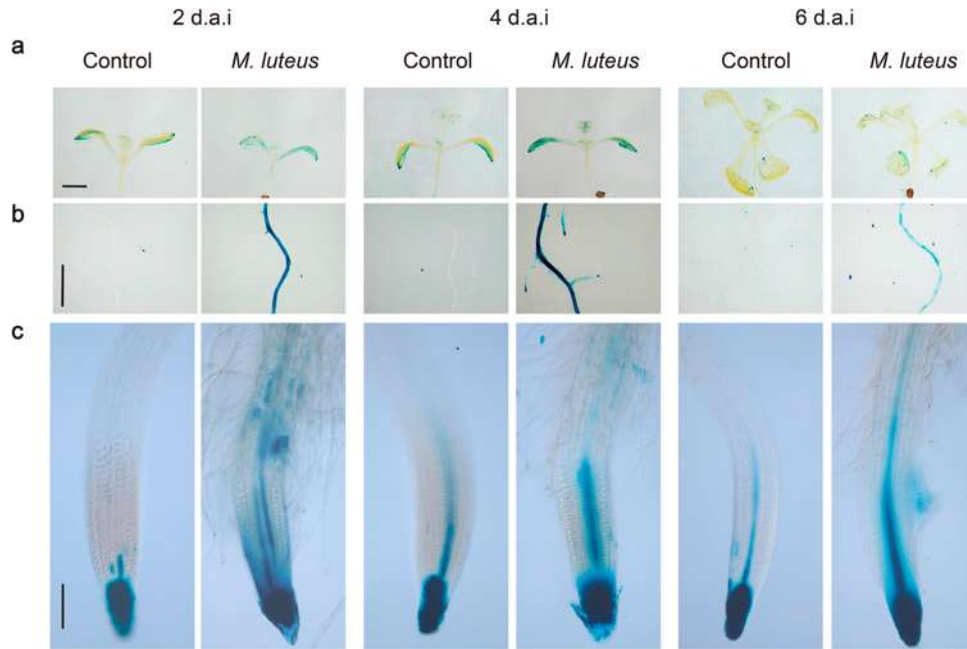


Fig. 6 Effect of *M. luteus* LS570 on *DR5:GUS* expression in *Arabidopsis* shoot and root tissues. Transgenic seedlings expressing the *DR5:GUS* construct were germinated and grown for 4 days and then transferred over *M. luteus* streaks for 2, 4, and 6 days. Expression in shoots (a), lateral root forming zone (b), and root tip (c). Repre-

sentative photographs selected from six independent images taken from seedlings cultivated in three independent plates. Scale bars a–c: 0.1 cm, 500 μ m, 100 μ m respectively, $n=18$. The experiment was repeated three times with comparable results

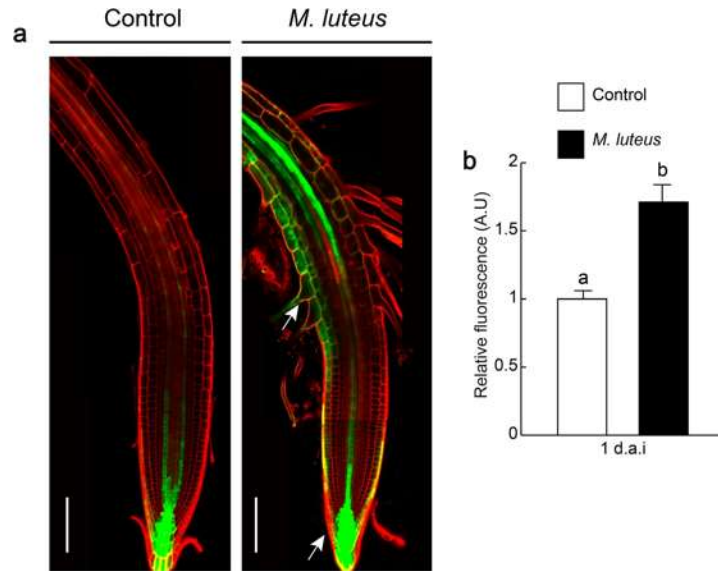
notable changes were observed during the first 2 to 4 days after inoculation, in which a strong and widespread shoot and root expression could be visualized, along with numerous, long root hairs being formed close to the meristem of the transgenic seedlings. After 6 days, these inducible expression patterns decreased, although the previously described events overall remained in the root system.

In vivo detection of the root auxin response using transgenic seedlings that express *DR5:GFP* confirmed the contrasting auxin-related expression patterns between axenic and bacterized seedlings, where increased green fluorescence could be appreciated in the roots in interaction with the bacterial streak, comprising the columella, the meristem, vascular system, and cortex layers (Fig. 7a, b). Quantification of the fluorescence further confirmed that *M. luteus* LS570 inoculation increases auxin-inducible gene expression within the root tip.

***Micrococcus luteus* LS570 strongly decreases the size of the meristem and cell elongation in *Arabidopsis* primary roots**

According to the previous results, we had a shortening of the primary root related to an auxinic response in the plant. Thus, the next question was if the decrease in root growth could be caused by altered cell division, elongation, or both. Quantification of the lengths of meristematic and elongation zone was done from confocal micrographs of seedlings at three times of interaction (1, 2, and 4 days) stained with propidium iodide. The images show the comparison of the phenotypical alterations in the primary root of axenically grown versus bacterized seedlings from the first day where a widening of the root tip occurs, and later on (4 days), where more and longer root hairs are formed closed to the root meristem (Fig. 8a). The first parameter measured comprised the length from the quiescent center (QS) to the first root hair formed in the differentiation zone, referred to as growth zone length. We also quantified the size of 10 cortical cells

Fig. 7 Effect of *M. luteus* LS570 on expression of the auxin-inducible marker *DR5::GFP* in *Arabidopsis* primary root tips. Confocal images of GFP fluorescence in a representative non-inoculated seedling (a) and after 1-day contact of the root with a streak of *M. luteus* LS570. Images of single root tips were selected from six independent images taken from seedlings cultivated in three independent plates. Relative fluorescence determination (b). Scale bar: 100 μ m, $n=18$. The experiment was repeated three times with comparable results



above the first root hair observed in the micrographs. The bacteria caused a remarkable reduction of growth zones, the meristematic zone decreased by threefold (Fig. 8b) while the length of the cortical cells was reduced by fivefold (Fig. 8c). These results show that the bacteria inhibit both the mitotic activity and cell elongation within primary root tips, which cause early differentiation events evidenced by the formation of longer and denser root hairs.

Re-configuration of root architecture by *M. luteus* LS570 involves ARF7 and ARF19 and auxin receptors

To test the involvement of genetic elements of the auxin signaling pathway in mediating the root phenotype in response to *M. luteus* LS570, *Arabidopsis* wild-type seedlings and single *tir1*, *slr1*, double *arf7/arf19*, and triple *tir1/afb2/afb3* mutants were transferred to axenic medium or exposed to bacterial streaks for 6 days. The growth of the primary root and lateral root number was quantified. Regarding the primary root length, we could note the inhibition with the bacterial treatment in the wild-type seedlings, which contrasts with the double mutant *arf7/arf19*, in which *M. luteus* neither repressed root growth nor promoted root branching (Fig. 9a). Moreover, a mutant with a gain of function in an auxin repressor (*slr1*) or disruption of one (*tir1*) or three (*tir1/afb2/afb3*) auxin receptors clearly diminished the effects of the bacteria in the reconfiguration

of root architecture (Fig. 9b, c). These data unveils the critical role played by ARF7 and ARF19 and auxin receptors in mediating plant bacteria communication responsible of root organogenesis.

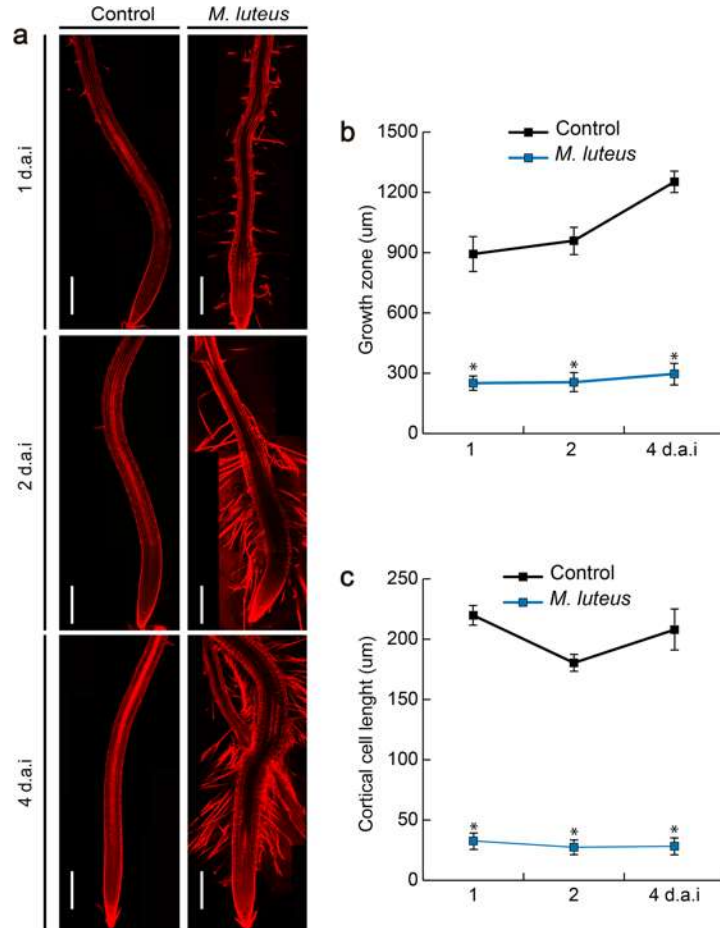
Plant co-cultivation did not affect bacterial growth

Considering that the *Arabidopsis*-*M. luteus* LS570 is a two-side interaction, next we asked whether the roots could have any effect on bacterial growth via determining optical density from bacterial streaks and cultivating culture dilutions on fresh media to count colony-forming units. The results show that bacterial growth was not modified after 5 days in contact with *Arabidopsis* roots since both the optical density (Fig. 10a) and the number of colony-forming units per plate (Fig. 10b) was comparable to the bacterial cultures in LB or MS 0.2 $\times$ media without plants. It means that under the conditions and time period investigated, the growth of *M. luteus* LS570 was not enhanced or repressed in the presence of *Arabidopsis* roots.

Discussion

A major goal of agriculture is to provide safe plant products for human consumption including fruits, grains, and fibers in a sustainable and environment-friendly manner. It requires a better understanding of the relationships established by

Fig. 8 Effect of *M. luteus* LS570 on root tip structure, meristem length and cell elongation. Representative images of axenically or bacterized seedlings stained with propidium iodide at 1, 2, and 4 days after interaction (d.a.i.) with the bacterial streak (a). Lengths of the root apical meristem (b) and cortical cell (c). Scale bar: 100 μ m. The results were analyzed with Student's *t* test when comparing two means, or with an ANOVA test and Tukey's means separation test for multiple comparisons ($p < 0.05$). Asterisks represent statistically significant differences. The experiment was repeated three times with comparable results



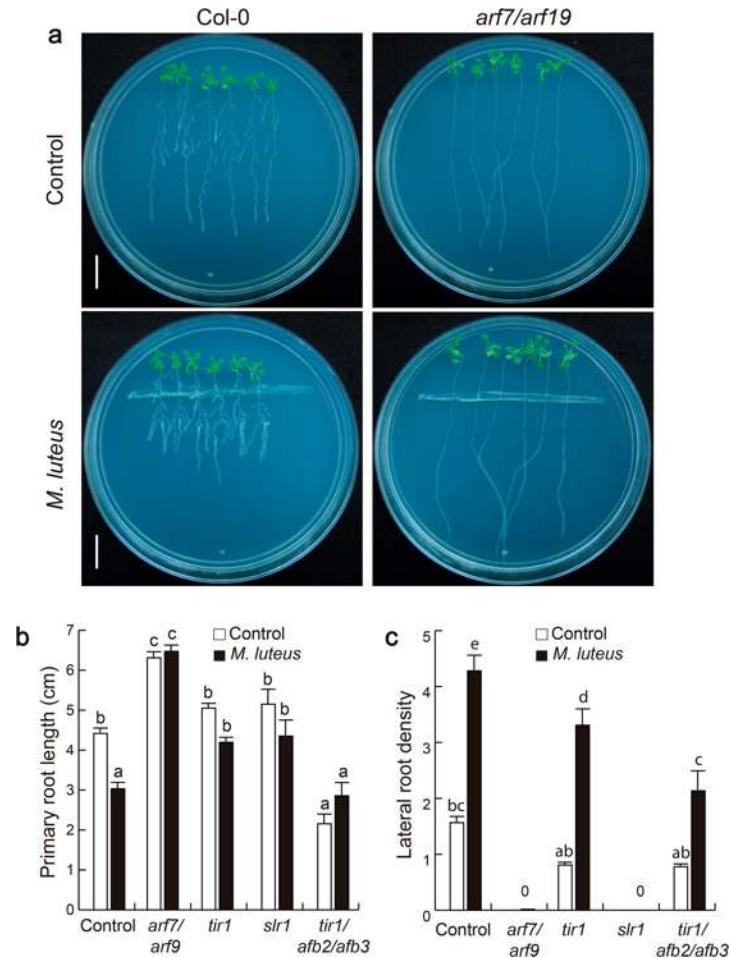
beneficial bacteria with roots, towards formulation of bio-inoculants aimed at enhancing plant growth, nutrient availability, tolerance to biotic and abiotic stress and, ultimately, to improve productivity (Busby et al. 2017).

The use of model plants such as *Arabidopsis thaliana* and systems for testing its interactions with pure bacterial isolates under axenic conditions has enormously increased our understanding of the molecular mechanisms mediating plant-bacteria recognition and determine functional mechanisms of plant-microbiome interactions in order to manage the interactions under variable environmental contexts (Zhou et al. 2020; Salas-González et al. 2021). Research with *Arabidopsis* shed light into the developmental processes affected by rhizobacteria by means of structural, diffusible,

and volatile molecules (Ortiz-Castro and López-Bucio 2019; Fitzpatrick et al. 2020).

Here, through screening the microbiome of a maize rhizosphere grown in the field termed “The Lometón,” we were able to isolate and characterize a bacterial isolate typified by DNA16s sequencing as *Micrococcus luteus* LS570. An analysis of bacterial colonies by electronic microscopy evidenced that the pure cultures were composed of round cells of around 2 μ m in diameter devoid of flagella, which is consistent with the phenotype of this genus. Detailed analysis of root, shoot, and total biomass indicated the root contact with a bacterial streak drives more biomass for root growth, increasing branching patterns and root hair formation in the first- and second-order lateral roots. Although the

Fig. 9 Comparison of root growth of WT and auxin-related mutants in response to *M. luteus* LS570. *Arabidopsis* seedlings were germinated and grown for 4 days and transferred to axenic 0.2×MS medium with or without a bacterial streak. Representative images of WT and the double mutant *arf7/arf19* grown in Petri plates 8 days after interaction (a). Primary root length (b), lateral root density (c), of WT, single *tir1*, *slr1*, double *arf7/arf19*, and triple *tir1/afb2/afb3* *Arabidopsis* mutants. Scale bar: 1 cm. The results were analyzed with Student's *t* test when comparing two means, or with an ANOVA test and Tukey's means separation test for multiple comparisons ($p < 0.05$). $n = 18$. Different letters represent statistically significant differences. The experiment was repeated three times with comparable results



more biomass in roots did not correlate with an enhanced shoot growth at the developmental stages of the seedlings analyzed, we did not observe any deleterious symptom in leaves such as growth retardation, chlorosis, and/or necrosis at least during 8 days that the seedlings were maintained in interaction with the bacterial streak, which indicates that LS570 did not behave as a plant pathogen. In support of this assumption, using a comparable assay for testing the interaction of the plant pathogen *Pseudomonas aeruginosa* PAO1 and *Arabidopsis* seedlings, a strong growth repression of primary root growth, lateral root formation, and overall root and shoot biomass production was caused by the root contact with a bacterial streak, which was missing in the

nearly isogenic *P. aeruginosa* LasI mutants that are defective in the production of the major quorum-sensing signal 3-oxo-*N*-dodecanoyl homoserine lactone (Ortiz-Castro et al. 2014). In this case, the pathogenic PAO1 strain decreased the auxin response at the primary root tip. Thus, disruption of QS communication in *P. aeruginosa* changes the relationship from a pathogenic to a growth-promoting one.

From our plant phenotypical analysis and marked effects towards enhancing root branching and biomass, we conclude that *Micrococcus luteus* LS570 behaves as a plant growth-promoting rhizobacterium. Comparable properties of another *M. luteus* strain, namely *chp37*, have been demonstrated in pot experiments with maize plants using both a

sandy soil and soil samples from a natural field. In 90-day-old plants, chp37 inoculation increased by 8.5, 13, 23, and 54% the number of leaves, length of shoots and roots, and the fresh weights, respectively, as compared to uninoculated controls (Raza and Faisal 2013). Phosphate-solubilizing, auxin production, and ACC deaminase activities have also been attributed to a *Micrococcus* sp. strain NII-0909 isolated from a forest soil in India, and its inoculation to cowpea roots increased growth and yield (Dastager et al. 2010). These results suggest that the plant probiotic traits of *Micrococcus*, validated in our research using axenic, in vitro systems go beyond the Petri dish assays and support promising applications to the greenhouse and the field.

Different plant species can harbor rhizobacteria that produce IAA, IAA precursors, or indole, and the profiles of these compounds may vary to give specificity for the integration of the microbiota into the root developmental programme (Gilbert et al. 2018). This might be a general rule for species that interfere with the host hormonal status, since comparable to the effects exerted on auxin homeostasis, several plant pathogens and symbiotic microbes can affect the host ethylene levels or degrade it through ACC deaminase, thus enhancing root elongation. Accompanying the halted primary root growth that happens by the inoculation with *Micrococcus luteus* LS570, we observed the acceleration of differentiation at the root epidermis giving rise to more and longer root hairs. In this regard, several Cactaceae family members follow a determinate root system program in response to environmental stimuli such as drought, temperature stress, and nutritional deficiency (Shishkova et al. 2008). Under these conditions, cell division in root meristems is compromised and rapid differentiation events take place in the root tip, undergoing the formation of secondary branches and epidermal root hairs that assist in water and nutrient acquisition (Shishkova et al. 2008). In this context, we observed a fast reconfiguration of the meristem and elongation zones of the primary root under physical contact with LS570 streaks, such that the formation of long hairs occurred since the first day in the interaction. We cannot exclude that these developmental modifications account to better plant performance for plants in the field.

Microbes modulate endogen phyto-hormone levels modifying the cellular homeostasis and consequently, plant architecture. Thus, it is reasonable to think that the LS270 isolate might produce a still unknown metabolite responsible of root branching induction. However, it is not easy to define the molecular identity (ies) of the bioactive compounds as these may be of diffusible or volatile origin. A few species of the *Pseudomonas* genus including *P. aeruginosa* PAO1, *P. putida*, and *P. fluorescens* have been characterized in detail and their effects in *Arabidopsis* analyzed using Petri plates with axenic media. Irrespective of the relationships established with the plant host, either pathogenic or symbiotic, these bacteria are

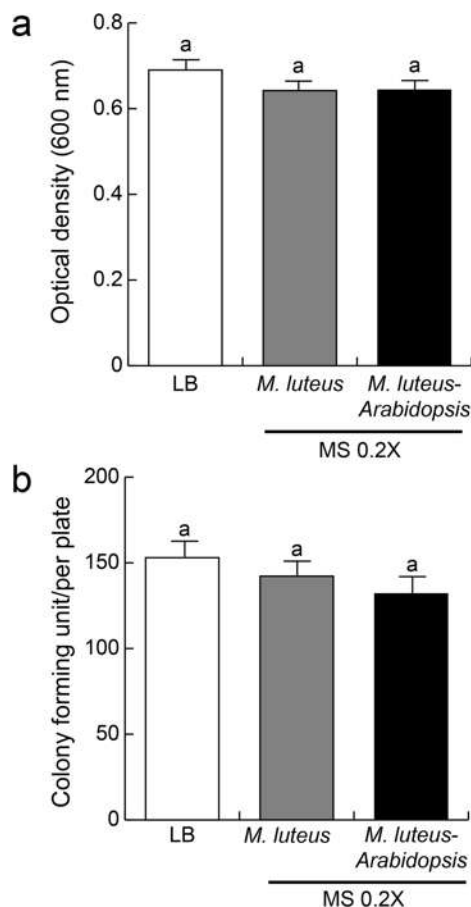


Fig. 10 Growth of *M. luteus* LS570 in axenic media or in co-cultivation with *Arabidopsis* seedlings. Bacterial streaks were recovered from the media after five days in interaction with roots or grown over agar plates with MS 0.2× media, or over LB solid media as a positive control for comparable times. (a) Optical density, (b) colony-forming units per plate from 1/10,000 dilutions of the total bacterial streak recovered after 5 days in interaction with plants. Data show the means and standard deviations from six independent streaks per treatment. The experiment was repeated two times with comparable results

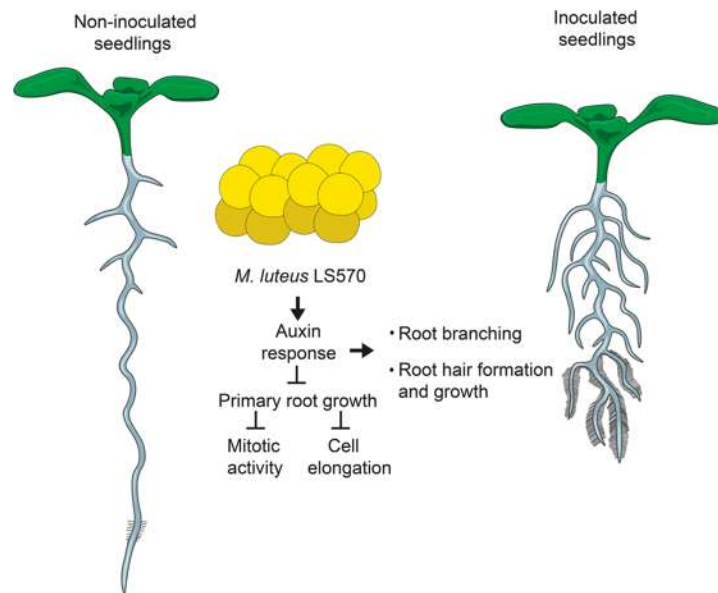
major triggers of developmental plasticity of the root system by inhibiting primary root elongation and promoting lateral root and root hair formation (Ortiz-Castro et al. 2011, 2020; Zamioudis et al. 2013). The regulation of root architecture by *Pseudomonas* has been tightly linked to bacterial production of cyclodipeptides, which may act both as quorum-sensing

signals and as auxin signal mimics (Ortiz-Castro et al. 2011, 2020). On the other hand, the rhizobacterial species *Bacillus methylotrophicus* M4-96 isolated from maize roots promoted the growth of *Arabidopsis* seedlings when cultured on opposite sides of divided Petri plates, and enhanced primary root growth, root branching, leaf formation, and shoot biomass, which was assisted by an auxin response driven by bacterial volatiles, as reported by the *DR5::GUS* gene marker, and was antagonized by the auxin transport inhibitor 1-naphthylphthalamic acid (Pérez-Flores et al. 2017).

IAA production is a trait highly manifested in bacteria that live as plant endophytes (Gilbert et al. 2018). However, the auxin role in plant-bacteria interactions appears to be very complex, possibly acting as an effector molecule or a hormonal driver of organogenesis. A positive function of IAA in the formation of lateral roots and root hairs, as well biomass production, was reported for *Azospirillum brasilense* Sp245, which also targets the target of rapamycin kinase to activate root meristems and overall plant growth (Spaepen et al. 2014; Méndez-Gómez et al. 2020). However, auxin signaling can be exploited by pathogens to colonize roots and spread into inner tissues forming tumors or driven development to the formation of massive amounts of lateral roots, and thus, in an ecological context bacteria can alter the plant organogenic program via auxin production and/or degradation in a such manner that specific operons have been identified for the *Variovorax* genus that encode auxin-degrading enzymes and help these bacteria to dominate in

synthetic communities (Fu and Wang 2011; Finkel et al. 2020). Auxins are also important for plant bacterial recognition and plant colonization. Ganin et al. (2019) showed that the plant growth-promoting rhizobacterium *Bacillus subtilis* colonizes roots and excludes pathogens through forming biofilms with a protective role. Interestingly, accumulation of specific indole derivatives at the plant root interface antagonizes biofilm formation and disruption of auxin sensing in the bacterium makes it pathogenic to plants. Instead of focusing our analysis in the metabolites being produced by LS570, we analyzed the plant auxin responsiveness using the auxin-inducible constructs *DR5::GUS* and *DR5::GFP*, via histochemical and confocal microscopy strategies. For both markers, the most pronounced induction occurred in the first days in the whole plant, which suggests that the reconfiguration of root architecture is tightly related to an enhanced auxin response. Several components in the auxin signaling pathway have been identified, including the coreceptors TRANSPORT INHIBITOR RESPONSE1 (TIR1) and AUXIN SIGNALING F-BOX PROTEINS (AFBs), the transcriptional repressors Auxin/INDOLE ACETIC ACID (Aux/IAA) that interact with the SCF complex for protein degradation through the proteasome 26S, and the transcriptional activators called AUXIN RESPONSE FACTORS (ARFs) that drive the auxin-responsive genes to be transcribed (Parry et al. 2009; Prigge et al. 2020). The comparison of the phenotypes of WT and mutants in this signaling pathway including *tir1*, *slr1*, *arf7/arf19*, and *tir1/afb2/*

Fig. 11 Schematic model to explain the influence of *M. luteus* LS570 on growth and development of *Arabidopsis* seedlings. Bacterial inoculation induces the shift from long primary roots to short, more branched root systems via inducing a canonical auxin response. In the primary root, major effects are the inhibition of mitosis in the meristem and cell elongation. This correlates with promotion of root hairs and lateral root formation, which can be explained through the induction of the auxin response



afb3 helped us to define their importance in decoding the chemical message triggered by *M. luteus* LS570 to trigger changes in primary root and lateral root formation processes. Our results clearly show the involvement of the ARF7 and ARF19 transcriptional activators, since the corresponding double mutant was totally resistant to inhibition of primary root growth and lateral root induction by bacterial streaking. This also happened for the auxin receptor mutants, although the deviation of the response was lower than for the *arf7/arf19* double mutants. To the best of our knowledge, this is the first time that the ARF7 and ARF19 proteins are involved in the control of the primary root growth, as these were primarily involved in the lateral root formation program activated by diverse stimuli (Okushima et al. 2007; Huang et al. 2018; Lee et al. 2019).

The beneficial role of root exudates for bacterial growth is well known. Therefore, we hypothesized that the growth of *Micrococcus luteus* LS570 could be positively influenced after 5 days in direct contact with *Arabidopsis* roots. This was not the case, since optical density and viable count estimations of bacterial streak cultures manifested comparable responses to the bacteria streaked over LB medium or MS 0.2× media. One possibility is that the plant-interaction medium (MS 0.2×) provides appropriate levels of nutrients to support bacterial growth and then, the effect of root exudates was negligible. It would be of interest to investigate the influence of *Arabidopsis* and other plant species in conditions of nutrient deprivation or environmental stress to further determine if the interaction with plants may provide an adaptive advantage to the bacterium to survive and thrive.

Collectively, our data provide a series of evidence summarized in Fig. 11, which helps to understand the molecular mechanisms by which *Micrococcus luteus* LS570 interaction with roots affects *Arabidopsis* seedlings. Our report points to an important role of auxin signaling supported by auxin-inducible gene expression and genetic studies in the phytostimulation program driven by this bacterium; thus, the bacterium can be regarded as a plant growth promoting rhizobacteria and opens the possibility for applications in agriculture as a growth promoter.

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Author contribution E.G.C., R.O.C., L.F.R.H., and E.V.C. performed the experiments. E.G.C., R.O.C., L.F.R.H., E.V.C., and J.L.B. analyzed the data. E.G.C. and J.L.B. designed the experiments and wrote the manuscript. J.L.B. applied for funding. All authors approved the manuscript.

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Declarations

Competing interests The authors declare no competing interests.

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Capítulo II



***Bacillus* sp. LC390B from the Maize Rhizosphere Improves Plant Biomass, Root Elongation, and Branching and Requires the Phytochromes PHYA and PHYB for Phytostimulation**

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Abstract

The plant microbiomes include root-associated beneficial species, which rely on carbon supplements to survive and exert their diverse attributes. Here, we isolated and identified through 16S rRNA analysis a novel plant growth promoting rhizobacterium belonging to the *Bacillus* genus (*Bacillus* sp. LC390B) from a collection from root isolates of maize plants grown in an alkaline-calcareous soil. The LC390B strain enhanced shoot and root fresh weights and chlorophyll content in leaves upon direct cocultivation with *Arabidopsis* roots in medium lacking sucrose or supplemented with 0.6% sucrose, and upon transfer to artificial soil led plants to produce more fruits. These effects coincided with faster cell elongation, enhanced primary root growth and higher branching capacity, and increase in local auxin response within lateral root primordia. Mutant analysis indicated that specific elements in the auxin pathway control either primary root elongation or branching in bacterized plants, while most of the probiotic attributes were missing in a double mutant defective on the phytochromes *PHYA* and *PHYB*. The bacteria remained viable in interactions with roots up to seven days, supporting its long lasting effects to plants. Our data show that the newly isolated LC390 strain has potential to stimulate the growth of plants and unveil a critical function of specific elements in the auxin and phytochrome signaling network in plant–bacteria interaction.

Keywords Phytochromes · Rhizobacteria · Auxin · Sucrose · Phytostimulation

Introduction

The physiology and activities of plants are tightly linked to microbial influence at the rhizosphere, where a myriad of bacterial species proliferate throughout the entire root system and enhance mutual adaptation. Root exudates provide a large number of carbon and nitrogen rich substances

that shape the microbiome and help establishing mutualist symbiotic relationships with the so-called plant growth promoting rhizobacteria (PGPR) (Berg and Smalla 2009; Kawasaki et al. 2021). These beneficial bacteria fine-tune plant architecture, adjust phytohormone homeostasis, produce plant growth regulators, quorum-sensing signals and secondary metabolites that drive host morphogenesis, reinforce immunity and help survive in hostile environments (Ortiz-Castro et al. 2011; Backer et al. 2018; Ortiz-Castro and López-Bucio 2019; Ortiz-Castro et al. 2020).

Bacteria-driven changes in lateral root development, root hair formation and primary root elongation are crucial features for plant nutrition, water absorption and soil anchorage. These developmental traits are regulated by auxin biosynthesis, transport and signaling, which are critically influenced by bacteria inhabiting roots either via auxin production or degradation (Alarcón et al. 2019; Finkel et al. 2020; García-Cárdenas et al. 2021). Within the most prolific rhizobacterial communities, the *Bacillus* genus can be distinguished by their production of secondary metabolites

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and volatile organic compounds (VOCs) (Akinrinlola et al. 2018). Many efforts have been conducted to assess how *Bacillus* infochemicals affect plants, but only a few studies have explored how direct root bacterization modulates plant physiology and morphogenesis.

VOCs improve plant growth and exert biological control properties, thus diminishing detrimental effects under biotic and abiotic stress (Li et al. 2021). Acetoin and 2–3 butanediol act as positive elicitors of plant growth by *Bacillus* species, triggering changes in phytohormone levels, modulating gene expression, and augmenting photosynthetic efficiency (Zhang et al. 2008; Tahir et al. 2017; Silva Dias et al. 2021). Accordingly, mutants affected in acetoin biosynthesis were unable to enhance plant growth (Ryu et al. 2003). Recent research demonstrated that the *Bacillus* VOCs improve lateral root development, increasing the formation of root primordia and their emergence via modulating auxin homeostasis in *Arabidopsis* (Li et al. 2021). Similarly, *Bacillus megatherium* WW1211 VOCs had notable biostimulation outcomes augmenting shoot fresh weight and lateral root development, but decreasing primary root length, the mechanisms underlying this response were again related to auxins (Wang et al. 2021). Single *Bacillus* species and consortia, as well as endophytes, have a strong influence in plant physiology, stimulating germination and yield traits in horticultural plants, which underscores their high potential to be applied as probiotics in agriculture (Tamošiūnė et al. 2018; Hernández-Soberano et al. 2019).

Sugars derived from photosynthesis have a dual role in metabolism and act as chemical attractants for bacteria, entailing mutualist symbiosis in plant–microbe interactions. Sucrose synthesis encompasses enzymatic reactions catalyzed by sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP). Then, sugar transporters translocate sucrose to sink organs, including roots, for metabolic processes impacting the biostimulation process (Malony et al. 2015; Solís-Guzmán et al. 2017; Hennion et al. 2019). Sucrose enables *Bacillus* mobilization inducing surfactin synthesis and *Arabidopsis* mutants affected in sucrose transporters *suc2* and *suc3* had decreased bacterial colonization of roots (Tian et al. 2021), which suggests that photosynthesis and sugar root exudation are amongst the driving forces for symbiosis events.

Photosynthesis involves light reactions to synthesize sugars, however, light is also the prime signal perceived by photoreceptors and triggers growth responses such as germination, organ formation, and the transition from vegetative stage to flowering (Kraiselburg et al. 2017; Legris et al. 2019). Currently, the involvement of phytochromes in plant–PGPR interactions remains to be investigated. To gather information into this important aspect of cross-kingdom signaling, we isolated and characterized a novel plant growth promoting *Bacillus* sp. LC390B strain. An

outstanding biostimulation effect could be observed in *Arabidopsis* seedlings whose roots were placed in direct contact with a bacterial streak, where shoot and root biomass and the promotion of root growth and branching could be correlated with auxin-sucrose networks and required phytochromes A and B. These data imply that different mechanisms of phytostimulation are manifested by *Bacillus* species where the LC390B strain can strongly promote lateral root development with no need to halt primary root growth, which may be advantageous for soil exploration.

Materials and Methods

Isolation and Molecular Characterization of *Bacillus* sp. LC390B

Bacillus sp. LC390B was isolated from an alkaline soil (pH 8.6). Samples were collected from the rhizosphere of maize plants in the community of Lometón, Tarímbaro, Michoacán state, México (19°42' N and 101°8' O) location. The strain grew well in Luria–Bertani (LB) solid media at 28 °C for 24 h forming irregular, smooth, undulated, creamy, opaque, and white bacterial colonies. Genomic DNA extraction from pure cultures of *Bacillus* sp. LC390B was carried out following the methodology described by Tzec-Interián et al. (2020). The 16S rRNA gene amplification was followed using the primers (F27: AGAGTTTGATCCTGGCTCAG and R1494: TACGGCTACCTGTTACGAC) through Qiagen specifications. Finally, the samples were sent to the National Laboratory of Genomics for Biodiversity LANGE-BIO-CINVESTAV for sequencing, the dendrogram construction included the sequences obtained from the GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the MEGA 7 software (Tamura et al. 2011). The evolutionary history was inferred by Maximum Likelihood method and Tamura–Nei model (Tamura and Nei 1993). The GenBank accession number of *Bacillus* sp. LC390B is MZ531890.

Seedling Treatment and Growth Conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) wild-type, auxin-related mutants *aux1-7* (Picket et al. 1990), *tir1/afb2/afb3* (Parry et al. 2009), *str1* (Fukaki et al. 2002), *arf7/arf19* (Okushima et al. 2007) and *axr1-3* (Estelle and Somerville 1987), a phytochrome double mutant *phyA/phyB* (Reed et al. 1994) and the transgenic line expressing the auxin inducible marker *DR5::GFP* (Ottenschläger et al. 2003) were used in this report. Cell proliferation and elongation were monitored in the primary root by using *CycB1::uidA* (Colón-Carmona et al. 1999), or *Exp7::uidA* (Cho and Cosgrove 2002), respectively. Seeds were disinfected using ethanol 96% (v/v), chlorine 20% (v/v), and

distilled, sterilized water for 5, 7 and 1 min, respectively, repeating five times the water washing. Seeds were stored at 4 °C for 48 h into a refrigerator and later on were placed into 0.2×MS solid medium, at 0.9 g/L basal salts concentration, and phytagar (PhytoTechnology Laboratories). Sucrose treatments were adjusted to the above described formulation. The plates were positioned vertically into a Percival growth chamber (Percival Scientific AR95L) at 22 °C, with 80% humidity, 16/8 h light/darkness photoperiod. The Petri dishes were randomized and placed vertically at 42 cm from the light source, under cool white fluorescent lamps (PHILIPS F17T8/TL841, 17 WATT) and Photosynthetic Photon Flux Density of 100 $\mu\text{mol}/\text{m}^2/\text{s}^{-1}$.

Plant–Microbe Interaction Assays

Bacillus sp. LC390B is an aerobic bacterium capable to grow in Luria–Bertani (LB) broth at 28 °C for 24 h as optimal conditions supplying bacto agar 1.5% as solidifying agent. Four day-old *Arabidopsis* seedlings were transferred to agar solidified 0.2×MS medium in vitro in non-inoculated plates, which served as controls. To analyze the effects of the LC390B strain, a horizontal bacterial streak was made over the 0.2×MS medium taking an isolated colony grown under axenic conditions. Then, seedlings were placed over the bacterial inoculum, allowing their main roots to establish direct root-bacteria interaction and plant growth parameters were measured after 6–8 days in the interaction. The plant traits analyzed included primary root length (growth after bacterization), lateral root number, lateral root length and lateral root density. Root, shoot and total fresh weights were obtained dissecting the root system from the aerial tissues, and measured using a high precision analytical scale.

Bacterial Growth Conditions and Maintenance Across the Plant–Microbe Interaction

Previously to *Arabidopsis* seedlings cocultivation, the *Bacillus* sp. LC390B strain was grown in LB liquid media at 30 °C and 110 rpm to determine the initial inoculum. In the exponential phase (12 h), optical density at 600 nm wavelength, and colony forming units were obtained by serial dilutions. The 4-days-old *Arabidopsis* seedlings were placed over a horizontal streak using a calibrated loop in 0.2×MS solid media with a concentration of 3.6×10^7 CFU/mL, and 1.100 OD₆₀₀. From the first to seven days of bacterial cocultivation with plants, density of the inoculum was analyzed recovering the bacterial culture from the media, which was resuspended in 1 mL of deionized water in axenic conditions to estimate OD₆₀₀ and cell viability by CFU assays. All these experiments were performed in triplicate.

Plant Growth in Artificial Soil

Bacterized and non-inoculated *Arabidopsis* seedlings were transferred into plastic boxes containing a mixture of peat moss, perlite and vermiculite in 3:1:1 proportion. Non-inoculated and bacterized seedlings were placed side by side into the same box, two lines per treatment with three plants each per triplicate. Vegetative and reproductive growth parameters were measured during six weeks into a Percival chamber at 22 °C, with 80% humidity, 16/8 h light/darkness photoperiod, and light intensity of 100 $\mu\text{mol}/\text{m}^2/\text{s}^{-1}$. Sterilized, distilled water was used for irrigation twice a week, to maintain the plants at field capacity and representative photographs and growth parameters were recorded weekly.

Chlorophyll Content Estimation

The shoot system was submerged in ethanol (96% v/v) for 30 min, covering the samples with aluminum foil to avoid degradation. Spectrum absorbance values were recorded at 649 and 664 nm wavelengths for chlorophyll “b and a”, respectively, using ethanol as a blank. The results were calculated according to Lichtenthaler (1987) equation as follows, $((5.24 \times \text{Absorbance } 664) + (22.24 \times \text{Absorbance } 649)) / (1000 \times (\text{shoot fresh weight}))$, the units were shown in (mg/g fresh weight).

Histochemical Procedure for GUS and GFP Activity Detection

Transgenic seedlings expressing the β -glucuronidase reporter gene *uidA* (GUS), were incubated into 5-bromo-4-chloro-3-indolyl- β -D-glucuronide in sodium phosphate 100 mM buffer, pH 7, 0.5 M, and incubated at 37 °C, overnight. To visualize the GUS activity, the stained plants were immersed into HCl 0.24 N and methanol 20% (v/v) solutions, at 60 °C for 45 min for pigment elimination. Then, NaOH 7% and ethanol 60% (v/v) were supplemented for 30 min at room temperature. Finally, plants were submerged in ethanol at 40%, 20%, and 10% and stored with glycerol (50% v/v). For visualization purposes, the seedlings were fixed on glass slides with glycerol (50% v/v), images were taken using Nomarski microscopy in 20× and 40× magnification. GFP reporter gene was detected by confocal microscopy (Olympus FV1000) at 568 nm excitation and 585–610 nm emission, the samples were previously treated with propidium iodide over clean glass slides and visualized in 20×, and 40× magnifications.

Lateral Root Primordia Analysis

All the development stages in lateral root formation were followed with transgenic plants expressing the auxin inducible

marker *DR5::GFP*. Seedlings were transferred at 4 days after germination to 0.2×MS solid media with or without bacteria. 24 h after transfer, the control and bacterized seedlings were analyzed by confocal microscope, placing the primary root in clean glass slides with propidium iodide dilution. Each lateral root primordia and those emerged from the primary root were registered per plant during 6 days. Representative images were taken at 20X magnification.

Data Processing, Statistics and Software

All experiments were carried out per triplicate. The statistical analysis was made using univariate and factorial ANOVA through STATISTICA 10 StatSoft program with Tukey's HSD test. Different letters mean the treatments are statistically significant with ($p < 0.05$) value. Images and graphics were processed, measured and analyzed with Microsoft Excel, Image J, Adobe Photoshop and Adobe Illustrator CS6.

Results

Isolation of the Plant Growth Promoting *Bacillus* sp. LC390B Strain from the Maize Rhizosphere

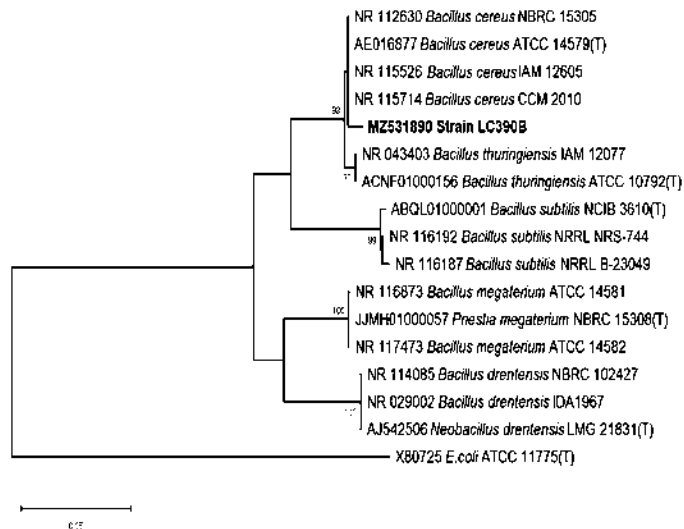
With the aim to identify plant growth promoting rhizobacteria, we scrutinized a group of isolates collected from the

rhizospheres of maize (*Zea mays*) plants grown in a field termed "The Lometón", Tarimbaro, Mich, Mex. The strain LC390B displayed the greatest biostimulation effects in cocultivation with *Arabidopsis* seedlings, using molecular 16S rRNA and evolutionary analysis through maximum likelihood method, the strain was positioned as *Bacillus* sp., which was closely related to *Bacillus cereus* (Fig. 1).

Bacillus sp. LC390B Promotes *Arabidopsis* Seedling Growth In Vitro

To elucidate the beneficial properties of the bacterial strain in *Arabidopsis* seedlings, we designed a co-inoculation method in which *Arabidopsis* seedlings were grown and transferred at 4 days after germination (d.a.g) to 0.2×MS solid media. Isolated bacterial colonies were streaked horizontally into the Petri dish, placing the primary root in contact to the inoculation zone and plant growth parameters were analyzed 6 days after inoculation (Fig. 2a, b). Plant-bacterium cocultivation strongly promoted plant growth and development, which correlated with increased shoot and root biomass (Fig. 2c, d), enhanced primary root elongation (Fig. 2e), and increased by twofold lateral root number, density and lateral root length compared to uninoculated seedlings (Fig. 2f–h). These data show that the *Bacillus* sp. LC390B strain behaves as a plant growth promoting rhizobacterium.

Fig. 1 Phylogenetic tree of *Bacillus* sp. LC390B. Dendrogram construction was inferred by molecular 16S rRNA analysis. *E. coli* was used as the outer group, the strain LC390B, was closely related with *B. cereus* with 96% of identity



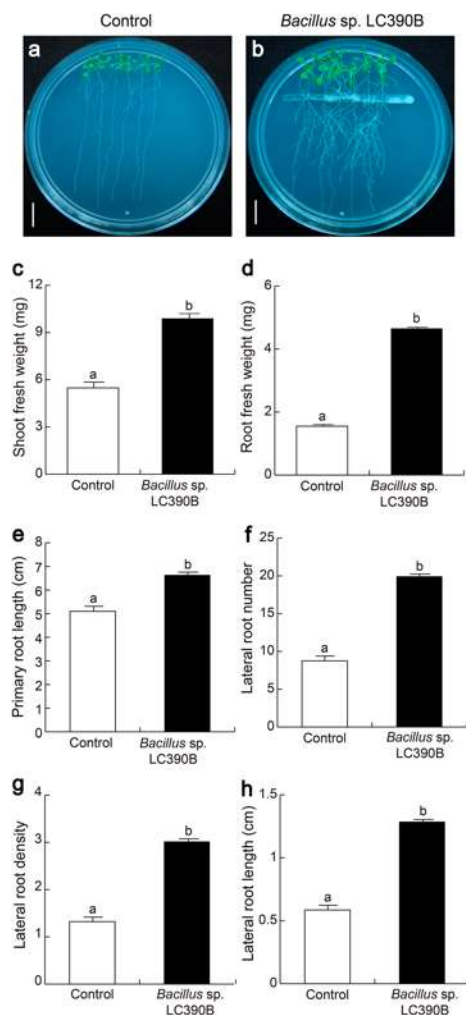


Fig. 2 Plant growth promotion by *Bacillus sp. LC390B* in Petri dishes. **a** Effects of the bacterium in *Arabidopsis* seedlings. **b** Representative Petri plates showing the effects of LC390B inoculation. **c** Shoot fresh weight. **d** Root fresh weight. **e** Primary root length. **f** Lateral root number. **g** Lateral root density. **h** Lateral root length. Different letters are used to indicate means that differ statistically through Tukey post hoc analysis with $p < 0.05$ significance. Scale bar: 1 cm

***Bacillus sp. LC390B* Promotes Fruit Production in an Artificial Soil**

To evaluate if the prominent effects by the strain LC390B

in vitro are maintained over time, we transferred axenic and bacterized *Arabidopsis* seedlings after 6 days in interaction in the Petri plates to plastic boxes containing an artificial soil substrate made by a combination 3:1:1 of peat moss, perlite and vermiculite, respectively, which is amenable for *Arabidopsis* growth under laboratory conditions. Six non-inoculated seedlings were placed in two rows towards the left side of the box and six bacterized seedlings were placed at the right side of the box in order to compare their growth side by side. Representative images from the boxes were taken during the duration of the experiment (Fig. 3a), and rosette diameter, stem number per plant, main stem length and number of siliques (fruits) per plant were recorded weekly (Fig. 3b–e). The unique and major trait modulated by the bacteria was the number of fruits, reaching significant differences by the fourth to the last week, augmenting around 150 and 200% silique production per plant (Fig. 3e). Although, we did not observe clear promoting effects in vegetative growth under these conditions, the beneficial effects of the strain LC390B achieved in vitro correlated with more fruit production in an artificial soil system along the entire life cycle of the plants.

***Bacillus sp. LC390B* Inoculation Increases Root Cell Elongation**

An outstanding effect of the bacterium in roots was the promotion of primary root elongation, a trait not frequently observed in plant bacteria interactions. We first hypothesized that the primary root length was longer in bacterized plants due to major changes in cell proliferation or elongation. To test this possibility, we evaluated the changes in expression of the mitotic reporter *CycB1::uidA* in the root apical meristem and measured meristematic zone length from the quiescent center to the first rapidly elongating cell. We did not observe any change in the size of the root meristems during the bacterial interaction period that lasted six days (Fig. 4a). We next assessed cell elongation using the *Exp7::uidA* marker, by measuring the size of the region from the transition zone to the first root hair formed. Interestingly, this zone was longer in bacterized seedlings than in the axenically grown seedlings, since from the second day after interaction to the sixth, the primary root and their cells elongated faster than uninoculated seedlings (Fig. 4b). These data suggest that a major trait influenced by *Bacillus sp. LC390B* in primary roots is the cell elongation process.

***Bacillus sp. LC390B* did not Enhance Auxin-Responsive Gene Expression in *Arabidopsis* Primary Root Tips**

Auxins are master plant growth regulators that can be produced by bacteria and influence root architecture. We

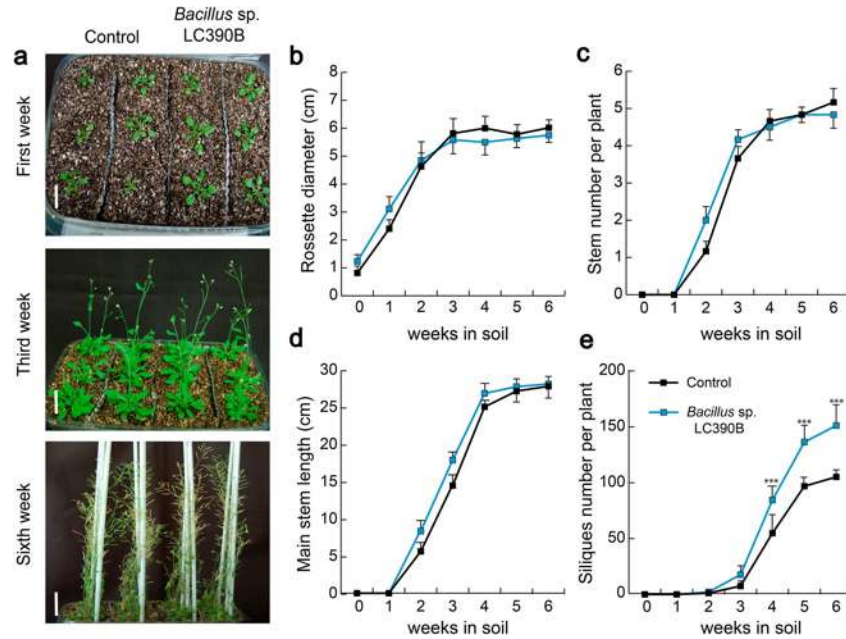


Fig. 3 Effect of *Bacillus* sp. LC390B in *Arabidopsis* seedlings in soil conditions. Plant growth parameters were recorded during the *Arabidopsis* life cycle. **a** Representative photographs were taken at the first, third and sixth week after transfer. **b** Rosette diameter. **c** Stem number

per plant. **d** Main stem length. **e** Siliques number per plant. Scale bar: 3 cm ($n=12$). Different letters are used to indicate statistically significant differences through Tukey post hoc analysis with $p<0.05$ significance

tested the auxin response in primary root tips of *Arabidopsis* seedlings expressing the auxin inducible gene construct *DR5::GFP* in uninoculated or *Bacillus* sp. LC390B bacterized roots. In uninoculated seedlings the green fluorescence recorded by confocal microscopy, could be detected in the quiescent center, the columella cells and proxylematic tissue. Comparable expression patterns were observed in root tips in interaction with *Bacillus* sp. LC390B (Fig. 5). These data indicate that the growth promoting effect in primary roots elicited by the bacterium cannot be attributed to bacterially produced auxins or to an enhanced auxin response within the primary root tip.

***Bacillus* sp. LC390B Accelerates Lateral Root Development and Enhances Auxin Response Within Lateral Root Primordia**

The strong promoting effect of bacterization in root branching opens the possibility that *Bacillus* sp. LC390B could increase lateral root primordia initiation or

accelerate the emergence of preformed lateral root primordia from the parent root. To test these possibilities, we counted the lateral root primordia in transgenic seedlings expressing *DR5::GFP*, which marks the developing primordia and enables quantitation of these structures. Confocal microscopy analyses showed an increased auxin expression in vascular tissues and developing lateral root primordia prior to emerge from the parent primary root of seedlings inoculated with *Bacillus* sp. LC390B (Fig. 6a). The bacterized seedlings did not develop more lateral root primordia de novo, instead the bacterium triggered by five-fold the maturation of primordia (stage E) compared to uninoculated seedlings (Fig. 6b). The promotion of lateral root growth by *Bacillus* sp. LC390B already occurred from two days after interaction and lasted from the six days of duration of the experiment (Fig. 6c). These data point to the local increase in auxin response in the bacterized seedlings, specifically during the transition of the lateral root primordia into a mature branch.

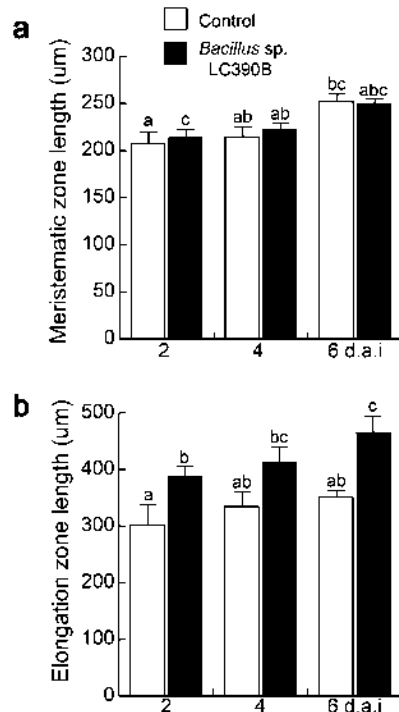


Fig. 4 *Bacillus sp.* LC390B enhances cell elongation in *Arabidopsis* primary roots. Cell proliferation and elongation were monitored by *CycB1::uidA* and *Exp7::uidA*, respectively. **a** Meristematic zone length and **b** elongation zone length were measured using Nomarski microscopy at 2, 4 and 6 days after interaction with the bacterium. Different letters are used to indicate means that statistically differ through Tukey post hoc analysis with $p < 0.05$ significance ($n = 18$). The experiment was repeated three times with comparable results

Promotion of Root Branching by *Bacillus sp.* LC390B Involves Auxin Signaling Components

The positive link between auxin and lateral root development in bacterized seedlings led us to compare the changes in primary root growth and branching in WT seedlings and auxin-related mutants uninoculated or inoculated with *Bacillus sp.* LC390B. Regarding the promotion of primary root elongation by the bacterium, a gain-of-function mutant in the SOLITARY ROOT gene (*slr1*) and a mutant defective in the auxin influx permease (*aux1-7*) did not manifest the growth promoting effect in primary root elongation of *Bacillus sp.* LC390B (Fig. 7a). Additionally, the triple mutant in the co-receptor complex *tir1/afb2/afb3*, a double mutant defective on the transcription factors *arf7/arf19* and *slr1* were unable to develop

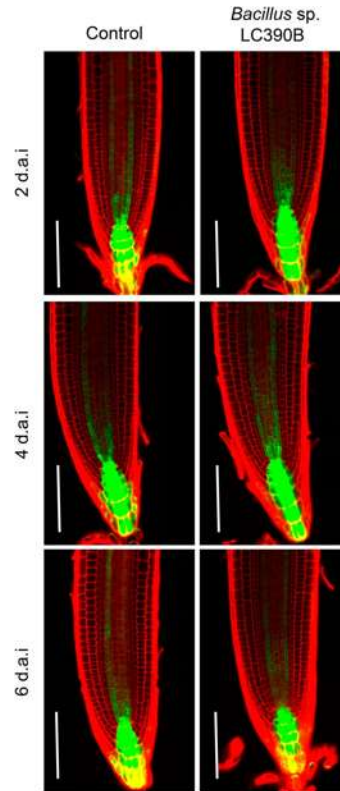


Fig. 5 Auxin inducible marker *DR5::GFP* expression pattern in the root apical meristem. Basal expression of *DR5::GFP* is observed from the quiescent center (QC) to the columella and protoxylem tissue in non-inoculated seedlings. Note the bacterial inoculation doesn't change auxin redistribution in the RAM throughout the interaction period, 6 d.a.i. Scale bar: 100 μm , ($n = 18$)

more lateral roots after bacterization, which was reflected in lateral root number and density (Fig. 7b, c). Together, these results indicate that specific, but not all components in auxin signaling are involved in the growth of the primary and lateral roots stimulated by *Bacillus sp.* LC390B.

Bacillus sp. LC390B Cocultivation Improves Plant Growth in Sugar Deprived Medium

Previous reports evidenced the dual role of sucrose in metabolism and signaling programs, modifying plant architecture (Aguilera-Alvarado and Sánchez-Nieto 2017).

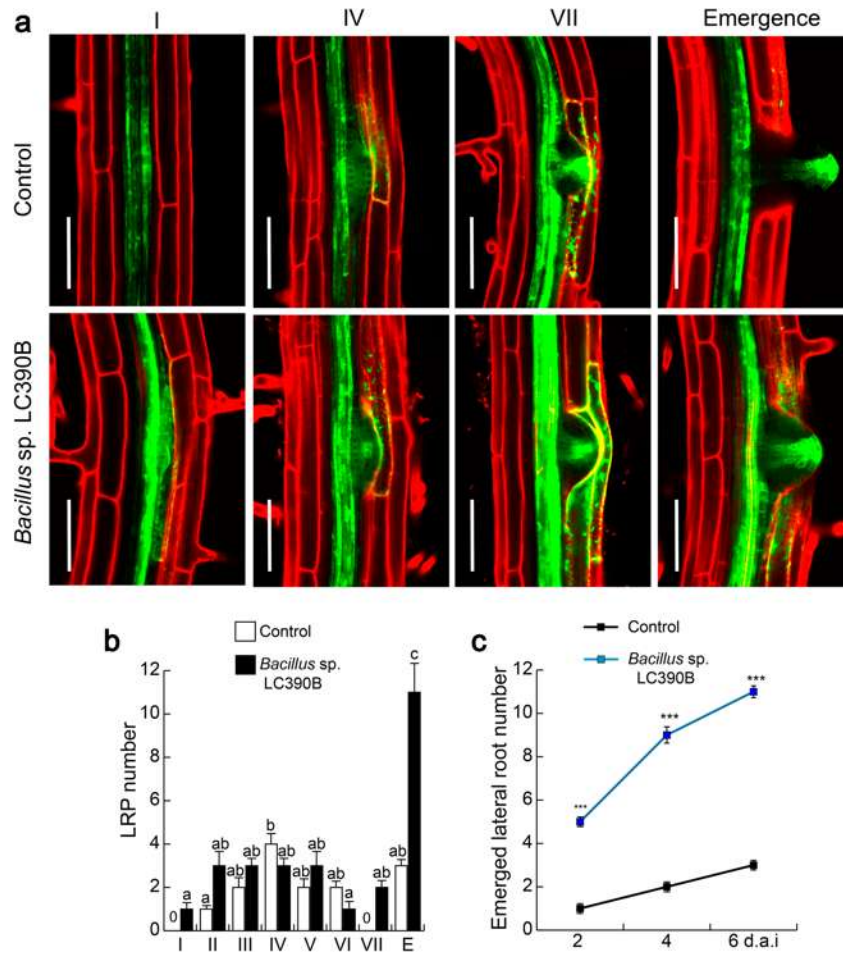


Fig. 6 Lateral root primordia developmental stages monitored via *DR5::GFP* expression. **a** *DR5::GFP* expression patterns. **b** Lateral root primordia number 2 d.a.i. **c** Emergent lateral root number. Scale bar: 100 μ m, ($n=18$). Different letters are used to indicate means that statistically differ through Tukey post hoc analysis with $p<0.05$ significance

In the previous experiments, the basal content of sucrose as a carbon source in the $0.2\times$ MS medium was adjusted to 0.6%, which supports early growth of *Arabidopsis* seedlings. The question was raised if under sugar unavailability, the bacterial phytostimulation could be also manifested or abrogated. To answer this question, the interaction of seedlings with *Bacillus* sp. LC390B was assessed in medium lacking sucrose or supplied with 0.6% sucrose. In sucrose deficient medium, non-inoculated seedlings grew

slowly showing delayed leaf formation and slow primary root growth and branching compared to plants grown in medium supplemented with 0.6% sucrose (Fig. 8a–d). Noteworthy, the bacterial interaction in medium devoid of sucrose could enhance the primary root elongation, root branching, biomass and chlorophyll content to the levels found in uninoculated seedlings supplied with sucrose (Fig. 8e–j). These results suggest that the bacterium is

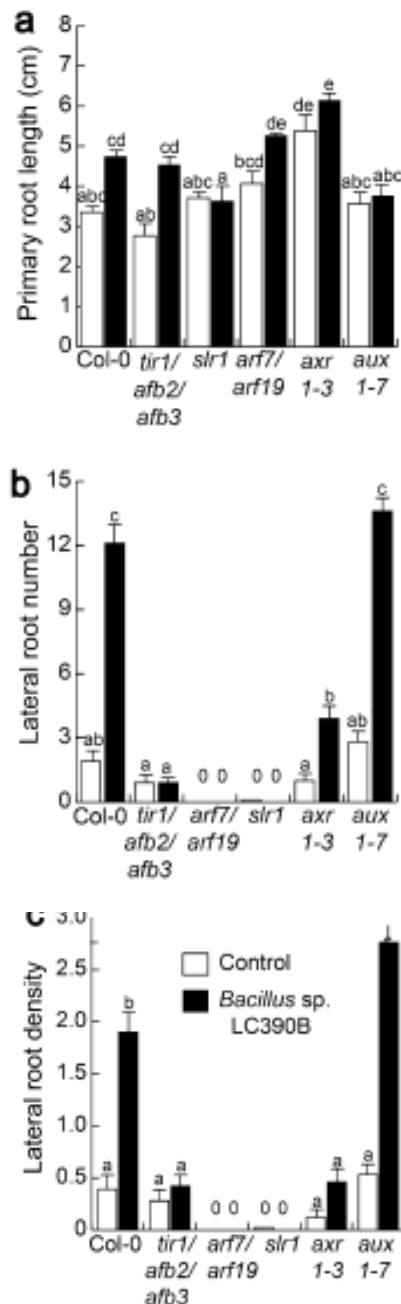


Fig. 7 Growth and development of auxin signaling mutants in response to *Bacillus* sp. LC390B inoculation. **a** Primary root length. **b** Lateral root number. **c** Lateral root density, ($n=18$). Different letters are used to indicate means that statistically differ through Tukey post hoc analysis with $p < 0.05$ significance

a promising biostimulant for plants under compromised carbon availability.

Bacillus sp. LC390B Driven Phytostimulation Is Disrupted in a *phyA/phyB* Double Mutant

Light perception is fundamental for plant morphogenesis. Seed germination, hypocotyl elongation, flowering and fruiting are critically influenced by light sensing proteins including the phytochromes (Smith 2000; Li et al. 2011). The *PHYA* and *PHYB* phytochromes act as photoreceptors perceiving specific light wavelengths and transduce the light signaling into cellular responses. Following the previous result where *Bacillus* sp. LC390B enhanced plant growth and chlorophyll content in medium devoid of sucrose, we examined the possible role of the phytochromes *PHYA/PHYB* by comparing the growth of the WT and the double mutant *phyA/phyB* side by side in medium supplemented with 0.6% sucrose. In axenic conditions, the phytochrome double mutant showed enhanced hypocotyl elongation and primary root shortening, and surprisingly, the bacterial treatment could neither restore, nor promote plant growth in the double mutant compared with the wild-type (Fig. 9a, b). The inoculation did not promote root and fresh weight and chlorophyll content in the double mutants (Fig. 9c, d, h), although, we could note that *Bacillus* increased 30% the primary root length and about 25% or 50% lateral root number and density in the mutants compared to the biostimulation attained in the WT (Fig. 9e–g). These data indicate that light perception via *PHYA* and *PHYB* is crucial for plant growth promotion by a beneficial bacterium.

The Growth of *Bacillus* sp. LC390B Is Maintained In Cocultivation with Plants

The *Arabidopsis*–*Bacillus* sp. LC390B interaction started after placing the plant roots over a horizontal bacterial streak. To determine the viability of the initial inoculum and how it was maintained across the time in the experiments, we determined optical density from bacterial streaks during 72 h monitoring bacterial growth and in $0.2 \times$ MS solid media during seven days with and without *Arabidopsis* seedlings. Determinations of optical density every 4 h indicate that the initial inoculum is viable (Fig. 10a). Moreover, in axenic LB medium without plants after seven days, the bacteria increased growth by twofold compared with the initial inoculum. However, regarding both optical density and colony forming units measurements, the bacterial biomass was maintained across the experiments in plates with and without *Arabidopsis* seedlings in $0.2 \times$ MS medium after seven days (Fig. 10b, c). These results indicate that the inoculum remained viable across the experiment and roots had neither positive nor negative influence over bacterial growth.

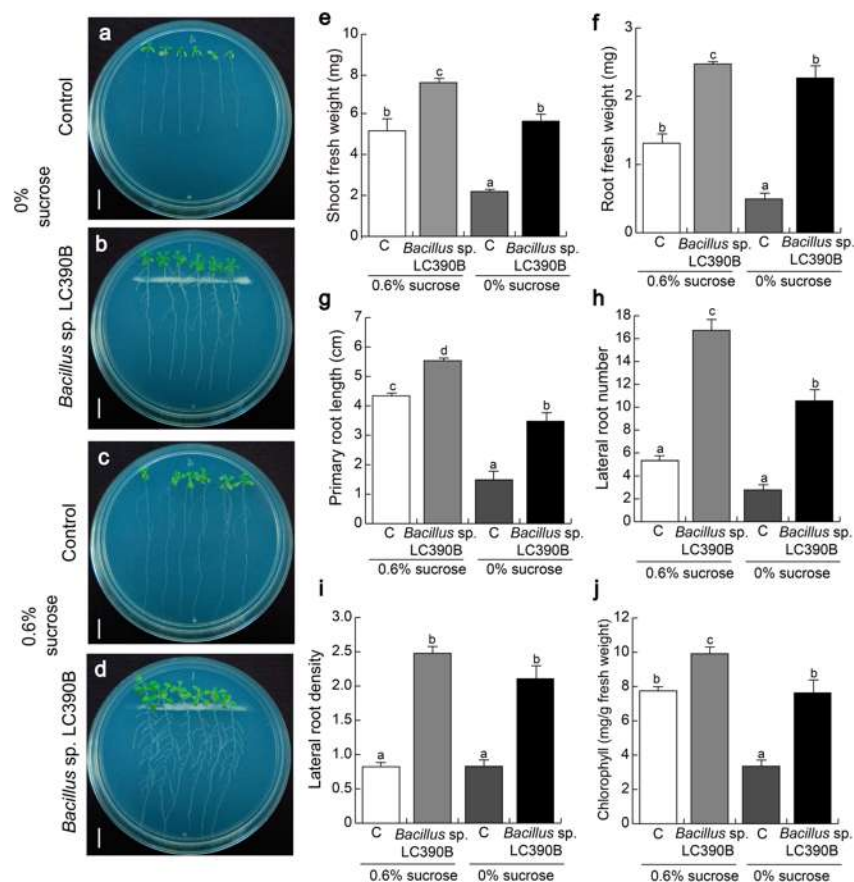


Fig. 8 *Bacillus sp. LC390B* promotes plant growth in medium lacking sugar as a carbon source. **a–d** Representative images of 10 days old *Arabidopsis* seedlings and a comparative analysis of plant architecture and biomass with and without sucrose with *Bacillus sp. LC390B* were performed. **e** Shoot fresh weight. **f** Root fresh weight.

g Primary root length. **h** Lateral root number. **i** Lateral root density. **j** Chlorophyll content. Scale bar: 1 cm, ($n=18$). Different letters are used to indicate means that statistically differ through Tukey post hoc analysis with $p<0.05$ significance

Discussion

The *Bacillus* genus comprises a large number of species that establish beneficial relations with plants with important biotechnological applications (Radhakrishnan et al. 2017). Nevertheless, the mechanisms by which these bacteria affect shoot or root development have been scarcely investigated and merit attention owing their contribution to overall plant biomass production. The present report provides new insights into the plant growth promotion elicited by the newly identified *Bacillus sp. LC390B* strain, isolated from

the rhizosphere of maize plants grown in an alkaline soil. Comparison of 16S rRNA sequence of LC390B strain placed it in close proximity to *B. cereus*, which has been previously reported to confer heavy metal and salt tolerance to plants, to aid in phosphate solubilization and to produce auxin (Hassan et al. 2018; Akhtar et al. 2021; Xu et al. 2021).

Our findings demonstrate that *Bacillus sp. LC390B* is a beneficial bacterium with a number of interesting effects in *Arabidopsis* grown in vitro and in an artificial soil. Direct root bacterization in Petri plates boosted shoot and root fresh weights, enhanced primary root growth and branching and

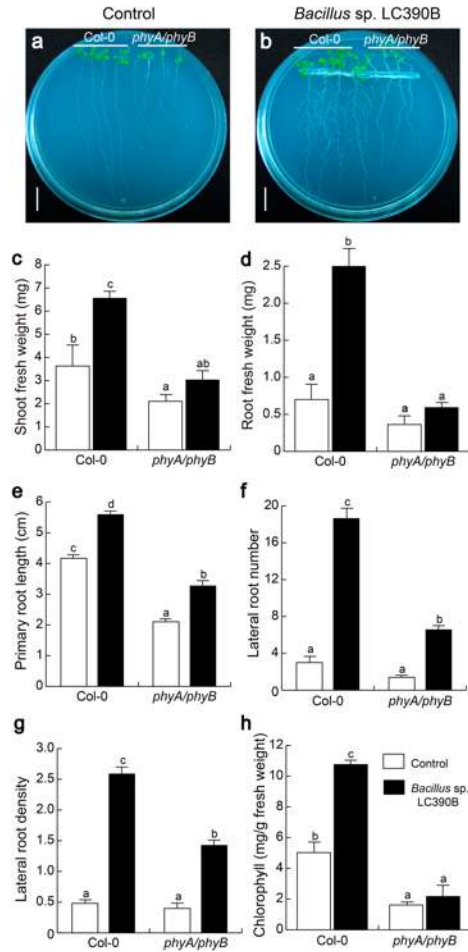


Fig. 9 *Bacillus* sp. LC390B phytostimulation is affected in *phyA/phyB* double mutants. **a, b** *Arabidopsis* wild-type Col-0 and *phyA/phyB* double mutants were transferred to 0.2×MS media with and without LC390B streaks. Plant growth parameters were measured after bacterization. **c** Shoot fresh weight. **d** Root fresh weight. **e** Primary root length. **f** Lateral root number. **g** Lateral root density. **h** Chlorophyll content. Scale bar: 1 cm, ($n=18$). Different letters mean statistical differences through Tukey post hoc analysis with $p<0.05$ significance

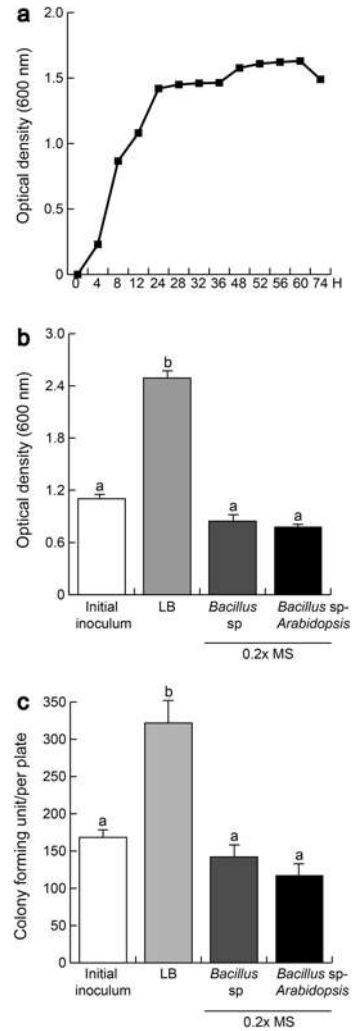


Fig. 10 Bacterial growth curve and performance of the inoculum. **a** *Bacillus* sp. LC390B initial inoculum at growth phases OD₆₀₀. **b** Mean OD₆₀₀ seven days after initial inoculum establishment in LB solid media, or 0.2×MS medium with or without *Arabidopsis* seedlings. **c** Mean colony forming unit/per plate as above described. Experiments were repeated three times with comparable results. Different letters mean statistical differences obtained through Tukey post hoc analysis with $p<0.05$ significance

increased fruit production in plants transferred to plastic boxes filled with an artificial soil substrate, highlighting the potential of the bacterium to support plant growth and biomass production through the life cycle of plants. This is an unusual phenotype manifested by *Arabidopsis* seedlings upon root contact with a bacterial streak, since most reported bacteria rather repress primary root growth, halt the apical dominance of the main root axis and trigger the formation of more branched root systems by means of auxin production. *Azospirillum brasilense* and *Micrococcus luteus* strongly repress *Arabidopsis* primary root growth through their production of indole-3-acetic-acid (IAA), the most abundant, natural auxin of plants, or through an exacerbated auxin response that drives the massive production of lateral roots and root hairs (Méndez-Gómez et al. 2020; García-Cárdenas et al. 2021).

It appears that the *Bacillus* sp. LC390B is not an auxin producing strain because it increased primary root length and cell elongation without affecting cell division, in contrast to bacterially produced auxins that normally repress primary root growth and halt mitosis (Méndez-Gómez et al. 2020; García-Cárdenas et al. 2021). Coincidentally, the expression domain of *DR5::GFP* at the root tip, did not change in plants whose roots remained in contact with the bacterial streak up to six days. These findings did not exclude that some components of the auxin machinery may be critical for the root organogenesis programme elicited by the bacterium, since a gain-of-function in the *slr1* mutant or the loss of the auxin intake permease AUX1 in the *aux1-7* mutant caused resistance to the primary root elongation effect driven by LC390B. The important role of AUX1 in the primary root elongation can be explained by a possible crosstalk with cytokinins, where it functions as a central modulator of root cell elongation (Street et al. 2016). Thus, it may be of interest to analyze if the bacterium triggers cell elongation and primary root growth via cytokinin signaling.

A recent study by Jiménez-Vázquez and coworkers (2020) detailed how the plant promoting bacterium *Achromobacter* sp. SB1 modulates primary root coiling through auxin transport, altering both efflux protein *PIN2* and influx mediator *AUX1-7*, whose mutants had exacerbate root coiling upon bacterial streak contact caused by deviation of auxin towards the concave side of the primary root tip (Jiménez-Vázquez et al. 2020). However, in the present research, the auxinic footprint in the primary root tip did not change by inoculation with *Bacillus* LC390B, and the bacterium failed to induce root waving and coiling, thus both processes are influenced by each bacterium in highly contrasting manners.

Sugars are both nutritional molecules, metabolic switches and signaling factors that in crosstalk with auxins control root growth. Sucrose supplementation to *Arabidopsis* seedlings grown in vitro increased primary root elongation and root branching (Freixes et al. 2002; Raya-González et al.

2017). Additionally, glucose modulates auxin-related gene expression in root developmental processes (Mishra et al. 2009, 2021). Other bioactive microbial molecules may affect cell elongation such as the *N*-acyl-L-homoserine lactones (AHLs) from Gram negative bacteria. Zhao et al. (2015) obtained evidence that *N*-3-oxo hexanoyl homoserine lactone improved primary root elongation in *Arabidopsis* (Zhao et al. 2015). Another possible driver of primary root elongation is microbially produced ACC deaminase, which degrades the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and thus diminishes ethylene levels (Thien et al. 2019).

Local homeostasis of auxin may be important for root architectural reconfiguration in plant cross-kingdom communication with bacteria. The promotion of shoot and root growth elicited by *Bacillus* sp. LC390B did not correlate with overall enhancement of the auxin response reported by *DR5::GFP*. However, auxin appears to be important for the induction of root branching in bacterized seedlings, which produced robust root systems through accelerating the emergence of preformed lateral root primordia. In this process, *DR5*-driven fluorescence increased within the developing lateral root primordia prior to their emergence from the primary root. MacGregor et al. (2008) demonstrated that sucrose availability is critical for lateral root formation and this may occur through induction of auxin biosynthesis. Comparable effects have been recently found in *Arabidopsis* seedlings exposed to volatiles emitted by *B. amyloliquefaciens*, which formed more primordia and accelerated their emergence (Li et al. 2021), the responsible molecules await identification.

In our research, auxin signaling components were involved in lateral root formation, since the *tir1/afb2/afb3*, *slr1*, and *arf7/arf19* mutants were defective in root branching in interaction with *Bacillus* sp. LC390B, suggesting their involvement in the root organogenesis process influenced by the bacteria in specific tissues, but not in whole plants. In addition, the bacterium could enhance fresh weight and chlorophyll content of plants under sucrose deprivation, which may occur through photosynthesis. Several microorganisms produce metabolites capable to manipulate plant sugar metabolism increasing photosynthesis, chlorophyll content and carbohydrate partitioning, leading to biostimulation in crosstalk with phytohormones (Zhang et al. 2008; Costa-Santos et al. 2021). The coordination between plant architecture, biostimulation and carbon status through an enhanced photosynthesis, reinforces the notion that the use of beneficial bacteria may improve plant growth in poor soils and take up of water and minerals through better explorations of the substrate and higher branching capacity.

There are distinct types of phytochromes and at least five genes have been identified in the *Arabidopsis* genome including *PHYA* and *PHYB* (Clack et al. 1994). The reason

of using *phyA/phyB* double mutants instead of the single mutants separately, or additional photoreceptor mutants was to address the question of whether the plant growth promotion by *Bacillus* sp. LC390B can involve a phytochrome other than PHYA or PHYB, which play a critical role in light perception, photosynthesis and organogenesis (Losi and Gärtner 2021). To our knowledge, whether plant photoreceptors could mediate plant growth in interactions with rhizobacteria had been scarcely investigated.

Our results with *Bacillus* sp. LC390B showed that inoculated seedlings affected in *phyA/phyB* did not manifest the increase in plant growth and chlorophyll content as seen in the wild-type. The phytochromes possess kinase activity, modulating in this manner the activity of transcriptional factors that bind to light-responsive elements in promoter regions activating the transcription related to photosynthesis, carotenoid and chlorophyll biosynthesis during photomorphogenesis (Quián-Ulloa and Stange 2021). The knockout *phyA/phyB* showed a 50% reduction on chlorophyll levels, which correlates with the fact that phytochromes are essential signaling molecules for photosynthetic pigment synthesis. In rice plants (*Oryza sativa*), *PHYB* is the main component modulating the chlorophyll synthesis (Inagaki et al. 2015). Bacterization could not reestablish optimal chlorophyll levels in the *phyA/phyB* double mutant, and the biostimulation was compromised regarding both shoot and root fresh production, hence, the bacterial potential to improve plant growth in *Arabidopsis* requires the phytochromes PHYA and PHYB, and this may be related to their critical functions in chlorophyll biosynthesis, hormone signaling and root development. For instance, PHYA and PHYB promote root elongation under different light regimes and both photoreceptors contribute to lateral root and root hair formation in *Arabidopsis* (Warnasooriya and Montgomery 2011). In natural systems, bacteria are likely to encounter a wide range of sources in a complex rhizosphere, which may depend on root exudation and branching patterns, ultimately influenced by the phytochromes. This suggests that the promoting effect triggered by *Bacillus* sp. LC390B relies not only in the photosynthesis, but also in the light sensing mechanism. Collectively, our data provide detailed evidence of the probiotic attributes of a *Bacillus* species in a model plant, translational biology would lead to novel and interesting approaches to manage plants in the field by reinforcing their rhizosphere with the LC390B strain.

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analysis tools; EGC, JLB wrote the manuscript. All authors read and approved the final draft.

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Availability of Data and Material The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interest The authors have no conflict of interest or competing interests to declare.

Code Availability (Software Application or Custom Code) Not applicable.

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Capítulo III

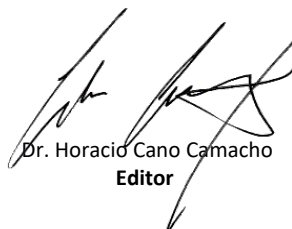


Saber Más: Revista de Divulgación
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Estimados Elizabeth García-Cárdenas y León Francisco Ruíz-Herrera:

Nos resulta muy grato comunicarles que el comité editorial de la **Revista Saber Más** ha tomado la decisión de **ACEPTAR** el artículo de divulgación científica **“El potencial de la microbiota vegetal para alimentar al mundo”** que será publicado en un próximo número de Saber Más. Agradecemos su colaboración y les invitamos a seguir colaborando, así como a ayudarnos a difundir Saber Más.



Dr. Horacio Cano Camacho
Editor

REVISTA DE DIVULGACIÓN Saber Más, ES UNA REVISTA SIN FINES DE LUCRO. LAS EXPRESIONES SON RESPONSABILIDAD DE LOS AUTORES. **COMITÉ EDITORIAL:** DR. RAFAEL SALGADO GARCIGLIA (DIRECTOR), DR. HORACIO CANO CAMACHO (EDITOR), DR. MARCO ANTONIO LANDAVAZO ARIAS, DR. CEDERIK LEÓN DE LEÓN ACUÑA, DRA. EK DEL VAL DE GORTARI, M.C. ANA CLAUDIA NEPOTE GONZÁLEZ, DR. LUIS MANUEL VILLASEÑOR CENDEJAS Y DR. JUAN CARLOS ARTEAGA VELÁZQUEZ.

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A QUIEN CORRESPONDA

Por este medio se extiende la **CONSTANCIA DE PUBLICACIÓN** del **ARTÍCULO DE DIVULGACIÓN CIENTÍFICA** titulado:

“EL POTENCIAL DE LA MICROBIOTA VEGETAL PARA ALIMENTAR AL MUNDO” ARTÍCULO DE PORTADA

Siendo autores **Elizabeth García-Cárdenas y León Francisco Ruíz-Herrera**, a quienes se les conservan y respetan sus derechos intelectuales de autor, y son responsables del contenido del **artículo de portada**, publicado en el **Número 65 (Año 11:34-39)** de la revista de divulgación **SABER MÁS**, con fecha de publicación del **3 de noviembre de 2022** (Registro ISSN: 20077041).

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Se extiende la presente **CONSTANCIA** para los fines legales que convengan a los autores correspondientes.



Dr. Horacio Cano Camacho
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ARTÍCULO DE PORTADA

El potencial de la microbiota vegetal para alimentar al mundo

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sabermás

La antesala de un desastre

Actualmente, el **cambio climático global** es un factor que ha modificado rápidamente las condiciones de vida de todos los organismos del planeta; es un fenómeno ocasionado por la **acumulación de distintos gases en la atmósfera** que retienen el calor y, en consecuencia, modifican la temperatura de los ecosistemas. Esta situación es alarmante, principalmente porque el incremento de estos gases (dióxido de carbono, óxido nitroso, metano y otros compuestos industriales) está relacionado al aumento de la población humana y sus actividades.

Un evento preocupante es el incremento de las zonas áridas o desertificación que amenaza la productividad agrícola y, por tanto, la disponibilidad de alimentos para una población mundial en constante crecimiento. En este escenario tan preocupante, **¿qué tipo de soluciones podrían aplicarse para mejorar la adaptabilidad y productividad de los cultivos?**

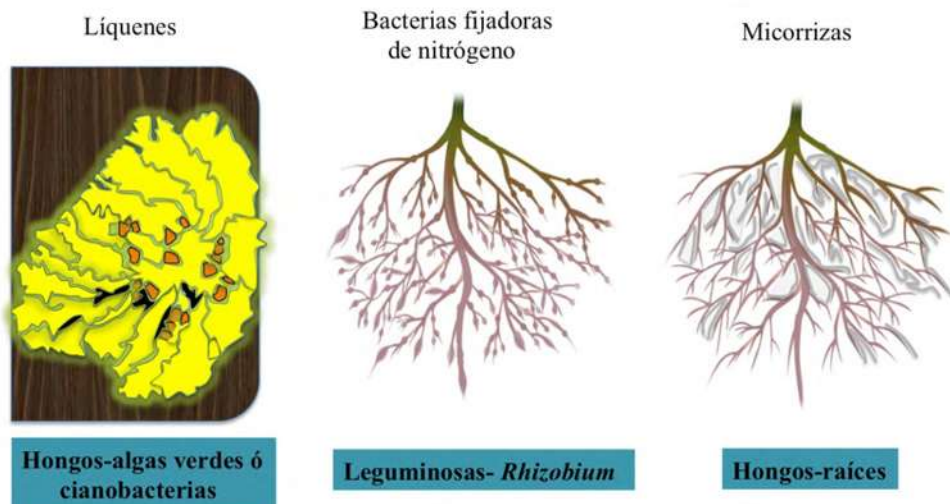
Imaginemos las condiciones ambientales de una zona hostil para la vegetación como lo es un desierto: altas temperaturas, deficiencia de agua, suelos arenosos, salinos y alcalinos. Considerando en conjunto estos factores que limitan las formas de vida, es sorprendente ver la presencia de plantas

en estos lugares, ¿cómo logran sobrevivir?, ¿cómo obtienen los nutrientes? La respuesta puede ser la solución ante el inminente cambio climático global.

La importancia de la microbiota vegetal

Las plantas poseen características distintas de acuerdo al tipo de ambiente en el que se desarrollan. En un desierto encontraremos que las plantas desarrollan hojas con forma de espinas, reducción del tamaño de sus órganos, desarrollo de raíces muy largas y actividad de fijación de dióxido de carbono al alba y al atardecer para disminuir la pérdida de agua; sin embargo, la clave de su supervivencia va más allá de lo que se puede ver a simple vista... ¡Está en el suelo!

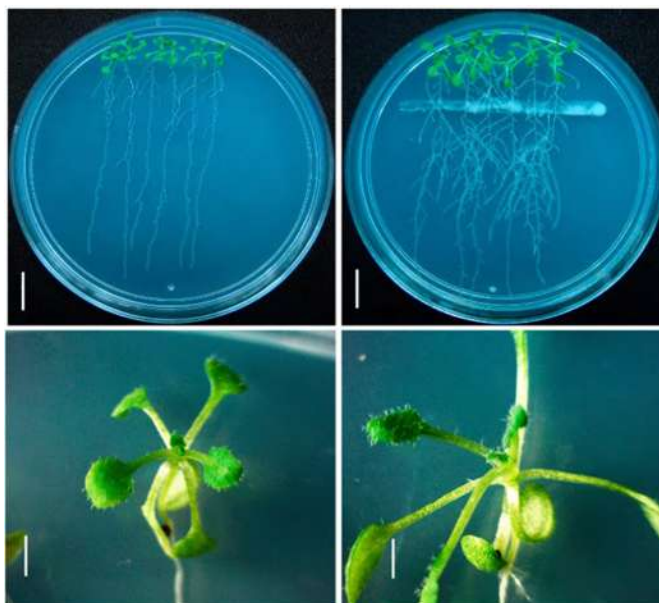
Dentro y fuera de los tejidos de las plantas podemos encontrar una gran diversidad de microorganismos, pero los que residen en las raíces son los más abundantes. Hay una estrecha **asociación de las raíces con los microorganismos que habitan el suelo**, entre ellos se pueden identificar bacterias, hongos y protistas, tanto benéficos como patógenos, los cuales conforman la «microbiota». El ser humano, las plantas y todo tipo de organismos multicelulares dependen de su microbiota para sobrevivir, conformando asociaciones simbióticas.



Fotografía de E. García Cárdenas.

La simbiosis como clave para la supervivencia

Una simbiosis es una interacción muy estrecha que ocurre entre poblaciones de especies distintas, en las que el huésped y su simbionte obtienen un beneficio que es imprescindible para su supervivencia. En las asociaciones simbióticas mutualistas ambos se benefician, por ejemplo, entre plantas y microorganismos benéficos podemos mencionar a los líquenes (hongos-algas), la fijación de nitrógeno en leguminosas por *Rhizobium* (raíces-bacterias) y a las micorrizas (raíces-hongos).



Efecto bioestimulante en plantas de *Arabidopsis thaliana* inoculadas con *Bacillus* sp. in vitro

Fotografía de E. García Cárdenas.

¿Qué hace la microbiota benéfica para estimular el crecimiento de la planta?

En la naturaleza, las asociaciones simbióticas mutualistas de las micorrizas y las rizobacterias, solubilizan nutrientes produciendo compuestos muy diversos y facilitando su captación por las plantas, a su vez, las plantas les proporcionan carbono orgánico en forma de carbohidratos, aminoácidos y otros compuestos orgánicos nutritivos a través de los exudados de las raíces. Numerosos estudios han demostrado que estos exudados actúan como señales químicas que funcionan como atrayentes de grupos específicos de bacterias benéficas, moldeando así su propia microbiota. Por ejemplo, las leguminosas exudan flavonoides para atraer bacterias del grupo de *Rhizobium*, al ser percibidos, *Rhizobium* expresa genes del factor NodD, que son los responsables del proceso de nodulación para iniciar la internalización de la bacteria en las raíces y fijar el nitrógeno atmosférico.

La superficie de suelos para la agricultura en el mundo es muy limitada

Los principales cultivos en el mundo son trigo, maíz y arroz, pero no todos los suelos son adecuados para cultivarlos. En América Latina, **solo siete países cuentan con suelos idóneos para la agricultura**; México se encuentra en segundo lugar

después de Argentina, por esta razón, es indispensable hacer un buen uso de esos suelos para cubrir la demanda de alimentos. Mundialmente, los suelos presentan deficiencias nutrimentales que limitan la productividad agrícola, siendo los principales nutrientes carentes el nitrato, el amonio, el hierro y el fosfato, por esta razón, los fertilizantes los contienen y se aplican en grandes cantidades. Así, el impacto por el uso prolongado y abundante de agroquímicos, ha puesto en la balanza la productividad agrícola contra la integridad de los recursos naturales y la diversidad microbiana de los suelos.

Debido a esta problemática, las soluciones van encaminadas al conocimiento de las interacciones planta-microorganismo en su ambiente natural para utilizar su potencial en las prácticas agrícolas. La facilitación de los cuatro macronutrientes esenciales mencionados, ocurre por la actividad microbiana de la siguiente manera:

- a) *Rhizobium* forma nódulos en las raíces de leguminosas para transformar el nitrógeno atmosférico en **nitrato y amonio**. Otras bacterias como *Azospirillum*, *Frankia* y *Azotobacter* fijan el nitrógeno sin internalizarse en la planta.
- b) La mayoría de las bacterias y hongos captan

el **hierro**, un elemento limitante para las plantas a través de la producción de sideróforos, compuestos orgánicos de bajo peso molecular de alta afinidad al hierro.

a) Para obtener **fosfato**, producen enzimas llamadas «fosfatasa» que lo liberan cuando está unido a otras moléculas o es solubilizado por ácidos orgánicos que acidifican el suelo, haciéndolo disponible. *Pseudomonas* sp., *Bacillus* sp. y las micorrizas, son buenos candidatos solubilizadores de este nutriente.

La aplicación de fertilizantes tiene limitaciones para continuar su uso debido al exceso de nutrientes que contienen, los cuales no son asimilados eficientemente por las plantas, lo que provoca con el tiempo la contaminación de los suelos y cuerpos de agua.

Herramientas de defensa

La microbiota del suelo es también responsable de **prevenir enfermedades y de mejorar el sistema inmune de las plantas**. La epidermis, tricomas, cutícula cerosa, pared celular y corteza son algunas barreras de protección que exhiben las plantas. Pueden detectar sustancias o componentes microbianos tóxicos (flagelina, quitina) a través de receptores, desencadenando una respuesta de

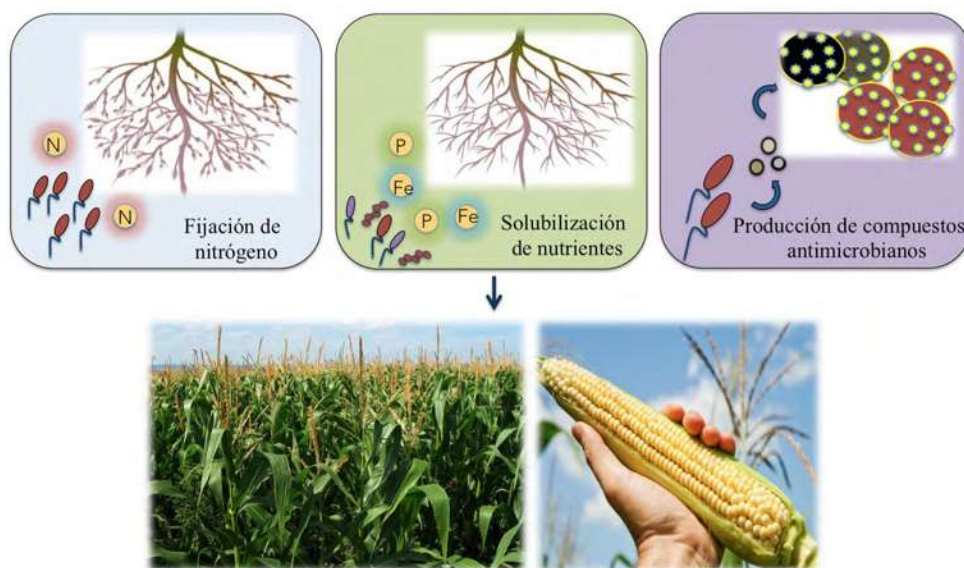
señalización celular para producir compuestos antimicrobianos y fortalecer su sistema inmune; sin embargo, la interacción con microorganismos benéficos les confiere una ventaja extra, ¡les permite activar su inmunidad y estar alerta antes de ser atacadas estimulando su defensa!

Los exudados de las raíces permiten reclutar microorganismos específicos que atacan a los patógenos a través de la producción de antibióticos y otros compuestos antimicrobianos. La planta de jitomate, por ejemplo, produce ácido láctico y ácido hexanoico para atraer *Bacillus cereus* y reducir el daño por la bacteria *Ralstonia solanacearum* que causa la marchitez.

Con base en estas características que posee la microbiota vegetal en los procesos de nutrición y defensa, se han aislado y estudiado microorganismos sobresalientes para aplicarse como *biofertilizantes en distintos cultivos*. Ejemplo de ello son las micorrizas del grupo Glomeromycota, las bacterias *Rhizobium* sp, *Azospirillum* sp, *Pseudomonas* sp, *Bacillus* sp y hongos del género *Trichoderma* sp.

Un futuro alentador

Los agroquímicos han mejorado el rendimiento agrícola con consecuencias negativas al ambiente a lo largo de su implementación, por lo



Propiedades microbianas que estimulan el crecimiento y defensa de las plantas

Fotografía de E. García Cárdenas.

que se tienen que considerar otras soluciones prácticas viables para reducirlos o sustituirlos. **Los biofertilizantes son excelentes herramientas de bajo costo y de mínimo impacto ambiental**, contienen agregados de microorganismos vivos que mejoran y estimulan la nutrición e inmunidad en las plantas. Por estas propiedades, necesitan ser explorados ampliamente para utilizarse como prioridad en el campo. Las **limitaciones del uso de biofertilizantes** radican en la composición del suelo, el pH, la salinidad, el tipo de cultivo y su composición microbiana, los cuales pueden afectar considerablemente su adecuado establecimiento y colonización en el suelo y la planta, disminuyendo su efectividad, razón por la cual su uso no ha logrado reemplazar totalmente la fertilización.

Reforzar la investigación para abarcar todas las variables que comprometan su eficiencia, resultará en **una nueva revolución verde** que cubra la demanda de alimento y conduzca a la preservación de los recursos naturales.

Ahora, cada vez que veas una planta crecer en ambientes desérticos, muy fríos, en suelos muy salinos y ácidos, sabrás que no están confrontando esas condiciones solas, es seguro que su microbiota es la responsable de ayudarlas a sobrevivir.

La naturaleza tiene todas las respuestas, adentrarse a descubrirlas y aplicarlos en la vida cotidiana es la forma más divertida e interesante del aprendizaje.

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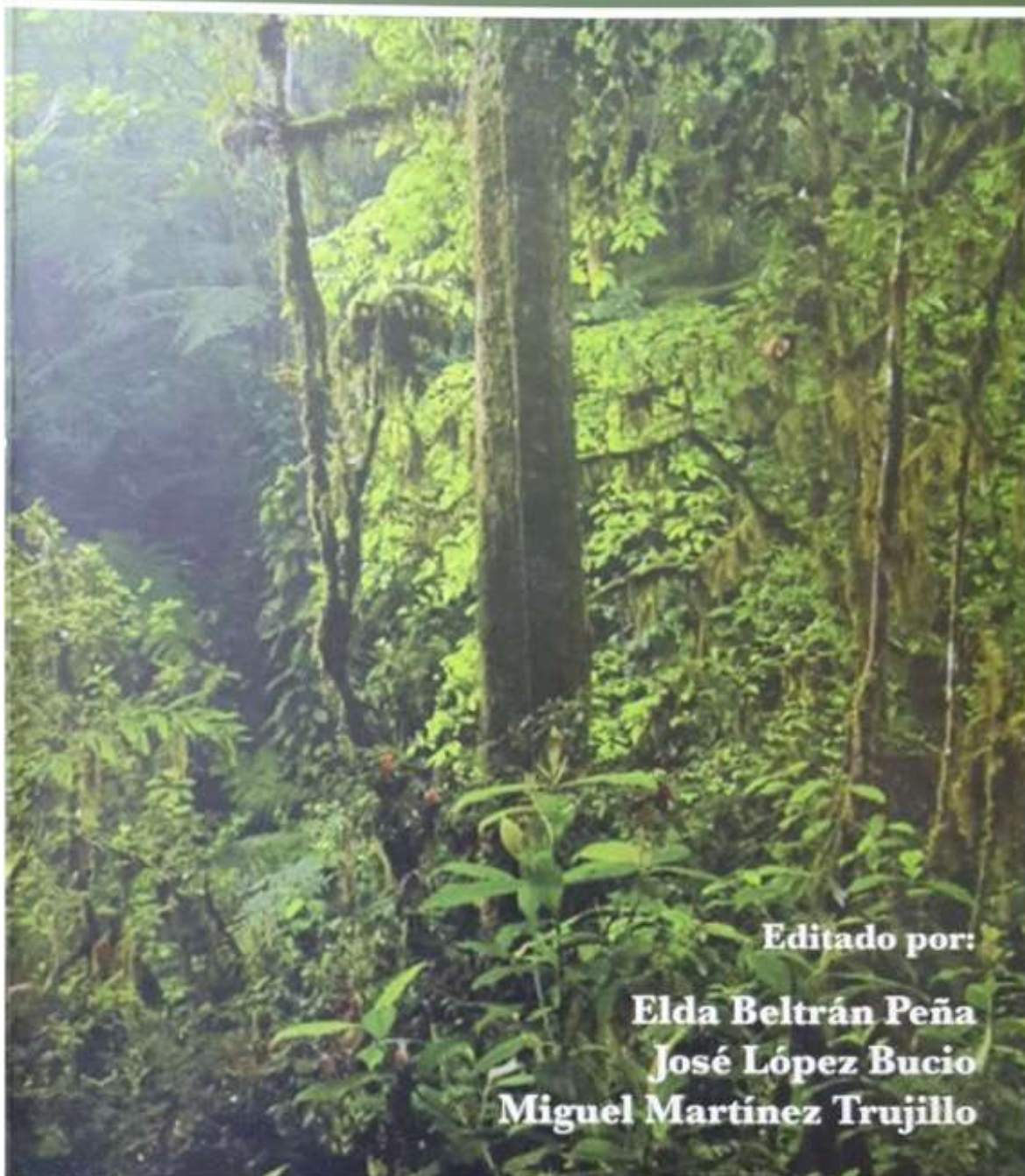


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Capítulo IV

FRONTERAS EN LA BIOLOGÍA:

SEÑALIZACIÓN Y COMUNICACIÓN DE LAS PLANTAS



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Las auxinas: hormonas que coordinan el crecimiento y desarrollo de las plantas y su interacción con el ambiente

Elizabeth García Cárdenas, Edith Muñoz Parra, José López Bucio y Elda Beltrán Peña

Las plantas son organismos multicelulares sésiles que responden a estímulos ambientales con una gran plasticidad fenotípica, modificando así la arquitectura de sus raíces y del follaje para optimizar los recursos disponibles en su ambiente. Las auxinas son los reguladores del crecimiento vegetal más estudiados, debido a que controlan las diferentes etapas del ciclo de vida incluyendo la embriogénesis, los tropismos, la organogénesis de la raíz y del follaje, la resistencia al ataque de patógenos, así como la senescencia. La auxina natural más abundante es el ácido indol-3-acético (AIA), molécula que se produce en los brotes, en los meristemos y en los ápices de las hojas y se transporta a diferentes tejidos por proteínas acarreadoras localizadas en la membrana plasmática. En este artículo se discute la información más relevante sobre los procesos del crecimiento y desarrollo de las plantas en los que participan las auxinas, así como sus mecanismos de transporte e interacciones a nivel molecular.

Introducción

Las plantas son organismos autótrofos, es decir, fabrican sus propios alimentos a partir de la luz del sol, agua y nutrientes minerales que toman del suelo. Aunque tradicionalmente se les ha diferenciado de los animales por su naturaleza sésil, cada vez resulta más evidente su enorme capacidad para adaptarse a las fluctuaciones en las condiciones ambientales cambiando su morfología. Esta propiedad se conoce como plasticidad fenotípica, e incluye la recepción, decodificación e integración de señales que provienen de factores abióticos y bióticos como la intensidad lumínica, la disponibilidad de agua y nutrientes, los cambios en la temperatura, las condiciones fisicoquímicas del suelo y la presencia de microorganismos (Vanneste y Friml, 2013; Satbhal *et al.*, 2015). Diversos compuestos también conocidos como fitohormonas, entre los que se incluye a las citocininas, los brasinoesteroides, el etileno, el ácido salicílico, el ácido abscísico, las estrigolactonas, el ácido jasmónico, las giberelinas y las auxinas coordinan las respuestas de los tejidos a las señales del ambiente con los procesos de crecimiento y desarrollo subyacentes (Depuydt y Hardtke, 2011). Las auxinas desempeñan un papel preponderante en la organogénesis (que va desde la germinación hasta la senescencia), ya que promueven la formación de las raíces laterales, el mantenimiento de los meristemas apical y radicular, la formación de yemas axilares y de los tejidos vasculares, los tropismos y el desarrollo del fruto. El concepto clásico de regulación hormonal implica que una molécula se sintetiza en un órgano desde donde se moviliza a otros tejidos dentro del organismo, estimulando una respuesta celular. En el caso de las auxinas, éstas se sintetizan en los meristemas y hojas jóvenes, y se transportan a zonas adyacentes o distantes mediante proteínas presentes en las membranas de las células.

La respuesta celular a las auxinas ocurre mediante su percepción por un grupo de proteínas que actúan como receptores, que están localizadas en el núcleo de las células. Sí, en el núcleo, este hallazgo fue sorprendente porque en la mayor parte de los organismos, los receptores de señales típicamente se localizan en la membrana plasmática. Los receptores de auxinas forman parte de un complejo cuya función consiste en modificar a otras proteínas (las Aux/IAA, represoras de los factores de transcripción ARF) mediante la adición de residuos de ubiquitina; este marcaje permite un recambio activo al degradarse las Aux/IAA por el proteosoma, un complejo enzimático ubicado en eucariontes. Particularmente relevante es el recambio de los factores de transcripción que determinan el fenotipo, las respuestas ambientales y la expresión de genes. Cuando los factores de

transcripción ARF quedan libres, se unen a los promotores de los genes cuyos productos regulan el crecimiento y desarrollo vegetal, modulando la división, elongación y diferenciación (Ortiz-Castro *et al.*, 2009).

Las auxinas naturales principales son el ácido indol-3-acético (AIA) y el ácido indol-3-butírico (AIB) (Korasick *et al.*, 2013). También existen diversos compuestos sintéticos con actividad auxínica que por sus propiedades se han usado tradicionalmente como herbicidas en la agricultura y horticultura, algunos ejemplos son el ácido 2,4-diclorofenoxiacético (2,4-D), el ácido 2,4,5-triclorofenoxiacético (2,4,5-T), el ácido 4-amino-3,5,6-tricloro-2-picolínico (picloram) y el ácido 3,6-dicloro-2-metoxibenzoico (dicamba) (Ljung, 2013). Las aplicaciones inherentes del conocimiento sobre el funcionamiento de las auxinas van desde la posibilidad de cultivar plantas o algunas de sus partes *in vitro*, hasta la formulación de agroquímicos con impacto en el manejo de los cultivos.

1. Homeostasis de las auxinas: biosíntesis, conjugación y degradación

Las auxinas son moléculas pequeñas y móviles, que se acumulan en los tejidos vegetales en respuesta a una amplia variedad de estímulos. Se han descrito dos rutas de biosíntesis del AIA, una dependiente del triptófano y otra independiente de este aminoácido. La primera vía involucra varios intermediarios como la indol-3-acetamida (IAM), el ácido indol-3-pirúvico (IPA), la indol-3-acetaldoxima (IAOX), y la triptamina (TAM). La vía independiente de triptófano no ha sido completamente dilucidada aunque se propone como precursor un compuesto indólico (Finet y Jaillais, 2012). Recientemente, se sugirió que una proteína que sintetiza indol, de localización citosólica, es clave en la vía independiente de triptófano, y tiene un papel preponderante en la embriogénesis de las plantas (Wang *et al.*, 2015). La inactivación mediante conjugación es una forma de regulación de los niveles de AIA, debido a que altas concentraciones de auxinas son tóxicas para la planta, lo que explica que diferentes compuestos se puedan usar como herbicidas. Las formas conjugadas del AIA se clasifican en tres grupos: i) de bajo peso molecular, incluye la adición de azúcares, ii) de bajo peso molecular, en cuyo caso la auxina se modifica con la adición de aminoácidos y iii) de alto peso molecular, lo cual ocurre mediante la adición de péptidos. La vía más conocida en la conjugación del AIA con aminoácidos involucra a la familia de genes *GRETCHEN HAGEN (GH3)* que codifican amido sintasas y amido hidrolasas (Kramer y Ackelsberg, 2015). Los genes *GH3* se encuentran ampliamente distribuidos en el reino vegetal y las mutaciones con ganancia o pérdida de función de

estos genes afectan el crecimiento y desarrollo, como sucede en la mutante con ganancia de función *ydK1-D*, que presenta una reducción en la raíz primaria, en el número de raíces laterales y en la dominancia apical (Westfall *et al.*, 2010). El AIB y el éster metil inactivo del AIA (MeAIA) pueden almacenarse e interconvertirse en auxinas activas (Korasick *et al.*, 2013). La familia de genes *ILR1/ILL*, que codifican para hidrolasas de aminoácidos, posibilitan el catabolismo de algunos conjugados, con lo que se mantiene un balance en los niveles de auxinas conjugadas y libres (Kramer y Ackelsberg, 2015). La inactivación irreversible de los conjugados con azúcares se lleva a cabo mediante oxidación cuando los niveles de auxinas se incrementan. Los metabolitos que son producto de dicha degradación se han identificado como el ácido 2-oxoindol-3-acético (oxAIA) y glucosa (oxAIA-Glu) (Ljung, 2013).

2. Distribución de las auxinas

El transporte de auxinas en las plantas se lleva a cabo mediante dos vías distintas: i) el AIA que se distribuye hacia zonas lejanas de los sitios de biosíntesis se mueve a través del floema con el flujo de fotosintatos, y ii) el transporte polar de auxinas (TPA) se lleva a cabo entre tejidos, célula a célula y ocurre por la acción de proteínas membranales de entrada y salida (Petrásek y Friml, 2009). Los acarreadores de entrada pertenecen a la familia AUX1/LIKE AUX1 (AUX1/LAX) y se identificaron con la caracterización de una mutante agravitropica llamada *auxin resistant 1* (*aux1*) de *Arabidopsis* (Taiz y Zeiger, 2002). Por otra parte, la salida de auxinas de las células está mediada por proteínas transportadoras de la familia PIN (PIN-FORMED), que contienen de 10 a 12 segmentos transmembranales separados por una región hidrofílica. En *Arabidopsis thaliana* se han reportado ocho proteínas PIN, las PIN 1, 3, 4 y 7 son importantes durante la organogénesis y en las respuestas gravitropicas de la raíz (Barbosa y Schwechheimer, 2014; Adamowski y Friml, 2015). A diferencia de las PIN de la membrana plasmática, las PIN 5, 6 y 8 se localizan en el retículo endoplásmico, junto con los transportadores denominados LIKE PIN (PILS) (Barbez *et al.*, 2012). Los métodos para estudiar la localización de las PIN y la determinación de las concentraciones endógenas de auxinas durante los procesos del desarrollo se basan en el uso de inhibidores del transporte de entrada a las células como el ácido 1-naftoxiacético (1-NOA), el ácido 2-naftoxiacético (2-NOA), el ácido 3-cloro-4-hidroxifenil acético (CHPAA) y la cromosaponina natural; así como de inhibidores de salida, entre los que se incluyen moléculas sintéticas como el ácido 1-naftilftalámico (NPA), ácido 2,3,5-triidobenzoico (TIBA) y algunos flavonoides naturales (Hosek *et al.*,

2012). Los flavonoides son un grupo de compuestos polifenólicos que resultan del metabolismo secundario de las plantas y que al modular el transporte de auxinas afectan los tropismos (Petrásek y Friml, 2009). Recientemente, se ha descrito otra familia de transportadores de salida de auxinas de las células denominada ABCB/MDR/PGP, que transportan diferentes tipos de moléculas y cuya sobreproducción confiere resistencia a drogas o toxinas; aquí se incluye a las proteínas ABCB 1, 4, 19, 15 y 21 involucradas en la formación de pelos radiculares, de raíces adventicias y el fototropismo (Retzer *et al.*, 2014). Las PGPs pertenecen a una subfamilia de los transportadores ABCB que se descubrieron inicialmente en líneas cancerígenas de mamíferos, donde su sobreexpresión confería resistencia a fármacos (Cho y Cho, 2013). En las plantas, se sugirió que dichos transportadores funcionan como bombas de eflujo con acción inespecífica, después se les atribuyó un papel en el transporte de auxinas en monocotiledóneas y dicotiledóneas. Como un ejemplo de la importancia de este grupo de proteínas, se resalta el hecho que la mutante *atpgp1*, deficiente en el transportador PGP1 presenta reducción en el desarrollo y en las mutantes *atpgp4* y *19* hay defectos en el desa de la raíz, transporte de auxinas disminuido y la pérdida del gravitropismo (Geisler y Murphy, 2006). El modelo vigente sugiere que las proteínas PIN se distribuyen desde organelos internos como el retículo endoplásmico hacia la membrana dentro de vesículas secretoras (Finet y Jaillais, 2012). La brefeldina A (BFA) es una toxina que inhibe el flujo de las vesículas y promueve así la acumulación del transportador PIN1 en compartimentos internos. Las proteínas ARF-GEF GNOM controlan el tráfico vesicular y en consecuencia el transporte de auxinas, causando diferentes anomalías en el desarrollo. Por ejemplo, las mutantes *gnom* muestran defectos desde la embriogénesis que conducen a la malformación de órganos (Adamowski y Friml, 2015). Existen otras proteínas que regulan la distribución vesicular de los transportadores como la cinasa PINOID (PID/WAG) y la fosfatasa PP2A, necesarias para re-direccionar el flujo de auxinas (Fig. 1).

3. Señalización de las auxinas

La percepción de las auxinas inicia con su unión al receptor TIR1 (TRANSPORT INHIBITOR RESPONSE1), localizado en el núcleo de las células. Estas moléculas actúan como un “pegamento molecular” y posibilitan la interacción del receptor con otras proteínas (Guilfoyle, 2007). En particular, TIR1 es un co-receptor asociado con otras proteínas para formar un complejo denominado SKP1-Cul1-F-Box (SCF^{TIR1/AFB}) (Calderón *et al.*, 2012). Cuando los

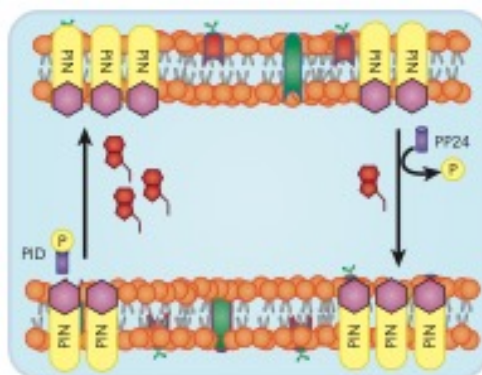


Figura 1. Localización de los transportadores de las auxinas de la familia PIN. El transporte de dichas proteínas desde los sitios de biosíntesis en el retículo endoplásmico hacia la membrana plasmática está mediado por reacciones de fosforilación que regulan la localización del transportador y por lo tanto la concentración intracelular de las auxinas.

niveles de auxinas son bajos, las proteínas represoras Aux/IAA forman multímeros con los factores de transcripción de respuesta a auxinas denominados ARFs (AUXIN RESPONSE FACTORS) mediante interacciones proteína-proteína, bloqueando así la transcripción de genes de respuesta a auxinas. Al aumentar la concentración de auxinas se promueve la ubiquitinación del represor Aux/IAA y entonces ocurre su degradación por el proteosoma 26S, dejando los ARF libres para la activación de los promotores de los genes de respuesta a auxinas. Interesantemente, se encontró un dominio de dimerización (DD) en los ARFs que al unirse a la secuencia AuxREs de los promotores forman dímeros para activar la expresión de genes correspondiente a las familias *GRETCHEN HAGEN* (*GH3*), *Small Auxin Up Regulated* (*SAUR*) y *Aux/IAA* (Tian *et al.*, 2002). Recientemente se reportó que los componentes en la señalización de auxinas también pueden presentar modificaciones pos-traduccionales que afectan la vía. La nitración de TIR1 conduce a la degradación de los represores Aux/IAA, en tanto que los ARFs pueden ser fosforilados por la cinasa BIN2 (BRASSINOSTEROID-INSENSITIVE2) suprimiendo con ello su interacción con los represores Aux/IAA y promoviendo entonces la transcripción de los genes de respuesta a auxinas (Wang y Estelle, 2014) (Fig. 2). A nivel transcripcional, el co-receptor TIR1/AFB-Aux/IAA controla la vía de señalización auxínica, sin embargo, existen procesos que requieren respuestas rápidas a las auxinas que no involucran la transcripción de uno de los genes (Vanneste y Friml, 2013). ABP1 es una proteína de 22 kDa que contiene un péptido señal de retención en el retículo

endoplásmico; estudios iniciales sugirieron un papel esencial para esta proteína en casi todos los aspectos del desarrollo de las plantas, controlando la división y expansión celular (Sauer y Kleine, 2011). Sin embargo, un estudio controversial publicado por Gao *et al.* (2015), mostró que ABP1 no era un componente en la señalización de auxinas ni tampoco jugaba un papel importante en la regulación del desarrollo, debido a que las mutantes *abp1* presentaban un fenotipo normal. En conclusión, parece ser que las investigaciones de la función de ABP1 han regresado al siglo XX, por lo que valdría la pena revisar la hipótesis propuesta desde la década de los 60's de que los transportadores PIN podrían ser los receptores de las auxinas (Strader y Zhao, 2016).

4. Eventos del crecimiento y desarrollo vegetal regulados por auxinas

4.1. Embriogénesis

La formación del embrión se lleva a cabo por divisiones anticlinales del cigoto fertilizado. Desde esta etapa, la distribución de auxinas en forma de un gradiente apical-basal en la hipófisis conduce a la formación del meristemo radicular. En el estadio de corazón del embrión, la acumulación de auxinas se presenta mayoritariamente en los sitios de formación de los cotiledones, donde se diferencian grupos de células para formar el meristemo del follaje (Wendrich y Weijers, 2013). Durante la germinación, la primera estructura en salir de la testa de la semilla es la radícula, en la que ya se distinguen tres zonas bien definidas: meristemática, de elongación y de diferenciación. En el meristemo, las células se encuentran en división constante para posteriormente crecer y aumentar de volumen. En la zona de diferenciación se forman los pelos radiculares a partir de los tricoblastos de la epidermis y las raíces laterales que emergen de algunas células del periciclo (Sathbal *et al.*, 2015).

Las raíces laterales permiten al sistema radical una mejor exploración del suelo para hacer más eficiente la captación de agua y nutrientes y son componentes clave para la adaptación de las plantas a los cambios en el ambiente (Lavenus *et al.*, 2013). En el desarrollo de estos órganos, el transporte de auxinas mediado por los transportadores PIN1 y PIN3 es necesario para regular el reflujo de auxinas entre la epidermis y el periciclo. Cascada abajo en la señalización, el factor de transcripción (ARF5)/MONOPTEROS (MP) participa en la formación de las raíces laterales y activa la expresión de PIN1 a través de una retroalimentación positiva (Tian *et al.*, 2014).

4.2. Tropismos

Las auxinas están involucradas en la orientación

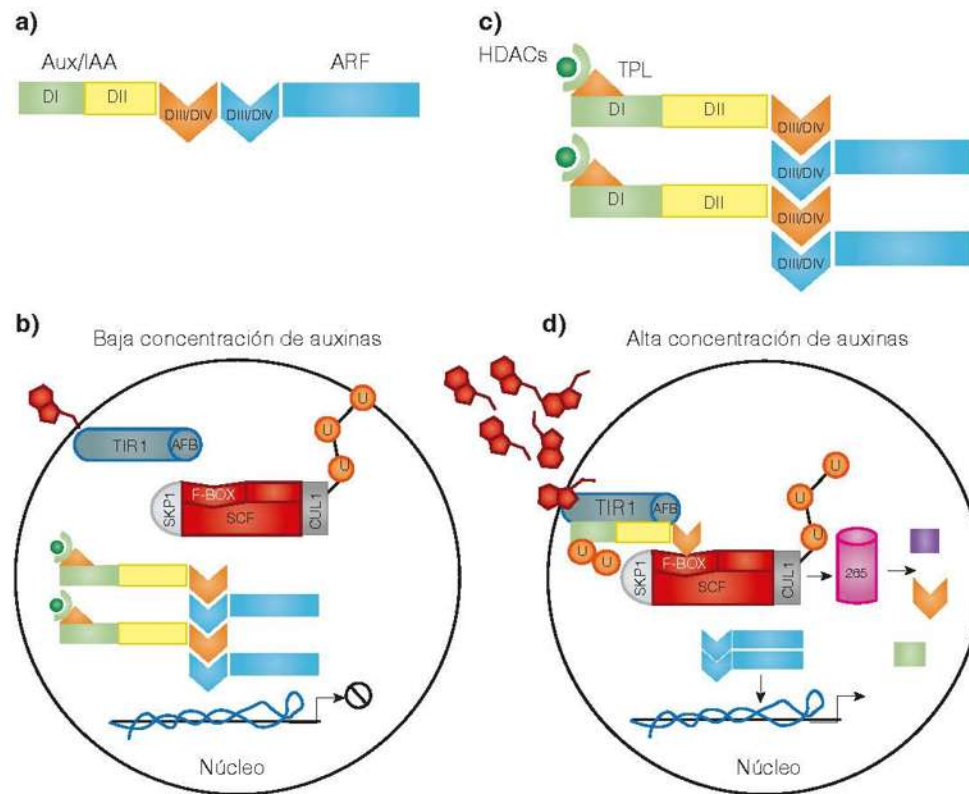


Figura 2. Señalización de las auxinas. a) Interacción del represor Aux/IAA y el activador transcripcional ARF a través del dominio DIII/IV. b) Las auxinas, en bajas concentraciones, no son percibidas por el co-receptor nuclear TIR1/AFB/Aux-IAA. c) El represor forma un complejo multimérico con el activador que interactúa por el dominio I con el co-receptor TOPLESS (TPL), constituido por desacetilasas de histonas (HDACs) lo que resulta en la represión de la transcripción de genes de respuesta a auxinas. d) El co-receptor percibe una alta concentración de auxinas y permite el acoplamiento y ubiquitinación del represor para su degradación en el proteosoma 26S, el activador queda libre y se dimeriza para unirse a los elementos AuxRE de los promotores para activar la transcripción de genes de respuesta a auxinas.

del crecimiento de las plantas a estímulos de luz (fototropismo), tacto (tigmotropismo) y gravedad (gravitropismo) (Taiz y Zeiger, 2002). La gravedad es percibida por la raíz en las células de la cofia que contienen almidón, es ahí donde PIN3 se localiza después de dicha percepción. El flujo auxínico se dirige entonces desde la punta de la raíz hacia la zona de elongación. PIN2 y AUX1 son requeridos en esta ruta de transporte. Esto se dedujo al observar que las mutantes de *Arabidopsis* deficientes en cada uno de estos genes forman raíces agravitrópicas. Por otra parte, se ha propuesto que los transportadores ABCB podrían estar implicados en la respuesta a la gravedad, ya que las mutantes *abcb4*, *abcb19* y *abcb1* son agravitrópicas (Petrásek y Friml, 2009). El calcio

participa como un mensajero intracelular durante el gravitropismo inducido por auxinas, debido a que su acumulación colocaliza en la misma dirección del flujo auxínico conducido por PIN3 y PIN7 (Vanneste y Friml, 2013).

4.3. Funcionamiento de los meristemos

Para el mantenimiento del meristemo la tasa de diferenciación debe ser igual a la generación de nuevas células. Poco después de la germinación, el meristemo de la raíz alcanza un tamaño estable que se conserva en el tiempo, permitiendo el crecimiento indeterminado. El aumento de los niveles de la proteína SHY2, inducible por auxinas y su interacción con diversos reguladores del crecimiento como las citocininas,

estrigolactonas, ácido abscísico, y brassinosteroides permite el balance entre la división y la diferenciación celular requerido para el crecimiento y funcionalidad de la raíz (Pacífico *et al.*, 2015).

La formación de órganos a partir del meristemo apical del brote involucra a las auxinas (Vernoux *et al.*, 2010). Este meristemo apical está formado por tres capas, un centro organizacional (OC) rodeado por un grupo de células fuente con poca actividad proliferativa ubicado en la base de la zona central (CZ). También se reportan una zona basal/médula (BZ) y otra periférica (PZ) donde los nuevos órganos laterales se forman, incluyendo las yemas axilares y los primordios de hojas. La función que tienen las auxinas en la iniciación de primordios de raíces laterales y/o de las hojas depende de la localización de los transportadores PIN1 y AUX/LAX (Käthe *et al.*, 2014). Las auxinas también se coordinan con la señalización por citoquininas a través de uno de los complejos que regulan el ciclo celular, CYCD/CDK, que al fosforilar a la proteína represora RB (RETINOBLASTOMA), libera al factor de transcripción E2F y entonces se induce la progresión de la fase G1 a la S (síntesis de DNA) del ciclo celular (Schaller *et al.*, 2015).

4.4. Interacción de la vía auxínica con el complejo MEDIADOR

La transcripción es un proceso catalizado por diversas proteínas que funcionan en sincronía. En eucariotes, las principales proteínas implicadas son la enzima ARN Polimerasa II, los Factores de Transcripción Generales (GTFs por sus siglas en inglés 'General Transcription Factors'), así como activadores y represores que actúan en *trans*, y el Complejo MEDIADOR (MED) (Kim *et al.*, 1994; Koleske y Young, 1994; Malik *et al.*, 2005). Desde su descubrimiento en *Saccharomyces cerevisiae*, las proteínas MED se han encontrado en casi todos los eucariotes (Bonhe *et al.*, 2002; Bourbon *et al.*, 2004; Bourbon, 2008). En *Arabidopsis thaliana*, se conservan 21 subunidades incluyendo las del dominio catalítico formado por MED12, MED13 y CDK8 (Wang y Chen, 2004; Backstrom *et al.*, 2007; Gillmor *et al.*, 2010; Ito *et al.*, 2011). Además de controlar el desarrollo de la planta, se ha demostrado que las subunidades MED modulan las respuestas a estrés biótico y abiótico, la floración y las vías de señalización hormonal, o contribuyen en funciones esenciales de los núcleos de las células como, la actividad de la helicasa del ADN, así como el empalme de intrones y exones del ARN mensajero denominado "splicing" (Fig. 3). Con los análisis de las mutantes afectadas en MED14, se ha evidenciado la participación del Complejo Mediador en la organización del tamaño de órganos vegetales y en la diferenciación celular, ya

que dichas mutaciones ocasionan una desorganización del meristemo apical del brote (Antrun *et al.*, 2002; Sundaravelpandian *et al.*, 2012). Además de MED14, las mutantes del módulo cinasa *med12*, *med13* y *cdk8* muestran fenotipos de diferenciación celular alterada durante el desarrollo (Wang y Chen, 2004; Gillmor *et al.*, 2010; Ito *et al.*, 2011) y se ven afectadas en la transición del embrión de la etapa globular al estadio de corazón (Gillmor *et al.*, 2010). El efecto de la mutación en MED13 sobre la diferenciación celular ha sido explicado por una respuesta defectuosa a las auxinas, cuyo flujo direccional mediado por PIN1 es visiblemente anormal en la zona de división celular y en los coiledones en desarrollo (Ito *et al.*, 2011). El gen MED25, también conocido como *PHYTOCHROME 1 AND FLOWERING TIME 1 (PFT1)*, fue descrito como un regulador del proceso de adaptación conocido como Síndrome de Evasión de la Sombra (SAS por sus siglas en inglés 'Shade Avoidance Syndrome') que promueve la floración en respuesta a cambios en la calidad de la luz (Gordon y Chory, 2003) y podría estar relacionado directamente a una respuesta auxínica a través de la participación de PIN3 (Kenskremp *et al.*, 2010). El reporte más reciente de la participación de PFT1/MED25 en el desarrollo de las plantas mostró que la pérdida de su función estimula el crecimiento de la raíz primaria y raíces laterales, así como la formación *de novo* de raíces laterales y adventicias por el contrario, y coincidentemente la sobreexpresión de PFT1/MED25 redujo estas respuestas, lo que sugiere que PFT1/MED25 es un elemento importante en la proliferación celular del meristemo y en el control del tamaño celular en la raíz primaria y las raíces laterales (Raya-González *et al.*, 2014). Estos autores, también demostraron que PFT1/MED25 regula negativamente el transporte de auxinas a través de la modulación de la expresión y distribución del transportador PIN1, y la expresión de genes de respuesta a auxinas en toda la planta (Raya-González *et al.*, 2014).

Conclusiones

Las plantas pueden cambiar la arquitectura del follaje y de la raíz ante las fluctuaciones en las condiciones ambientales aumentando o disminuyendo las concentraciones endógenas de los reguladores del crecimiento vegetal como las auxinas. El ácido indol-3-acético puede ser producido por plantas y por diversos microorganismos que forman parte del microbioma vegetal, por lo que su participación en la interacción entre organismos de diferentes reinos es un área de investigación activa. La homeostasis de las auxinas en los tejidos vegetales involucra aspectos de biosíntesis, transporte, transducción de señales y metabolismo, cada uno de los cuáles resulta esencial

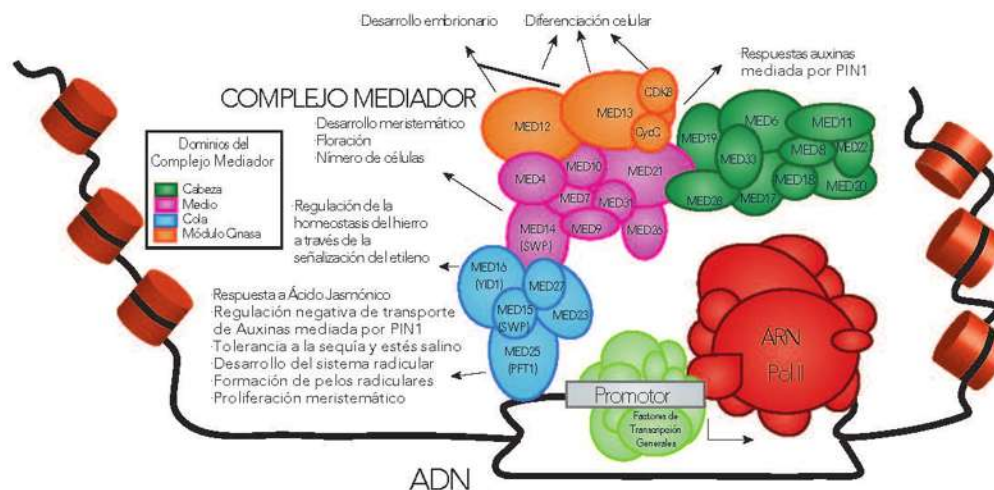


Figura 3. El Complejo MEDIADOR como componente importante de la maquinaria transcripcional eucariota. Algunas subunidades específicas son importantes para la regulación de diversos procesos del desarrollo vegetal. En particular, la subunidad MED25 participa en la respuesta auxínica y en los procesos celulares subyacentes, como la formación de los pelos radiculares y de las raíces laterales.

para el desarrollo de las plantas. Con el advenimiento de técnicas de secuenciación masiva de transcritos y de identificación de proteínas y metabolitos a gran escala, se abre un panorama amplio para dilucidar la forma como las auxinas interactúan con otras fitohormonas para coordinar los programas de organogénesis que determinan la forma y función de los vegetales y su adaptación al ambiente.

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DISCUSIÓN Y CONCLUSIONES

El suelo es un ecosistema dinámico, constituido por una gran diversidad de microorganismos que desempeñan numerosas funciones ecológicas. Una de ellas es el reciclaje de los nutrientes de los que dependen las plantas para realizar procesos metabólicos y de crecimiento para proveernos de alimentos. La productividad agrícola, por lo tanto, depende de diversos factores bióticos y abióticos presentes en el suelo y estas características físico-químicas pueden limitar su actividad y afectar el rendimiento de los cultivos. A partir de la estrategia implementada durante la revolución verde, en la que se optó por incrementar la producción para satisfacer la demanda de alimentos mediante el uso indiscriminado de agroquímicos, los impactos negativos al medio ambiente hacen necesario el planteamiento de nuevas estrategias. Actualmente, el cambio climático global y el incremento acelerado de la población propician la intensificación de la explotación de suelos arables y la sobre-aplicación de fertilizantes. Ante esta situación, es urgente buscar y aplicar prácticas sustentables en la agricultura moderna para producir alimentos seguros para el consumo humano.

Las propiedades que presentan los microorganismos benéficos asociados a las plantas, son de gran utilidad para ayudar a mejorar el crecimiento, adquirir tolerancia a distintos tipos de estrés y protegerlas del ataque por patógenos que causan enfermedades, las cuales representan importantes pérdidas a nivel mundial. A pesar del esfuerzo en la obtención de aislados sobresalientes en estas características para la formulación de bio-inoculantes, los efectos en campo pueden variar o verse atenuados por múltiples causas, por lo que requiere de un mejor conocimiento de las interacciones planta microorganismo y de los mecanismos de acción de las PGPR para su aplicación efectiva.

En este trabajo, uno de los objetivos principales fue la obtención e identificación de microorganismos rizosféricos con gran potencial para promover el crecimiento vegetal en el modelo de investigación *Arabidopsis thaliana*. Este sistema nos permitió evaluar el efecto benéfico de las rizobacterias a través de un diseño experimental por contacto directo con las plántulas, en el que se pueden observar

cambios en la arquitectura de la raíz, posibles síntomas de estrés como clorosis y necrosis, y en general podemos cuantificar el impacto en la producción de biomasa. En sistemas de interacción *in vitro*, se determinaron los elementos moleculares que participan en la reprogramación de la arquitectura radical, en los ensayos de contacto directo, dos cepas presentaron efectos bio-estimulantes destacables, la cepa LS570 tipificada como una actinobacteria, *Micrococcus luteus*, y *Bacillus* sp, LC390B. Ambas fueron obtenidas de un cultivo de maíz a partir de suelo alcalino (pH 8.5) en la localidad de “Lometón”. Los efectos de cada cepa fueron diferenciales, lo que sugiere que los mecanismos, así como las propiedades de cada rizobacteria son únicas. Un análisis de microscopía electrónica de barrido (MEB) a partir de un cultivo puro de *Micrococcus*, demostró que las células de forma esférica están desprovistas de estructuras de motilidad o flagelos, su tamaño, oscila entre los 2 μm de diámetro y se agrupan en tétradas formando cadenas largas o aglomerados. Este resultado es consistente con la morfología descrita de la bacteria y con el análisis filogenético se confirma que el aislado es *Micrococcus luteus*.

El análisis detallado del peso fresco de la raíz, del follaje y la biomasa total indicó que en contacto directo con el inóculo bacteriano conlleva a una mayor biomasa de la raíz, incrementando el patrón de ramificación, la formación de pelos radicales y raíces laterales de primer y segundo orden. En el sistema aéreo no hubo un efecto promotor, aunque tampoco presentaron síntomas de clorosis o deletéreos. Las auxinas no solamente son producidas por bacterias benéficas, los patógenos como *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa* o *Pseudomonas syringae* intervienen en la homeostasis de auxinas y señalización en los tejidos vegetales (Nunkel y Johnson, 2021).

Al respecto, en estudios con una cepa mutante en la sintasa de AHLs de *Pseudomonas aeruginosa* PAO1, ΔlasI , se encontró que la mutación revierte los efectos patogénicos, promoviendo la ramificación de la raíz y el peso fresco total en *Arabidopsis*. Las moléculas responsables de la bio-estimulación se identificaron como ciclodipéptidos, los cuales presentaron una actividad auxínica disminuida, comparada con el ácido indol-3-acético (Ortiz-Castro *et al.* 2011).

Consistente con este resultado, el marcador inducible por auxinas, *DR5::GUS*, se induce fuertemente en las plantas en co-cultivo, sin embargo, no presentan signos de clorosis o necrosis durante los días de interacción. Estos resultados refuerzan el resultado de que la cepa LS570 es promotora del crecimiento y la respuesta auxínica incrementada no se correlaciona con efectos represores. Resultará interesante verificar los compuestos que produce la bacteria, para conocer su estructura química y poder o no relacionarlos con las auxinas u otras moléculas que tienen afinidad para unirse a receptores de la vía de señalización auxínica, como sucede con los ciclodipéptidos de *P. aeruginosa*.

En otros aislados de *Micrococcus luteus* también se evaluó la producción de auxinas, en donde las cepas AKAD 3-5, chp37, NII-0909, RWL-3, y 4.43 demostraron la capacidad de producir ácido indol-3-acético con triptófano suplementado (Dubey *et al.* 2021). Esto sugiere que la producción de compuestos indólicos es una propiedad común en esta especie, aunque falta por investigar las consecuencias de su interacción con plantas. En otro estudio con *M. luteus*, la cepa chp37, incrementó el número de hojas en tratamientos con suelo natural y arenoso, hasta en un 54%, así como la longitud y el peso fresco del follaje y de las raíces, comparado con controles no inoculados (Raza y Faisal, 2013).

La respuesta fenotípica en la raíz primaria fue una inhibición del crecimiento, idéntico a la aplicación de ácido indol-3-acético. La proliferación celular, así como la elongación se encuentran reprimidas, correlacionándose con la inducción de la respuesta auxínica y la diferenciación de pelos radicales en el meristemo apical. Cuando la concentración de auxinas es alta, se favorece la biosíntesis de etileno, lo que explica por qué algunas bacterias expresan la enzima ACC desaminasa para degradar al precursor del etileno y disminuir los síntomas de estrés (Gupta y Pandey, 2019).

La interacción con *Bacillus* sp. LC390B, a diferencia de *Micrococcus*, mejoró el peso fresco del follaje y raíz, la longitud, el número y la densidad de raíces laterales, promoviendo la elongación celular. Una hipótesis es que, los compuestos orgánicos volátiles podrían participar en esta respuesta promotora, ya que el género *Bacillus*,

es prolífico en la producción de compuestos que promueven el crecimiento y potencian la inmunidad (Ryu *et al.* 2003).

Durante las etapas del desarrollo de las raíces laterales, la bacteria aceleró el proceso de maduración y la emergencia de estas estructuras. Los azúcares son moléculas con funciones metabólicas y nutricionales, interruptores metabólicos y factores de señalización que controlan el crecimiento radical. La suplementación de glucosa a plántulas de *Arabidopsis in vitro* incrementaron la elongación de la raíz primaria, la ramificación de la raíz y la respuesta auxínica (Freixes *et al.*, 2002; Raya-González *et al.*, 2017; (Mishra *et al.*, 2009, 2021). MacGregor y col. (2008) demostraron que la disponibilidad de sacarosa es crítica para la formación de raíces laterales y puede ocurrir por la inducción de la biosíntesis de auxinas. Recientemente se han encontrado efectos comparables en plántulas de *Arabidopsis* expuestas a los volátiles emitidos por *B. amyloliquefaciens*, los cuales formaron más primordios y aceleraron su emergencia (Li *et al.* 2021), sin embargo, las moléculas responsables aún no han sido identificadas.

La comparación de los fenotipos silvestres y mutantes en la vía auxínica, incluyendo *tir1*, *slr1*, *arf7/arf19*, y *tir1/afb2/afb3* nos ayudaron a definir su importancia en decodificar el mensaje químico causado por *M. luteus* LS570 y *Bacillus* sp. LC390B, sobre la raíz primaria y la formación de raíces laterales. Nuestros resultados muestran claramente la participación de los activadores transcripcionales ARF7 y ARF19, debido a que la doble mutante fue totalmente resistente a la inhibición de la raíz primaria y la inducción de raíces laterales con *M. luteus* LS570. Esta respuesta también ocurrió en mutantes del receptor de auxinas, aunque la desviación de la respuesta fue menor que con la doble mutante *arf7/arf19*. Esta es la primera vez que las proteínas ARF7 y ARF19 se relacionan con el control del crecimiento de la raíz primaria, ya que su función primordial ocurre en la formación de raíces laterales activada por diversos estímulos (Okushima *et al.* 2007; Huang *et al.* 2019; Lee *et al.* 2019). El incremento en la ramificación de la raíz por *Micrococcus luteus* y *Bacillus* sp. requirió del co-receptor TIR1/AFB2/AFB3, el represor y los activadores transcripcionales SLR1 y ARF7/ARF19, respectivamente, lo que indica que

elementos de la señalización por las auxinas actúan como moduladores maestros necesarios para el proceso de organogénesis por rizobacterias.

Finalmente, se evaluaron los efectos de las plántulas de *Arabidopsis* sobre el crecimiento y viabilidad de las bacterias durante el periodo de interacción. El papel benéfico de los exudados radicales sobre el crecimiento bacteriano ha sido ampliamente reportado. Por lo que, hipotetizamos que el crecimiento de *Micrococcus luteus* LS570 podría estar influenciado positivamente después de 5 días en contacto directo con las raíces de *Arabidopsis*. Este no fue el caso, debido a que las estimaciones de densidad óptica y viabilidad celular de los cultivos bacterianos manifestaron respuestas comparables a los cultivos estriados sobre medio LB o MS 0.2x. Los ensayos con *Bacillus*, a diferencia de la cepa LS570, demostraron una mejor viabilidad celular. Este comportamiento puede explicarse por la producción de endoesporas en el grupo de los *Bacilliales*, y representa una ventaja para su supervivencia y mantenimiento en distintos ambientes si se aplica como un bio-inoculante. Otra posibilidad por la que no se observe un efecto sobre el crecimiento de las bacterias, puede atribuirse a que el medio MS 0.2x de interacción con la planta provee un apropiado nivel de nutrientes a la bacteria, por lo que el efecto de los exudados radicales no se evidencia directamente. Es interesante investigar la influencia de *Arabidopsis* y otras especies de plantas en condiciones de escasez de nutrientes y bajo estrés ambiental para determinar si la interacción con plantas puede proveer una ventaja adaptativa a la supervivencia de la bacteria.

Una ventaja adicional de *Bacillus* sp. LC390 sobre *Micrococcus luteus*, es la inducción de un sistema aéreo mucho más robusto, en el que se incrementa el contenido de clorofila. Diversos microorganismos producen metabolitos con capacidad de manipular el metabolismo de azúcares en la planta incrementando la eficiencia fotosintética, el contenido de clorofila y el metabolismo de carbohidratos, en interacción con auxinas y otras fitohormonas (Zhang *et al.* 2008). La coordinación entre la arquitectura de la planta, la bio-estimulación y el nivel de carbono a través de una fotosíntesis más eficiente, sostiene el concepto de que el uso de bacterias benéficas pueden promover el crecimiento vegetal en suelos pobres de nutrientes y

la toma de agua y minerales mejorando la exploración en el sustrato por un sistema radical con mayor capacidad de ramificación.

Debido a la función que presentan los fitocromos en los programas de fotomorfogénesis, se les han atribuido funciones en la síntesis de clorofila y carotenoides, principalmente *PHYA* y *PHYB* (Losi y Gärtner, 2021). Nuestros resultados con *Bacillus* sp. LC390B mostraron que las plántulas inoculadas afectadas en *phyA/phyB* no manifestaron un incremento en el crecimiento y el contenido de clorofila comparado con el fenotipo silvestre. Los fitocromos presentan actividad de cinasas, modulando de esta manera la actividad de factores transcripcionales que se unen a elementos en la región del promotor activando la transcripción en relación a la fotosíntesis, biosíntesis de clorofila y carotenoides durante la fotomorfogénesis (Quian-Ulloa y Stange, 2021).

Vistos en conjunto, nuestros resultados indican que las rizobacterias *Micrococcus luteus* LS570 y *Bacillus* sp. LC390B, activan mecanismos en los que se reprograman el crecimiento y desarrollo de las plantas. Las respuestas diferenciales de cada una nos indica que presentan propiedades únicas y refleja la diversidad de mecanismos involucrados en la bio-estimulación en los distintos grupos taxonómicos. En los estudios de interacción se relacionó la respuesta auxínica con programas de diferenciación en las raíces laterales y pelos radicales con *Micrococcus*, sin embargo, las auxinas también estuvieron involucradas en el desarrollo de primordios y raíces laterales con *Bacillus* sp. LC390B, lo cual indica que las auxinas son moduladores cruciales indispensables para esos eventos de organogénesis.

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9. ADDENDA

Las contribuciones adicionales al proyecto de investigación de Doctorado se incluyen en los siguientes apartados.

9.1. Publicaciones en colaboración de arbitraje Internacional

1. Jiménez-Vázquez KR, **García Cárdenas E**, Barrera-Ortiz S, Ortiz-Castro R, Ruíz-Herrera LF, Ramos-Acosta BP, Coria-Arellano JL, Sáenz-Mata J, López-Bucio J. 2020. The plant beneficial rhizobacterium *Achromobacter* sp. 5B1 influences root development through auxin signaling and redistribution. *The Plant Journal*. 103, 1639-1654.
2. López-Hernández J, **García-Cárdenas E**, López-Bucio JS, Jiménez-Vázquez KR, Reyes de la Cruz H, Ferrera-Rodríguez O, Santos-Rodríguez DL, Ortiz-Castro R, López-Bucio J. 2022. Screening of phosphate solubilization identifies six *Pseudomonas* species with contrasting phytostimulation properties in *Arabidopsis* seedlings. *Microbial Ecology*. 1-15.

Publicaciones de arbitraje Internacional

The plant beneficial rhizobacterium *Achromobacter* sp. 5B1 influences root development through auxin signaling and redistribution

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SUMMARY

Roots provide physical and nutritional support to plant organs that are above ground and play critical roles for adaptation via intricate movements and growth patterns. Through screening the effects of bacterial isolates from roots of halophyte Mesquite (*Prosopis* sp.) on *Arabidopsis thaliana*, we identified *Achromobacter* sp. 5B1 as a probiotic bacterium that influences plant functional traits. Detailed genetic and architectural analyses in *Arabidopsis* grown *in vitro* and in soil, cell division measurements, auxin transport and response gene expression and brefeldin A treatments demonstrated that root colonization with *Achromobacter* sp. 5B1 changes the growth and branching patterns of roots, which were related to auxin perception and redistribution. Expression analysis of auxin transport and signaling revealed a redistribution of auxin within the primary root tip of wild-type seedlings by *Achromobacter* sp. 5B1 that is disrupted by brefeldin A and correlates with repression of auxin transporters PIN1 and PIN7 in root provascular, and PIN2 in the epidermis and cortex of the root tip, whereas expression of PIN3 was enhanced in the columella. In seedlings harboring *AUX1*, *EIR1*, *AXR1*, *ARF7ARF19*, *TIR1AFB2AFB3* single, double or triple loss-of-function mutations, or in a dominant (gain-of-function) mutant of *SLR1*, the bacterium caused primary roots to form supercoils that are devoid of lateral roots. The changes in growth and root architecture elicited by the bacterium helped *Arabidopsis* seedlings to resist salt stress better. Thus, *Achromobacter* sp. 5B1 fine tunes both root movements and the auxin response, which may be important for plant growth and environmental adaptation.

Keywords: *Arabidopsis*, auxin signaling, development, root biology, meristems.

Linked article: This paper is the subject of a Research Highlight article. To view this Research Highlight article visit <https://doi.org/10.1111/tpj.14941>.

INTRODUCTION

Roots support plants via soil attachment, exploration and environmental sensing. Their spatial configuration, defined as root architecture, is an important agronomic trait of increasing relevance due to its direct role in water and nutrient acquisition. The study of the mechanisms that determine root architecture has been difficult because roots grow deeply into the ground. To solve this limitation, several experimental systems have been established,

which allow the earlier stages of growth to be analyzed *in vitro*. Advances in this field were boosted by the use of *Arabidopsis thaliana*, which develops a tap root system, in which the primary root grows indeterminately due to the activity of the root apical meristem and for which genetic stocks and growth procedures are well established (Smith and De Smet, 2012; Atkinson *et al.*, 2014).

Roots are very sensitive and move towards water (hydrotropism) and gravity (gravitropism), and react to touch

and hard substrates (thigmotropism) such that these dynamic responses are important for plant foraging of resources (Ruiz-Herrera *et al.*, 2015; Su *et al.*, 2017; Dietrich, 2018). Root movements are typically slow and difficult to observe as they occur below ground. However, root navigation circumvents physical obstacles and is integral to plant function and adaptation (Falik *et al.*, 2005; Oliva and Dunand, 2007; Harmer and Brooks, 2018). The underlying molecular and physiological mechanisms remain unknown.

Phytohormones are implicated in root movements with auxin playing a critical role in gravitropism and abscisic acid in hydrotropism (Su *et al.*, 2017; Dietrich, 2018). In *Arabidopsis* mutants that are defective in the auxin intake permease AUX1 or the efflux transporter gene *EIR1/AGR1/PIN2*, the gravity response is lost and this leads to the formation of circles called coils (Chen *et al.*, 1998; Luschnig *et al.*, 1998; Müller *et al.*, 1998; Marchant *et al.*, 1999; Swarup *et al.*, 2005). Coil formation also involves AXR4, a modulator of AUX1 (Dharmasiri *et al.*, 2006), and *WAG1* and *WAG2*, which code for protein kinases that influence PIN protein activity (Santner and Watson, 2006). Gravitropism triggers auxin redistribution from the center of the primary root to external cell layers within minutes to promote directional growth towards the gravity vector, thus auxin transport is tightly linked to root tip navigation (Abas *et al.*, 2006; Rahman *et al.*, 2010; Geisler *et al.*, 2014).

The formation of coils on primary roots of *patatin-related phospholipase-AT-1 (pplal-1)* *Arabidopsis* mutants was exacerbated following application of the auxin efflux transport inhibitor 1-naphthylphthalamic acid or the influx transport inhibitor 1-naphthoxyacetic acid, as well as in response to far red light in a process mediated by *PHYB* (Effendi *et al.*, 2014; Perrineau *et al.*, 2016). Moreover, the influence of light and sugars on coiling has already been studied by Singh *et al.* (2014), who showed that glucose affects root navigation. Thus, light provision via photosynthesis and/or sugar availability modulates auxin redistribution within the root tip to instruct the direction of growth, which may help soil exploration and/or penetration.

The root-associated microbial communities play fundamental roles in plant nutrition, development and defense (Vandenkoornhuyse *et al.*, 2015; Lemanceau *et al.*, 2017). The term plant growth promoting rhizobacteria was coined to typify the bacterial strains isolated from roots in their natural environment, which improve growth, development and productivity (Lugtenberg and Kamilova, 2009; Verbon and Liberman, 2016). The mechanisms by which these beneficial bacteria influence host physiology include the production of phytohormones and quorum-sensing signals, which diffuse between cells, volatile blends perceived at long distances and/or macromolecular constituents that are recognized upon bacterial–root contact (López-Bucio *et al.*, 2007; Ortiz-Castro *et al.*, 2008; Ortiz-Castro *et al.*, 2011; Pérez-Flores

et al., 2017; Stringlis *et al.*, 2018). Despite the knowledge gained in recent years on plant growth promoting rhizobacteria research, the influence of bacteria on root behavior or tropic responses remains largely unexplored.

Root tropisms are controlled by environmental perception and tactile exploration that rely on endogenous genetic factors, stimuli integration and cellular responses for rapid cell division and elongation readjustments. Here, we discovered and characterized an *Achromobacter* sp. 5B1 strain that show several probiotic attributes both *in vitro* and in soil and noteworthy, deviates root growth from the gravitropic vector promoting lateral root development. Through a combination of cellular, molecular and genetic analysis, we show that the bacterial-induced root tip navigation and architectural reconfiguration is a dynamic process involving auxin-dependent and -independent mechanisms. These results point to a critical role of rhizobacteria not only on plant performance, but also on root behavior.

RESULTS

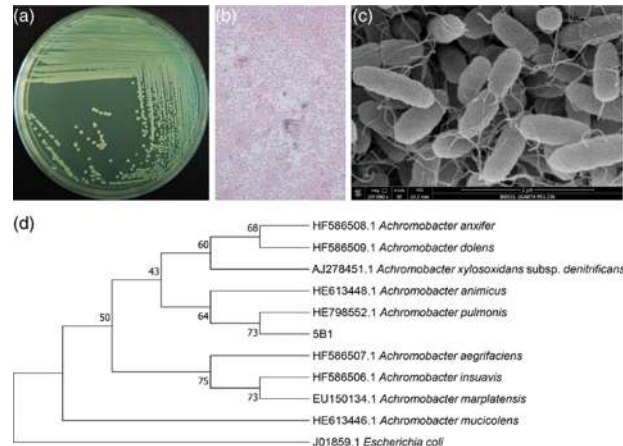
Isolation and characterization of *Achromobacter* sp. 5B1

Plants harbor very diverse and rich microbiomes that support adaptation to adverse conditions (Bulgarelli *et al.*, 2012). An exploration to the “Poza Salada,” a salty pool located in the Sobaco Valley, southeast of the Chihuahuan Desert in the state of Coahuila, México, showed several healthy Mesquite trees growing at the sides of the pool, despite the high salt content of the soil. Root samples were collected from a single tree and 43 bacterial isolates were recovered, which were co-cultivated in direct contact with *Arabidopsis* roots *in vitro* to assess their growth promotion potential. A single isolate termed 5B1 was obtained from the rhizosphere, cultivated on free nitrogen medium with 3% NaCl and successfully propagated on Luria-Bertani (LB) medium (Figure 1a) and both gram-staining and electronic microscopy analyses, respectively, grouped it as a gram-negative (Figure 1b), flagellated bacterium (Figure 1c). 16S rRNA gene sequencing and sequence comparisons using the BLAST algorithm positioned it as part of the *Achromobacter* genus, closely to *A. pulmonis* (Figure 1d).

Achromobacter sp. 5B1 is a plant growth promoting rhizobacterium

Arabidopsis thaliana is a useful plant model to assess the molecular and physiological responses to rhizobacteria. Co-cultivation experiments using agar plates were initially performed to unravel the plant responses to root colonization with *Achromobacter* sp. 5B1 *in vivo*. The effects of bacterization on root architecture and biomass production were analyzed in *Arabidopsis* wild-type (WT) (Col-0) seedlings whose roots were placed over axenic, agar-solidified medium, or over a bacterial streak and grown for six additional days. *Achromobacter* sp. 5B1 promoted primary root

Figure 1. Characterization of *Achromobacter* sp. 5B1. (a) Representative image of bacterial colonies grown on a Luria-Bertani solidified medium. Isolated colonies were streaked on MS 0.2× media to interact with *Arabidopsis* seedlings. (b) Gram staining of bacterial cells in a bright-field micrograph showing gram-negative rods. (c) Scanning electron microscopy of bacterial cells with flagella. (d) Phylogenetic tree of the 16S rRNA sequence of the 5B1 strain and nine additional sequences with 99% similarity. 16S rRNA sequence of J01859.1 *Escherichia coli* was used as the outgroup.



growth, and increased the lateral root number and density approximately fourfold, which correlated with a twofold increase in total fresh weight and shoot fresh weight and with a threefold increased root fresh weight (Figure 2a–f). The bacterial effects could be confirmed in experiments, including many more samples and up to 11 Petri plates with six seedlings each (Figure S1).

Two additional plant–bacteria modes of interaction were assessed, (i) one in which the plants and the bacteria were grown on two separate sides of Petri plates, enabling communication via volatiles only, and (ii) another where the bacterial streak was placed into the front of the primary root and for which sensing of the bacteria occurs as the root tip approaches the inoculum site. In both cases, bacterization triggered growth of plants while promoting primary root growth and branching (Figures S2 and S3). These beneficial effects could be reproduced in soil grown plants, in which root inoculation with *Achromobacter* sp. 5B1 increased whole plant height, size and productivity (Figure 3a–d).

***Achromobacter* sp. 5B1 promotes cell division in primary roots and causes changes in auxin response**

The plant hormone auxin is an important factor for root growth and architecture reconfiguration. The growth-promoting effect of the bacterium in primary roots and its relationship with cell division and auxin signaling was then analyzed using transgenic *Arabidopsis* seedlings that express *CycB1;1::uidA*, a cell cycle reporter construct and *DR5::GFP* (green-fluorescent-protein, GFP), an auxin-inducible gene marker, whose roots were placed over a bacterial streak and grown for six additional days. The results show that *Achromobacter* sp. 5B1 increases the number of cells expressing *CycB1;1::uidA* in primary root meristems

(Figure 4a,b). Interestingly, confocal imaging of *DR5::GFP* expression showed an increase in auxin responsiveness in lateral root primordia (Figure 4c), but a highly contrasting expression pattern occurred in primary root meristems, as bacterization enhanced auxin responsiveness at the very root tip while placing most fluorescence to the concave side of the coil (Figure 4d).

To gain more information on the step(s) of lateral root formation affected by inoculation with 5B1, an analysis of lateral root primordia development was performed using *Arabidopsis* transgenic seedlings that express *DR5::GUS*. Quantification of total lateral root primordia at 2, 4 and 6 days after inoculation showed that bacterization decreased the number of primordia with time, which correlated with an increased number of mature lateral roots (Figure S4). Representative images of the primordia at different developmental stages confirmed an increase in auxin responsiveness in inoculated seedlings at all the stages analyzed (Figure S4). These data suggest that an inoculation-induced increase in lateral root formation is due to an enhancement of primordium development, or emergence rate from the primary root, which are correlated with an auxin response.

***Achromobacter* sp. 5B1 causes primary root growth deviation from gravity and leads to asymmetrical *DR5::GFP* distribution expanding into one side within lateral cap cells**

The primary roots of *Arabidopsis* seedlings inoculated with *Achromobacter* sp. 5B1 deviate from the gravity vector and had an increased skewing/coiling (Figure S1). To determine if this growth pattern could be related to an altered gravitropic response, and clarify the role of auxin in this

process, a number of the *Arabidopsis* seedlings expressing *DR5:GFP* were inoculated with the 5B1 strain and the proportion of agravitropic roots quantified. While all seedlings manifested root deviation, nearly 80% of the bacterized

seedlings formed any root coil (Figure 5a–c). Interestingly, during root coiling, all seedlings manifested a *DR5:GFP* gradient preferentially expressed at the concave side of the coils. Moreover, an analysis of the roots at different stages

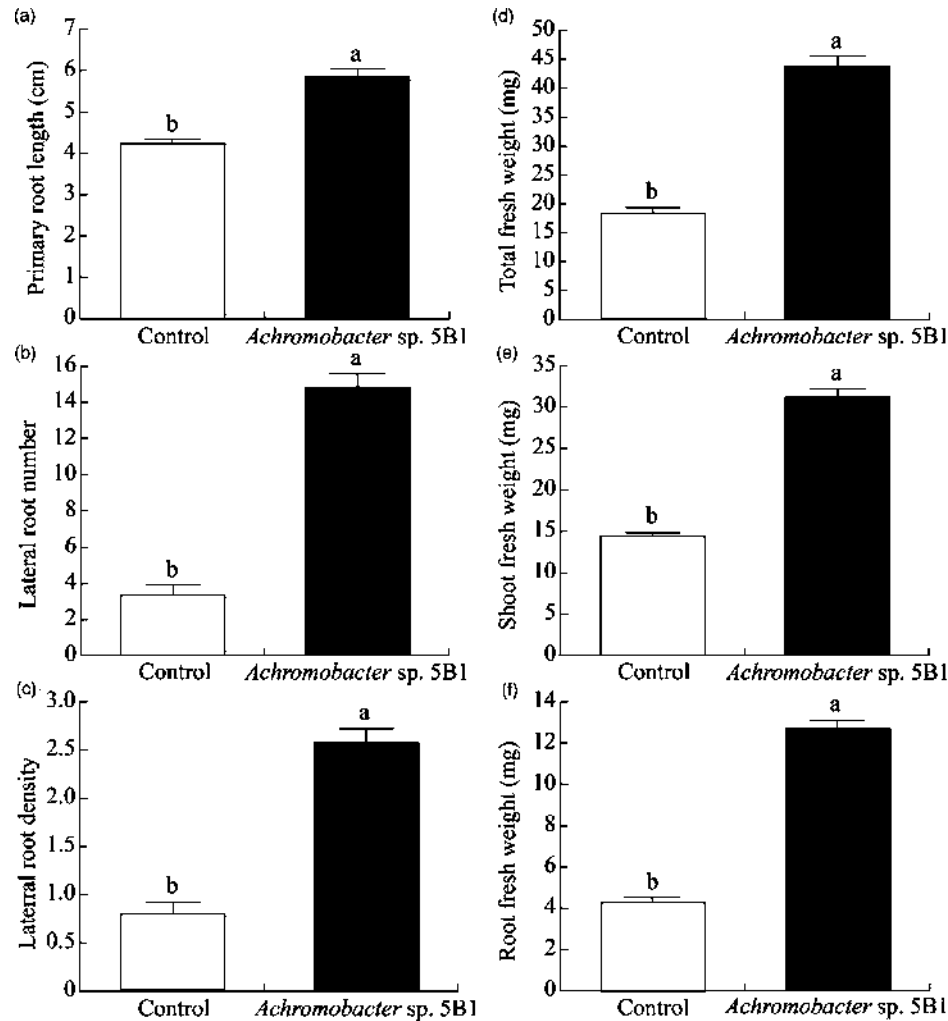


Figure 2. Effects of *Achromobacter* sp. 5B1 on root architecture and biomass production in *Arabidopsis thaliana* seedlings. *Arabidopsis* wild-type (Col-0) seedlings were germinated and grown on agar-solidified 0.2× MS medium, and 4 days after germination, transferred to fresh media by placing the roots over a streak of bacteria and grown for six additional days. (a) primary root length, (b) lateral root number, (c) lateral root density (number of roots per cm of root), (d) total fresh weight, (e) shoot fresh weight and (f) root fresh weight. Values shown represent the mean ± SD ($n = 18$). Different letters indicate statistical differences from a Tukey analysis ($P < 0.05$). Experiment was repeated three times with similar results.

Figure 3. Effect of *Achromobacter* sp. 5B1 on growth and seed production of *Arabidopsis* plants in soil.

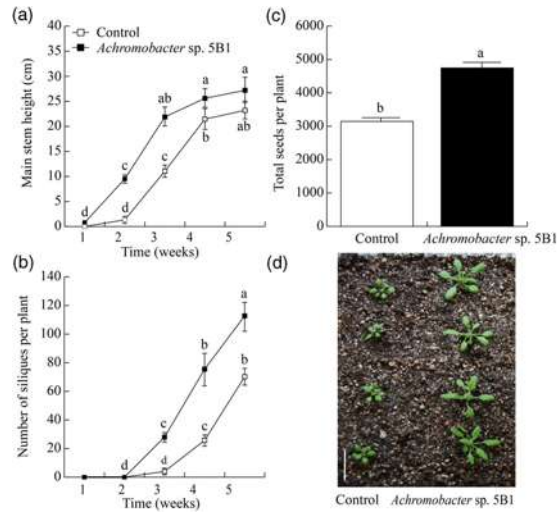
Wild-type (Col-0) seedlings were inoculated by placing their roots over a bacterial streak and maintained in the interaction during 6 days. Seedlings were transplanted into plastic boxes containing sterilized soil comprised of peat moss, perlite and vermiculite mix, and maintained to field capacity by irrigation with distilled water until the end of the life cycle.

(a) Main stem height.

(b) Number of siliques per plant.

(c) Total seeds per plant.

(d) Representative photograph of plants at the rosette stage, 7 days after transfer to soil. Values shown represent the mean $\pm$ SD ($n = 12$). Different letters indicate statistical differences from a Tukey analysis ($P < 0.05$). Experiment was repeated twice with similar results.



during the curvature clearly supported the correlation between changes in auxin-inducible gene expression and root coiling (Figure 5c). These data indicate that the altered pattern of *DR5:GUS* expression in primary root tips may be responsible for the skewing/coiling behavior displayed in the presence of the bacterium.

***Achromobacter* sp. 5B1 represses expression of auxin transporters PIN1, PIN2 and PIN7 in root tip and enhances expression of PIN3 in columella cells**

Auxin is redistributed within the meristem and root elongation zones through several members of the PIN family of auxin transporters (Blilou *et al.*, 2005). To define the possible roles of PIN1, PIN2, PIN3 and PIN7 in the redistribution of auxin following bacterial colonization, confocal imaging was performed in *Arabidopsis* transgenic seedlings expressing *pPIN1::PIN1-GFP*, *pPIN2::PIN2-GFP*, *pPIN3::PIN3-GFP* and *pPIN7::PIN7-GFP* in plants grown under axenic conditions or co-cultivated with *Achromobacter* sp. 5B1. Representative images of GFP expression (Figure 6a) and relative fluorescence intensity quantitation in the meristem and columella (Figure 6b,c) showed that root colonization with *Achromobacter* sp. 5B1 strongly repressed PIN1 and PIN7 expression in vascular tissue, and PIN2 in the epidermis and cortex of the root tip. Noteworthy, PIN3 expression was activated in the lower root cap columella cells relative to the control (Figure 6a,c). This result implies a complex influence of the bacterium on the PINs and did not rule out the possibility that 5B1 treatment changes the localization of these transporters.

Brefeldin A affects root curling and lateral root formation promoted by *Achromobacter* sp. 5B1 and disrupts bacterial-mediated auxin redistribution within the root tip

Root growth and architectural changes are mediated by the distribution of auxin transporters into the plasma membrane, which requires vesicle transport from the cytoplasm to the membrane (Geldner *et al.*, 2001, 2003). Brefeldin A (BFA) is an inhibitor widely used to alter PIN protein localization to root cell membranes by inducing their intracellular accumulation (Paponov *et al.*, 2019). Next, the interaction between *Arabidopsis* and *Achromobacter* sp. 5B1 was assessed in medium supplemented with 0, 5 and 10 μ M BFA. BFA treatment inhibited primary root growth, root coiling and lateral root formation in bacterized WT seedlings (Figure S5), which coincided with decreased expression of PIN1, PIN3 and PIN7 in provascular cells (Figure S6) and disrupted *DR5:GFP* distribution within the primary root cap (Figure 7a–d). These data suggest that major changes in root architecture elicited by the bacterium are affected by BFA, which blocks vesicle trafficking and auxin transport.

Disruption of auxin transport or signaling affects *Achromobacter* sp. 5B1 root growth promotion and enhances root curling

Auxin transport and signaling play critical roles in root growth and gravitropic responses (Brand *et al.*, 2012). To determine at the genetic level the contribution of the auxin transport and signaling pathways in root growth promotion and lateral root formation elicited by *Achromobacter*

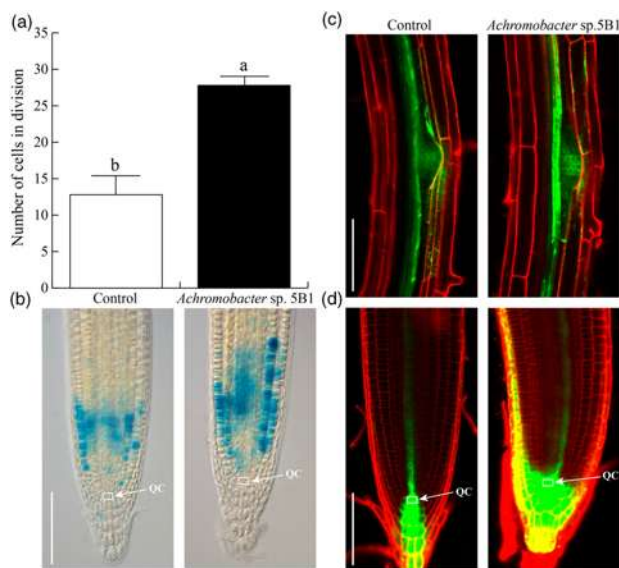


Figure 4. Effects of *Achromobacter* sp. 5B1 on cell division and auxin response in roots of *Arabidopsis* seedlings.

Transgenic *Arabidopsis* seedlings expressing *CycB1;1::uidA* and *DR5::GFP* were germinated and grown on agar-solidified 0.2× MS medium, and 4 days after germination, transferred to fresh media by placing the roots over a streak of bacteria and grown for six additional days, and then processed to analyze *GUS* and *GFP* expression.

(a) Number of cells expressing *CycB1;1::uidA* in primary root meristems.

(b) Representative images of root meristems showing *CycB1;1::uidA* expression.

(c) Confocal imaging of *DR5::GFP* expression in lateral root primordia and (d) the primary root tip of plants. Quiescent center (QC) position within the primary root tip is indicated by white arrow. Values shown represent the mean ± SD ($n = 20$). Different letters indicate statistically significant differences from a Tukey analysis ($P < 0.05$). Experiment was repeated three times with similar results.

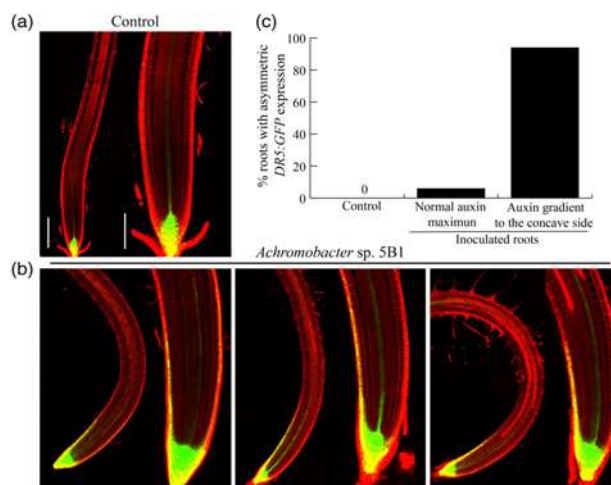
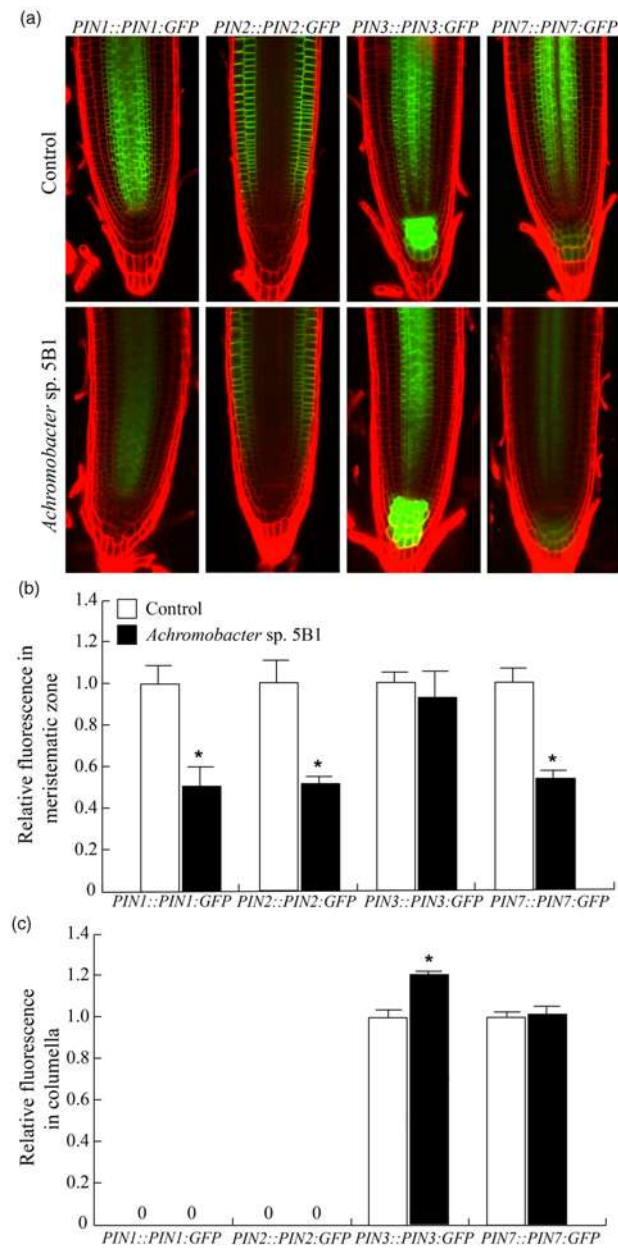


Figure 5. *DR5::GFP* expression in root tips of plants inoculated with *Achromobacter* sp. 5B1.

(a,b) Confocal photomicrographs of *DR5::GFP* expression pattern in (a) control plants and (b) in plants inoculated with *Achromobacter* sp. 5B1. Representative images in (b) show how the auxinic signal is progressively diverted to the concave side of the root tip during the coiling process. Images show independent roots during the coiling process, imaged at different times during growth. Close-up images are from the same root.

(c) Graph shows percentage of plants ($n = 18$) inoculated with the bacterium that exhibit this deviation of the auxinic signal towards the concave side of root coil. All inoculated roots showed asymmetrical expression gradient with activation on the concave side. Experiment was repeated in triplicate with similar results. Scale bars represent 200 and 100 μm , respectively.

Figure 6. *Achromobacter* sp. 5B1 represses expression of auxin transporters in provascular cells. (a) Representative images of Arabidopsis seedlings expressing *pPIN1::PIN1-GFP*, *pPIN2::PIN2-GFP*, *pPIN3::PIN3-GFP* and *pPIN7::PIN7-GFP* in plants grown under axenic conditions or co-cultivated with *Achromobacter* sp. 5B1. (b,c) Graphs show the relative fluorescence intensity at (b) the meristem and (c) columella cells. Photographs show representative individuals of at least 10 seedlings. Asterisks indicate that means are statistically different at $P < 0.05$. Experiment was repeated two times with similar results. Scale bar: 50 μ m.



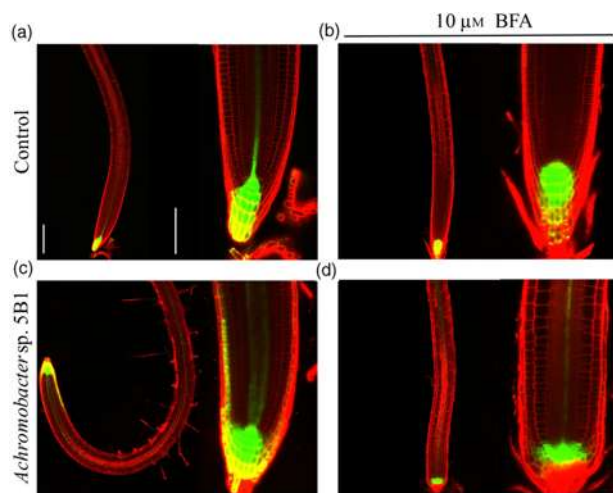


Figure 7. Effect of brefeldin A (BFA) and *Achromobacter* sp. 5B1 in the expression pattern of *DR5:GFP* at the root tip of *Arabidopsis thaliana*.

(a) In axenic conditions the expression pattern of *DR5:GFP* is observed in the provascular bundle, quiescent center and in columella.

(b) Brefeldin A (BFA) blocks auxin transport in provascular bundle and promotes accumulation in columella as evidenced by the increase in signal intensity in this area.

(c) *Achromobacter* sp. 5B1 changes the direction of growth and triggers the *DR5:GFP* signal redistribution to the concave side of coils.

(d) When plants are treated with BFA and co-inoculated with *Achromobacter* sp. 5B1, BFA prevents auxin redistribution. Images shown are representative of 18 plants analyzed per treatment. Scale bars shown represent 200 and 100 μm , respectively.

sp. 5B1, the response of WT (Col-0) seedlings and mutants defective on auxin transport *eir1-1* and *aux1-7*, or response *tir1-1*, *axr1-3*, *slr1-1*, *arf7-1arf19-1* and *tir1-1afb2-3afb3-4* were compared. In plants transferred to fresh media with or without a streak of bacterial inoculum for 6 days, the promotion of root branching was decreased in auxin transport-related mutants, *eir1-1* and *aux1-7* (Figure 8a–d). Nevertheless, these mutants still responded to bacterization and therefore the main difference between mutants and WT seedlings seems to be that the mutants display fewer lateral roots in the absence of an inoculum. A comparable lateral root response was observed for *tir1-1* mutants. Indeed, the bacterium repressed primary root growth in *tir1-1*, *axr1-3*, *slr1-1* and *tir1-1afb2-3afb3-4* mutants. Noteworthy, the primary roots of these mutants had an exaggerated deviation of growth from the gravity response vector and their root tips curled synchronously (Figures 8d and 9a–d). Close-up images from *Arabidopsis* WT and *tir1afb2afb3* primary root tips, show that in the WT as the root navigates over the medium, lateral roots are stimulated to emerge and grow, and thus more branches are formed, whereas in *tir1afb2afb3* mutants, the primary roots develop excessive curling and fail to form lateral roots (Figure S7). This latter response could simply be the consequence of a pleiotropic effect of mutating three auxin receptors on early lateral root primordium initiation.

***Achromobacter* sp. 5B1 improves growth of *Arabidopsis* seedlings under salt stress**

Achromobacter sp. 5B1 was isolated from roots of Mesquite trees that can grow under high salt conditions. To

investigate any possible relevance of the bacterium to plants growing under salt stress, the growth and development of *Arabidopsis* seedlings in medium with increasing salt concentrations was tested *in vitro*, using Petri plates. An initial screening of the growth response included concentrations of 0, 50, 100 and 150 mM NaCl. Salt supplements affected lateral root formation and shoot, root and total biomass production with the 150 mM concentration being highly toxic (Figure S8). From this analysis, we decided to test the responses of axenic and bacterized seedlings to 0 and 100 mM salt supplements. Although primary root growth was similarly inhibited by salt in control and inoculated seedlings, the formation of lateral roots was maintained and the shoot, root and total fresh weight was improved by the bacterium under salt conditions (Figure 10), which indicates a possible functional relevance of the plant probiotic effects of *Achromobacter* sp. 5B1 not only under standard growth conditions but also to resist salinity.

DISCUSSION

Achromobacter species are aerobic gram-negative bacteria with a wide distribution throughout freshwater and soil environments. *Achromobacter xylosoxidans* shows a certain level of nitrogenase activity, indole acetic acid production and P solubilization, and any or some of these traits may account for the plant growth properties of the genus (Jha and Kumar, 2009). However, little is known about how these microbes interact with plants to improve their adaptation to biotic and/or abiotic stress. A major plant response of *Arabidopsis* to root colonization with *Achromobacter* sp. 5B1 was the reconfiguration of root

architecture, characterized by an enhanced primary root growth followed by increasing formation of lateral roots, and these traits could be observed in three plant–bacteria co-cultivation systems: (i) in roots placed over a bacterial

streak, (ii) in plants and bacteria co-cultivated on separated sides of a divided Petri plate, and (iii) placing the bacteria in front of the growing primary root. Thus, plants would greatly benefit from inoculation with selected

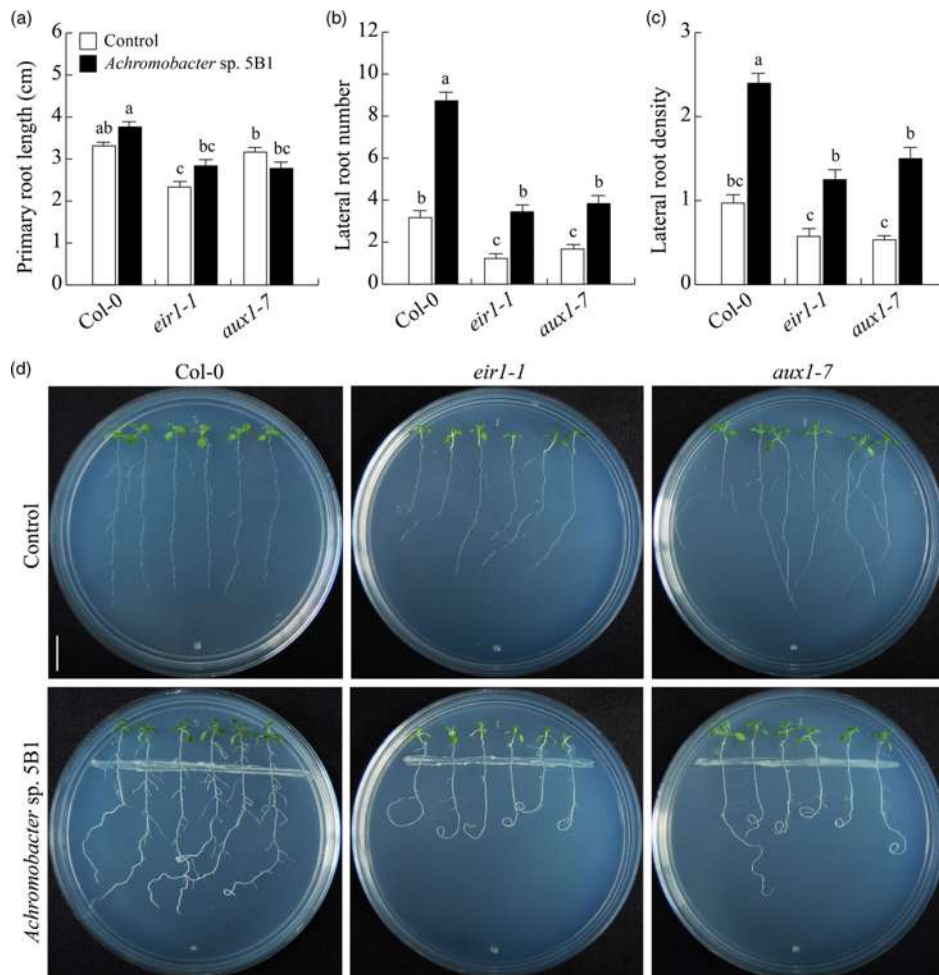


Figure 8. Root response of *Arabidopsis* wild-type and auxin transport-related mutants to colonization by *Achromobacter* sp. 5B1. Wild-type (Col-0) and mutants *eir1-1* and *aux1-7* were germinated and grown on agar-solidified 0.2× MS media, 4 days after germination, transferred to new media with or without a streak of bacterial inoculum for six additional days. (a) Primary root length. (b) Lateral root number. (c) Lateral root density. (d) Representative images of Col-0 and mutant seedlings in control condition and 5B1 treatment (scale bar = 1 cm). Values shown represent the mean ± SD ($n = 18$). Different letters indicate statistical differences from a Tukey analysis ($P < 0.05$). Experiment was repeated three times with similar results.

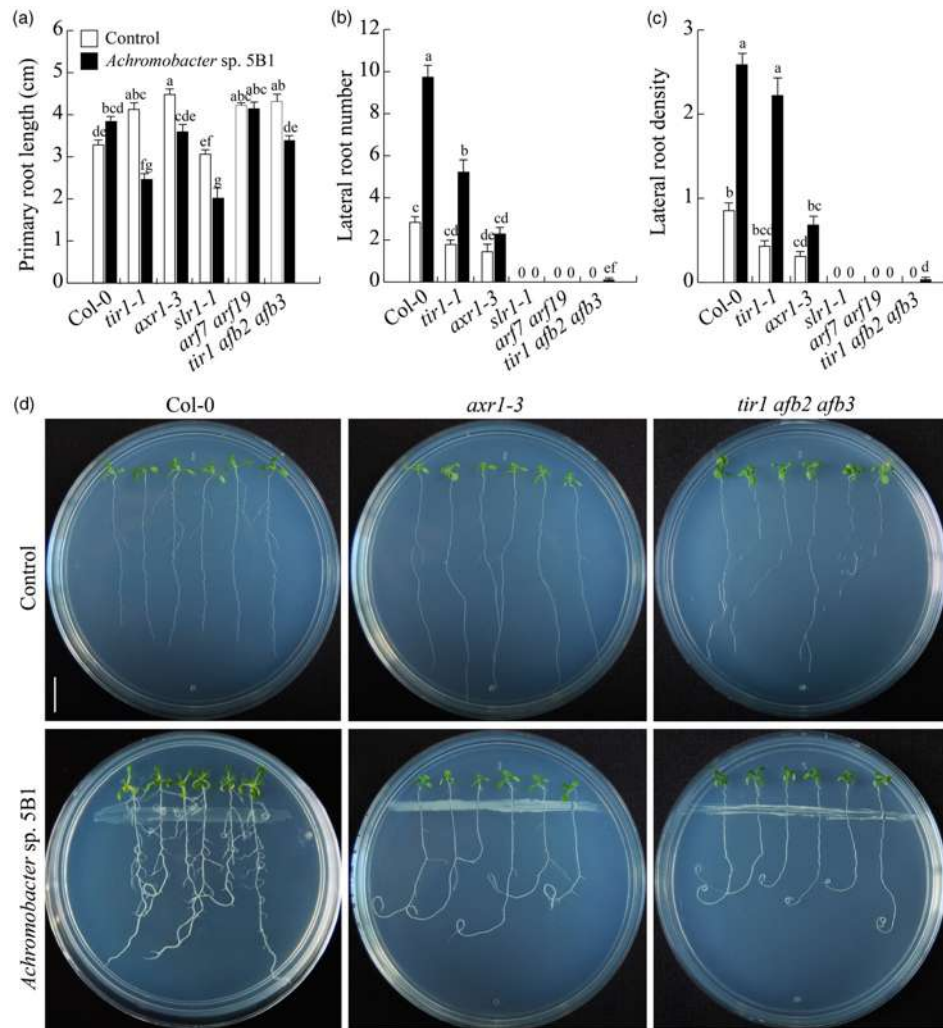


Figure 9. Root response of *Arabidopsis* wild-type and auxin signaling mutants to colonization by *Achromobacter* sp. 5B1. Wild-type (Col-0) and mutants *tir1-1*, *axr1-3*, *slr1-1*, *arf7 arf19-1* and *tir1-1afb2-3afb3-4* seedlings were germinated and grown on agar-solidified 0.2× MS media, 4 days after germination, transferred to new media with or without a streak of bacterial inoculum for six additional days. (a) Primary root length. (b) Lateral root number. (c) Lateral root density. (d) Representative images of Col-0, *tir1-1afb2-3afb3-4* and *axr1-3* in control condition and 5B1 treatment (scale bar = 1 cm). Values shown represent the mean ± SD ($n = 18$). "0" in graphs is used to indicate "0 values." Different letters indicate statistical differences from a Tukey analysis ($P < 0.05$). Experiment was repeated three times with similar results.

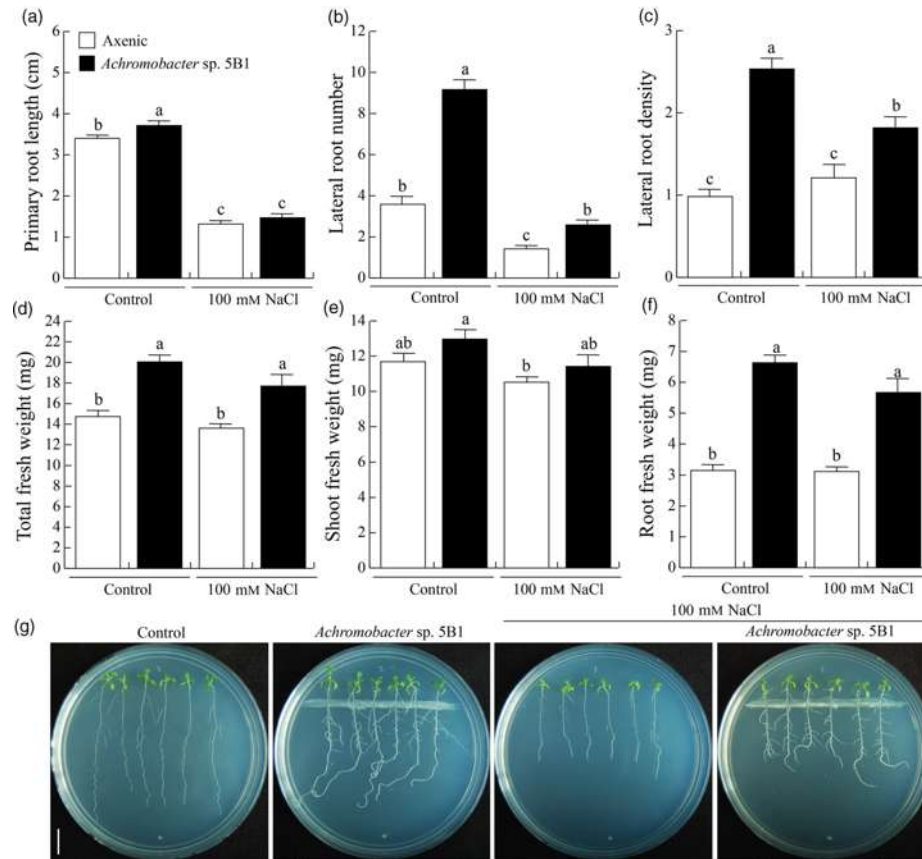


Figure 10. Root architectural response and biomass production of Arabidopsis plants in co-inoculation with *Achromobacter* sp. 5B1 in salinity conditions. Wild-type Arabidopsis (Col-0) seedlings were germinated and grown in solidified MS medium with 0.2% agar and supplemented with 100 mM NaCl, 4 days after germination, transferred to fresh media with or without a streak of bacterial inoculum for six additional days. (a) Primary root length, (b) lateral root number, (c) lateral root density, (d) total fresh weight, (e) shoot fresh weight and (f) root fresh weight. (g) Representative images of plates are shown. Values represent the mean $\pm$ SD ($n = 18$). Different letters indicate statistical differences from a Tukey analysis ($P < 0.05$). Experiment was repeated three times with similar results. Scale bar: 1 cm.

Achromobacter strains, which clearly behave as probiotics both *in vitro* and in an artificial soil as substrate.

Root branching is a highly desirable trait in the search of more cheap, efficient and sustainable agriculture and therefore rhizobacteria application is a promising option to reach this goal. Our results show that *Achromobacter* sp. 5B1 promotes primary root growth and at the same time, it increases root branching via inducing the maturation of lateral root primordia, aspects that differ from the halted

root growth caused by other root-associated bacteria. In previous reports, Arabidopsis seedlings were inoculated *in vitro* with rhizobacteria from several genera, including *Bacillus* and *Pseudomonas*, which manifest halted primary root growth upon sensing of bacterial volatiles or diffusible molecules, and then lateral root growth is stimulated as a compensatory mechanism (López-Bucio *et al.*, 2007; Gutiérrez-Luna *et al.*, 2010; Zamioudis *et al.*, 2013; Stringlis *et al.*, 2018). In this regard, the primary root growth

response to *Achromobacter* sp. 5B1 seems to be unique, and apparently related more to the changes in root navigation that primes roots for enhanced lateral root development, as the induction of lateral roots occurs even when the growth of the main root axis remains dominant.

Interestingly, agravitropic root tip behavior could be only evidenced in roots colonized by the bacteria, but not in plants exposed to volatile organic compounds released by the bacteria on divided Petri plates or when the bacterial streak was placed in front of their growing roots. These results did not support the notion that volatile or diffusible molecules were responsible for the deviation of root growth during the interaction, and therefore an unknown bacterial signature, possibly a microbe-associated molecular pattern or pathogen-associated molecular pattern already present upon physical plant–bacteria contact may explain this particular root behavior. This is based on the assumption that bacterial physiology is constant whether or not it colonizes roots. However, an alternative explanation is that the bacteria colonizing roots have a different physiology and may release compounds that induce root coiling only under this condition.

Root movements are difficult to appreciate as they occur below ground; nevertheless, the study of gravitropism and hydrotropism shed light on the molecular and physiological aspects of root behavior, and these are tightly linked to the phytohormones, auxin and abscisic acid, respectively (Falik *et al.*, 2005; Oliva and Dunand, 2007; Harmer and Brooks, 2018). *Achromobacter* sp. 5B1 influences at least two critical aspects during root architecture reconfiguration, i.e., (i) it enhances auxin responsiveness in lateral root primordia, which correlates with a more efficient emergence of the primordia from the primary root, and (ii) changes the expression of auxin transporters within the primary root tip. Although both responses rely to some level upon auxin signaling, they likely operate via at least two highly contrasting mechanisms, as comparison of primary root growth and lateral root formation in WT and auxin transport or signaling mutants *eir1-1*, *aux1-7*, *tir1-1*, *axr1-3*, *slr1-1*, *arf7-1arf19-1* and *tir1-1afb2-3afb3-4* showed that *eir1-1* and *aux1-7* still respond significantly to inoculation with 5B1 by increased production of lateral roots. The main difference appears to be that the mutants displayed fewer lateral roots even in the absence of an inoculum. Similarly, the *tir1* mutation seems to affect only minimally the induction of lateral root formation by 5B1 inoculation. On the other hand, the primary root growth response to 5B1 inoculation depends upon auxin transport and signaling through *PIN2* and *AUX1* because mutating these genes exacerbates primary root curling, a trait also manifested in auxin signaling mutants.

Auxin transport is possible through the coordinated action of several members from the PIN family of auxin transporters. Our study shows that auxin-responsive gene expression evidenced by *DR5:GFP* shifts from the center of

the primary root (columella initials) to the concave side of the curling root in inoculated plants and this certainly accounts for the deviation of root growth. Confocal imaging revealed the repression of *PIN1* and *PIN7* in the provascular tissue during root navigation elicited by *Achromobacter* sp. 5B1. BFA, a compound that represses the vesicle efflux necessary to place the PIN proteins to the plasma membrane, disrupted root coiling and lateral root formation induced by the 5B1 strain and this was correlated with the halt of *DR5:GFP* redistribution within the root cap. This suggests that the bacterium disrupts the gravitropic response in roots, which may depend on the complex network of PIN proteins acting in concert. Consistently, whereas detection of *PIN1* and *PIN7* is decreased in root provascular tissue, *PIN3* expression was activated in the lower root cap columella cells. It was somewhat surprising that long-term (6 days) BFA treatments decreased *PIN1*, *PIN2* and *PIN7* expression, which differs from previous reports in which BFA treatment induced internalization of *PIN1* and *PIN2* proteins by inhibiting recycling (Geldner *et al.*, 2003). Importantly, when the effects of BFA treatments on *PIN1* localization were investigated in previous reports, Arabidopsis seedlings were treated with BFA for about 1 h (Paciorek *et al.*, 2005; Paponov *et al.*, 2019), whereas in the current research the roots were exposed to BFA for 6 days, and thus this long-term effect is different to short time treatments. Thus, the possibility that changes in *PIN-GFP* signals were dependent on changes of localization of PIN proteins cannot be ruled out.

The auxin transport mutants *eir1-1* and *aux1-7*, which are defective in the *PIN2* auxin efflux transporter, or in the auxin intake permease *AUX1*, respectively, had a supercoiling phenotype, similar to that of auxin signaling mutants. These data indicate that the agravitropic root behavior may be caused by a defective auxin transport to growing parts of the root, namely the meristem and elongation zones, because auxin signaling mutants showed halted primary root growth response when compared with the WT, in which growth was promoted. The fact that the bacterium still causes an increase in root branching in *tir1-1* and *axr3-1*, the only two auxin response mutants that showed some capacity to form lateral roots in control conditions, indicates an auxin-independent mechanism in mediating lateral root maturation by 5B1. The results that some of the other mutants including *slr1-1*, *arf7-1arf19-1* and *tir1-1afb2-3afb3-4* did not show a promotion could simply be the consequence of a pleiotropic effect on early lateral root primordium initiation.

Achromobacter sp. 5B1 was isolated from Mesquite plants growing in a saline soil and it was possible that as part of the natural bacterial populations, it could contribute to an improved nutrient acquisition by modifying root signaling and/or growth patterning or even might confer salt or drought tolerance. Our data support this latter

hypothesis, as *Arabidopsis* seedlings inoculated with the bacterium grew better under salt supplements than the axenically grown seedlings. Recent research identified two halophilic bacteria as *Bacillus* sp. and *Pseudomonas lini* that promoted growth under standard and saline conditions in *Arabidopsis*, cucumber and water melon, and thus rhizobacteria from extreme environments may extend their probiotic traits to horticultural species (Palacio-Rodríguez *et al.*, 2017; Fitzpatrick *et al.*, 2018). As *Achromobacter* sp. 5B1 influences root tip navigation, plant behavior and environmental adaptation, it is possible that other functional adaptive processes such as obstacle avoidance may depend upon the composition of the microbiomes, further research is needed to unveil how these dynamic responses contribute to plant fitness.

EXPERIMENTAL PROCEDURES

Rhizosphere sampling and isolation of rhizobacteria

Bacterial isolates were collected from roots of Mesquite plants (*Prosopis* sp.) growing at a location called "Poza Salada" in the Sobaco Valley, southeast of the Chihuahuan Desert in Coahuila state, México (26°10'54" N and 102°42'24" W). The Poza Salada is a natural sinkhole with saltwater and the surrounding saline soil with EC 10.12 mS cm⁻¹ and pH 8.61. The sampling was conducted in April 2015. Roots were washed, disinfected with 1% sodium hypochlorite and 70% ethanol, rinsed five times with sterile 0.5× phosphate-buffered saline solution, and then macerated in a mortar with 9 ml of phosphate-buffered saline 0.5× solution for endosphere bacteria isolation. For rhizosphere sampling, 10-fold serial dilutions were made and aliquots (100 µl) were plated on media with a contrasting composition: free nitrogen, King B and LB supplemented with 3% NaCl. The plates were incubated at 30°C for 1–3 days, and bacteria were isolated based on colony morphology, propagated in their respective culture media and maintained in 30% glycerol solution at -80°C.

Analysis of the 16S rRNA gene from 5B1 strain

The bacterial isolate 5B1 was grown in LB broth overnight and subsequently DNA isolation was performed as previously described with some modifications (Sambrook *et al.*, 1989). The universal primers 341 forward (341Fw; 5'-AGCGGGGAGGAAGGGAGTAA-3') and 1061 reverse (1061Rv; 5'-ATCGTCAGCTCGTGTGTGA-3') were used to amplify the gene encoding the 16S subunit of ribosomal RNA (16S rRNA gene). The mixture for each reaction was prepared using 32.25 µl of H₂O, 10 µl of 5× Colorless GoTaq[®] Reaction Buffer, 2.5 µl of MgCl₂ (1.5 mM), 1 µl of 341Fw (10 µM), 1 µl of 1061Rv (10 µM), 1 µl of dNTPs (10 mM), 0.25 µl of Taq polymerase to obtain 2.5 units per reaction and 2 µl of DNA (100 ng µl⁻¹) from the bacterial 5B1 strain. The amplification conditions were a denaturation step at 95°C for 2 min, then for 30 cycles: a denaturation at 95°C for 1 min, an alignment at 60°C for 1 min, amplification at 72°C and finally an extension step at 72°C for 3 min (Weisburg *et al.*, 1991). Three microliters of each amplification product were mixed with 2 µl of Gel Red[™] and 5 µl of H₂O, each sample was charged on a 1% agarose horizontal slab gel with TAE 1× and then an electrophoresis was performed at 90 V for 45 min. Subsequently, polymerase chain reaction products were purified using the UltraClean polymerase chain reaction Clean-up Kit (Mo Bio Laboratories, Carlsbad, CA,

USA) and were sent for sequencing to Laboratorio Nacional de Genómica para la Biodiversidad (LANGEBIO, Irapuato, Gto, México). The sequence of 16S rRNA gene from 5B1 was used to download sequences with 99% similarity using the BLAST algorithm from NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>) and all sequences were employed to build the phylogenetic tree in MEGA7 software (http://www.megasoftware.net/download_form) using the neighbor-joining method with 100 bootstrap replications (Felsenstein, 1985; Saitou and Nei, 1987; Kumar *et al.*, 2016). Genetic distances among tree sequences were estimated with the Kimura two-parameter model (Kimura, 1980), and the trees were constructed by randomly adding 10 sequences (10 repetitions) (Nei and Kumar, 2000).

Plant material and growth conditions

WT *A. thaliana* seeds (ecotype Columbia-0, Col-0) and mutants *eir1-1* (Roman *et al.*, 1995), *aux1-7* (Pickett *et al.*, 1990), *tir1-1* (Parry *et al.*, 2009), *tir1-1afb2-3afb3-4* (Parry *et al.*, 2009), *axr1-3* (Estelle and Somerville, 1987), *slr1-1* (Fukaki *et al.*, 2002) and *arf7-1arf19-1* (Okushima *et al.*, 2005), besides transgenic lines *CycB1::uidA* (Colón-Carmona *et al.*, 1999), *DR5::GFP* (Ottenschläger *et al.*, 2003), *pPIN1::PIN1-GFP* (Benkova *et al.*, 2003), *pPIN2::PIN2-GFP*, *pPIN3::PIN3-GFP* and *pPIN7::PIN7-GFP* (Bilou *et al.*, 2005), were used in the different experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 4 min and 10% (v/v) commercial chloride solution for 4 min. After five washes with sterile distilled water, seeds were incubated in darkness at 4°C for 48 h in sterilized distilled water. Seeds were sown on plates containing 0.2× Murashige and Skoog Basal Salt Mixture (MS salts) supplemented with 0.6% (w/v) sucrose and 1% (w/v) phytagar, pH was adjusted at 7.0. The suggested formulation of MS salts is 4.3 g L⁻¹ for a 1× concentration, then, we used 0.9 g L⁻¹ for 0.2× concentration. MS salts and phytagar were purchased from Phytotechnology Laboratories (Shawnee Mission, KS, USA), and sucrose was purchased from J. T. Baker (Phillipsburg, NJ, USA). Plates with germinating seeds were incubated at a plant growth chamber (Percival Scientific AR-95L, Perry, IA, USA) with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 100 µmol m⁻² sec⁻¹, and temperature of 22°C.

Plant-bacteria co-cultivation

Seedlings of 4 days after germination were transferred to 0.2× MS media with or without a streak of bacterial inoculum from isolated colonies on LB solidified media. Bacteriological agar was purchased from BD Bioxon and LB broth was purchased from Phytotechnology Laboratories[®]. For direct contact, six seedlings per plate were located by placing their roots over the streak whereby the shoots were approximately 1 cm above the inoculum. For emission of volatile compounds, six seedlings were placed on the right side of a plate with one division and the streak on the left side. For diffusible compounds, six seedlings were located whereby their primary root tips were approximately 5 cm above the inoculum. Marks were placed on the plates at the location of the primary root tips at the transfer moment and the growth analyzed 6 days later. The plates were incubated in a plant growth chamber AR-95L (Percival Scientific) with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 100 µmol m⁻² sec⁻¹ and temperature of 22°C.

The interaction between *Arabidopsis* and *Achromobacter* sp. 5B1 was assessed in medium supplemented with 0, 5 and 10 µM BFA. In this case, the compound was supplemented directly to the 0.2× MS growth medium before the transfer of seedlings 4 days after germination over a streak of bacterial inoculum and growth allowed for 6 additional days.

Growth in artificial soil

Seedlings 10 days after germination grown in the direct contact system described in the previous section were transferred to boxes with an artificial soil mixture composed of peat moss (Sunshine®, Agawam, MA, USA), perlite and vermiculite with a 3:1:1 ratio, as well as 300 ml of MS 0.1× nutrient solution. Boxes with growing plants were incubated in a plant growth chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light and 8 h of darkness, light intensity of $100 \mu\text{mol m}^{-2} \text{sec}^{-1}$ and temperature of 22°C. During the incubation period, each box was watered once a week with 300 ml of distilled sterilized water.

Histochemical analysis of GUS activity

Transgenic Arabidopsis seedlings expressing the *uidA* (*GUS*) reporter gene were incubated in a microtiter plate with 0.1% 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) in a phosphate buffer at pH 7 (0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4) with 2 mM $\text{K}_4\text{Fe}(\text{CN})_6$ and 2 mM $\text{K}_3\text{Fe}(\text{CN})_6$ overnight at 37°C (Jefferson *et al.*, 1987). After removing X-Gluc solution, seedlings were incubated during 60 min at 62°C with an acid solution (0.24 N HCl in 20% methanol [v/v]). The acid solution was substituted by a basic solution (7% NaOH [w/v] in 60% ethanol [v/v]) for 20 min at room temperature. After throwing away the basic solution, seedlings were dehydrated with ethanol treatments at 40%, 20% and 10% (v/v) for 20 min each, and fixed with 50% glycerol (v/v) (Malamy and Benfey, 1997). X-Gluc was purchased from Phytotechnology Laboratories®. The fixed roots were placed on glass slips, covered with coverslips and sealed with commercial nail varnish; subsequently they were photographed with a Nomarski microscope (Leica DFC450C, Leica Microsystems, Wetzlar, Germany). For each treatment, 10 seedlings were analyzed.

Analysis of GFP activity

Transgenic Arabidopsis seedlings expressing the *GFP* reporter gene were incubated in 10 mg ml^{-1} propidium iodide over 1 min. Propidium iodide was purchased from Sigma-Aldrich (St. Louis, MO, USA). The seedlings were rinsed in water and mounted with 50% glycerol on glass slips and covered with coverslips. Each sample was analyzed separately for propidium iodide (with an excitation at 568 nm and an emission window at 585–610 nm) and GFP fluorescence (with an excitation at 488 nm and an emission window at 505–550 nm) using a confocal microscope (Olympus FV1200, Tokyo, Japan) after which the two micrographs were merged to produce a final image. For each treatment, 10 independent seedlings were analyzed.

Plant growth analysis

To evaluate *in vitro* growth, elongation of the primary roots during the interaction with 5B1 were measured with a ruler, and lateral roots present in this primary root segment were recorded using a stereomicroscope (Leica MZ6) at $10\times$ magnification, and the lateral root density was calculated by dividing the lateral root number by primary root length in 18 individuals of each growth condition. The total fresh weight, shoot fresh weight and root fresh weight of six seedlings grown on the same plate were measured with an analytical balance (Ohaus, Parsippany, NJ, USA) and three plates of each growth condition were evaluated. Ten micrographs (photographs acquired in the microscope) of each growth condition were analyzed in ImageJ software (<http://rsbweb.nih.gov/ij/>) to determine how many cells expressed the reporter gene. To evaluate the growth in

soil, stem length was measured with a ruler and siliques were counted, while total seed production was calculated from the average number of seeds in 10 siliques harvested in the middle zone of shoot and total siliques in four plants of each growth condition. In all experiments, the dataset was statistically analyzed with STATISTICA 10.0 program (Dell StatSoft, Austin, Texas, USA). One-way ANOVA or two-way ANOVA were performed and then data are analyzed with the Tukey's *post hoc* test. Different letters were used to indicate means that differ significantly ($P < 0.05$).

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AUTHOR CONTRIBUTIONS

KRJ and JLB planned and designed the research. BPPA, JLCA and JSM conducted fieldwork and analyzed the data. EGC, SBO, ROC and LFRH performed the experiments, and analyzed data. KRJV, SBO and JLB wrote the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its Supporting Information.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Phenotype of *Arabidopsis thaliana* seedlings co-inoculated with *Achromobacter* sp. 5B1.

Figure S2. Effects of *Achromobacter* sp. 5B1 on growth and root architecture of *Arabidopsis thaliana* seedlings grown in divided Petri plates.

Figure S3. Influence of *Achromobacter* sp. 5B1 co-cultivated in the front of the primary root in growth and root system architecture.

Figure S4. Development of the lateral root primordia (LRP) and lateral roots (RL) in *Arabidopsis* plants co-inoculated with *Achromobacter* sp. 5B1.

Figure S5. Effect of brefeldin A (BFA) on roots of *Arabidopsis thaliana* co-cultivated with *Achromobacter* sp. 5B1.

Figure S6. Expression pattern of PIN transporters in primary root tips of plants co-cultivated or not with *Achromobacter* sp. 5B1 and treated with brefeldin A.

Figure S7. *Achromobacter* sp. 5B1 on growth of *Arabidopsis* WT and *tir1afb2afb3* primary roots.

Figure S8. Effects of salt on root architecture and biomass production in *Arabidopsis thaliana* seedlings.

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Screening of Phosphate Solubilization Identifies Six *Pseudomonas* Species with Contrasting Phytostimulation Properties in *Arabidopsis* Seedlings

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Abstract

The interaction of plants with bacteria and the long-term success of their adaptation to challenging environments depend upon critical traits that include nutrient solubilization, remodeling of root architecture, and modulation of host hormonal status. To examine whether bacterial promotion of phosphate solubilization, root branching and the host auxin response may account for plant growth, we isolated and characterized ten bacterial strains based on their high capability to solubilize calcium phosphate. All strains could be grouped into six *Pseudomonas* species, namely *P. brassicae*, *P. baetica*, *P. laurylsulfatiphila*, *P. chlororaphis*, *P. lurida*, and *P. extremorientalis* via 16S rRNA molecular analyses. A *Solibacillus isronensis* strain was also identified, which remained neutral when interacting with *Arabidopsis* roots, and thus could be used as inoculation control. The interaction of *Arabidopsis* seedlings with bacterial streaks from pure cultures in vitro indicated that their phytostimulation properties largely differ, since *P. brassicae* and *P. laurylsulfatiphila* strongly increased shoot and root biomass, whereas the other species did not. Most bacterial isolates, except *P. chlororaphis* promoted lateral root formation, and *P. lurida* and *P. chlororaphis* strongly enhanced expression of the auxin-inducible gene construct *DR5::GUS* in roots, but the most bioactive probiotic bacterium *P. brassicae* could not enhance the auxin response. Inoculation with *P. brassicae* and *P. lurida* improved shoot and root growth in medium supplemented with calcium phosphate as the sole Pi source. Collectively, our data indicate the differential responses of *Arabidopsis* seedlings to inoculation with several *Pseudomonas* species and highlight the potential of *P. brassicae* to manage phosphate nutrition and plant growth in a more eco-friendly manner.

Keywords Phosphate-Solubilizing Bacteria · *Pseudomonas* spp · Plant Growth Promotion · Root Branching · *Arabidopsis thaliana*

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Introduction

Plants interact with bacteria in many ways and depend on their microbial partners to grow and resist environmental challenges. Among the several bacterial properties that account for an efficient phytostimulation, nutrient solubilization, modification of root growth and branching, and modulation of the hormonal status through production of metabolites and endogenous plant hormones may lead to an improved biomass production [1–4].

Among the essential macronutrients, phosphate (P_i) availability critically determines plant success in natural and agricultural ecosystems. In acid soils, P_i combines with aluminum and iron; whereas in alkaline-calcareous soils, P_i binds to calcium; in both cases, the corresponding cations precipitate P_i.

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making it unavailable for uptake by roots [5]. The potential of the bacterial microbiota to assist plants to more efficiently solubilize phosphate may be a highly desirable trait to improve not only plant nutrition, but also to optimize fertilizer usage and plant production in the long term [6, 7]. However, how phosphate-solubilizing bacteria may influence plant processes such as root growth and morphogenesis and their possible effects on plant hormonal status remain largely unknown.

Bacteria may produce quorum-sensing signals, secondary metabolites, and endogenous plant hormones, all of which contribute to root growth and branching [2, 8]. The production of auxin or molecules that mimic auxin action impairs primary root growth while improving root branching, and it appears to be the major mechanism by which *P. aeruginosa*, *P. putida*, and *P. fluorescens* affect plant growth and development [9, 10]. Comparable results have also been obtained in interactions with *Azospirillum brasilense* [11, 12] and *Micrococcus luteus* [13], which repress cell division and elongation of the primary root while triggering more pericycle cells to produce lateral roots that further extend the root absorptive potential.

In a recent study of the Gram negative bacteria *Achromobacter* sp. 5B1 that inhabits the rhizosphere of mesquite plants in a saline soil, root colonization could promote primary root growth, deviated the growth direction making waves and coils, and enhanced the initiation of lateral roots. These effects were attributed to auxin redistribution at the root meristem [14]. The slow root movements driven by the bacteria not only helped plants to better resist salt stress, but also contributed to an enhanced biomass production [14]. These results suggest that auxin transport and signaling are influenced by bacterial traits, which change root coiling and waving.

In this report, we regarded the P_i -solubilizing trait as a major bacterial property potentially responsible for plant growth promotion. We employed plant-bacteria co-cultivation systems to assess its relevance to improved *Arabidopsis* biomass production and altered auxin-directed root morphogenesis. In media designed to support optimal seedling growth or using calcium phosphate as the only P_i source, six bacterial species belonging to the *Pseudomonas* genus were discovered, which differ in their phyto-stimulation properties and their regulation of root branching irrespective of their common P_i -solubilizing attributes. Moreover, we identified *P. brassicae* as a plant growth-promoting species that boosts plant biomass production and growth in medium with sparingly available phosphate.

Materials and Methods

Plant Growth and Development

Arabidopsis thaliana wild-type Columbia-0 (Col-0) and transgenic line *DR5::uidA* [15] were used for experiments.

Arabidopsis seeds were superficially disinfected with ethanol 96% (v/v) for 7 min, followed by chlorine 20% (v/v) for 7 min and five rinses with sterilized distilled water. Seeds were stored at 4 °C for 48 h. Then, disinfected seeds were germinated and grown over the surface of 0.2×Murashige and Skoog (MS) agar medium [16] in Petri plates, which were incubated vertically in a Percival growth chamber (Percival Scientific AR95L) at 22 °C, with 80% humidity, 16-/8-h light/darkness photoperiod, and light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Four days after germination, the Petri plates were opened and the seedlings were transferred onto fresh 0.2×MS agar medium, previously streaked with pure cultures of each bacterial isolate under axenic conditions. The primary root was placed over the streak and biomass and root architecture analyzed 6 days after transfer. To assess growth of plants with sparingly soluble phosphate, the potassium phosphate from the original recipe was replaced by 1 mM calcium phosphate. Plant growth and developmental parameters were recorded 15 days after transfer over the bacterial streak and included measurements of primary and lateral root length using a ruler, whereas the lateral root number was counted using a Leica L2 stereomicroscope. The fresh weight of root and shoot systems was quantified using an OHAUS Adventurer™ analytical scale.

Bacterial Isolation

To obtain and identify the pure bacterial cultures, 100 g of soil was collected from 4 different sites showing different degrees of perturbation, near the community of Ajuno, Michoacán state, México (19°30'46"N, 101°43'20"W). The sampled zones included an avocado farmland, a preserved pine forest, a preserved oak forest, and a soil patch affected by forest fires. We also sampled earthworm humus enriched with disposed domestic residual fruit peels and vegetables, this latter as an organic, nutrient rich substrate that in principle would increase bacterial performance. All samples were stored at 4 °C. Serial dilutions were performed with 1 g of soil or substrate in 9 ml of distilled, sterilized water. A sample of 100 μl was taken from each dilution and streaked into Petri dishes containing Luria Bertani (LB) solid medium at 30 °C. The isolated colonies were collected and streaked into LB media at 30 °C for 72 h. Finally, pure cultures of the isolated bacteria were conserved in glycerol 20% (v/v) and liquid LB medium at −80 °C.

Phosphate-Solubilizing Assay

The phosphate-solubilizing capacity of the bacterial isolates was assayed in pure bacterial cultures pre-grown overnight in Luria–Bertani (LB) medium and then cultured on Pikovskaya-bromophenol blue (PKV-BMP) medium at 0.025 OD600 ($n = 3$) according to Tzecz-Interian

et al. (2020) [17]. The cultures were incubated at 30 °C, 180 rpm for 48 h, harvested by centrifugation at 8000 rpm for 5 min, and the supernatant analyzed at 590 nm by using an Epoch2 (Biotek) spectrophotometer. The experiment was repeated twice and included six biological replicates each.

Molecular Characterization and Phylogenetic Analysis

Extraction of genomic DNA from pure bacterial cultures was done following a standardized protocol [17]. The 16S rRNA gene was amplified with forward and reverse primers 27F (3'-AGAGTTTGATCCTGGCTCAG-5') and 1492R (3'-TAC CTTGTTACGACTT-5'). The amplicons were sequenced in the LABSERGEN capillary sequencing service unit of LANGE BIO, CINVESTAV, México. Chimeric sequences, as identified by DECIPHER v2.0 [18], were discarded. The closest homologues of the remaining sequences were identified using the NCBI BLAST algorithm [19] and SILVA rRNA database [20]. The phylogenetic relationships were further confirmed by using the Ribosomal Database Project (RDP) tools [21]. We used the Classifier [22] and the Sequence Match [23, 24] tools. Phylogenetic reconstructions were performed using MEGA7 [25] and the free trial of Geneious, Bioinformatics Software for Sequence Data Analysis (<https://www.geneious.com>). 16S rRNA sequences obtained from the isolates were deposited in GenBank (Accession numbers: OK031074-OK031083).

Histochemical Determination of GUS Activity

Arabidopsis seedlings expressing the synthetic auxin-responsive promoter *DR5::uidA* were used to study the auxin response in direct root-bacteria interactions. The transgenic seedlings were immersed in a solution of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) in sodium phosphate 100 mM buffer, pH 7, supplemented with 2 mM $K_4Fe(CN)_6$ and 2 mM $K_3Fe(CN)_6$ at 37 °C and incubated overnight. Tissue clearing was done by placing the seedlings in 2 ml solution with methanol 20% (v/v) and HCl 0.24 N final concentrations, incubating at 60 °C for 45 min; after careful removal of the solution using 1 ml micropipette tip, NaOH 7% was added to cover the seedlings and maintained at room temperature for 30 min, and then the solution was discarded and the seedlings were washed with ethanol 40, 20, and 10% (v/v) for 30 min each, and finally stored with glycerol 50% (v/v) at 4 °C [26]. Plant tissues were fixed on clean glass slides with glycerol 50% (v/v), and representative images were taken using the Nomarsky optics in a Leica DM5000B microscope at 20 $\times$ magnifications.

Statistical Analysis

All the treatments were performed in triplicate, and yielded comparable results. Data processing was made through STATISTICA 10 StatSoft program with factorial ANOVA, and Tukey's HSD method. Different letters are used to indicate means that statistically differ ($P < 0.05$).

Results

Screening for Phosphate-Solubilizing Bacteria from Different Environments

Phosphate-solubilizing attribute may be desirable for agriculture. To understand whether this property may be found in soil bacteria from different natural environments, we screened 266 bacterial isolates from pure cultures coming from five different environments: an agricultural soil with avocado trees, an undisturbed soil from an oak grove, a soil from a well-conserved pine forest, as well as sampling earthworm humus, and ash from a 2-year-old fire in a forest patch (Table 1). The idea was to collect as much as possible of the bacterial diversity inhabiting all these different environments. From the pure cultures, individual isolates were further selected using Pikovskaya-bromophenol blue (PKB-BMP) medium for screening P_i -solubilizing microorganisms [27, 28]. Nine strains, H4-34, H4-45, H4-16, ASH21, 11H4-5, 3T4-12, 3T4-20, 9B3-17, and 3T4-21, manifested strong phosphate solubilization when cultured on PKV-BMP agar medium at 30 °C for 48 h, as revealed by clear halos surrounding the cultures (Fig. 1a). The isolate 2C3-28 did not show clear halos when compared to the other strains and represents a good control for P_i -solubilizing properties (Fig. 1a). P_i -solubilizing trait was further confirmed in PKV-BMP liquid medium, where all strains except 2C3-28 manifested strong P_i solubilization (Fig. 1b). In time-course

Table 1 Description of bacterial isolates, their predicted species based on 16S rRNA analysis and site of origin

Isolate	Predicted species	Origin
H4-34	<i>P. brassicae</i>	Earthworm humus
H4-45	<i>P. brassicae</i>	Earthworm humus
H4-16	<i>P. brassicae</i>	Earthworm humus
2C3-28	<i>S. isronensis</i>	Avocado farmland
ASH-21	<i>P. lurida</i>	Soil patch affected by forest fires
11H4-5	<i>P. extremorientalis</i>	Earthworm humus
3T4-12	<i>P. chlororaphis</i>	Well-preserved oak forest
3T4-20	<i>P. chlororaphis</i>	Well-preserved oak forest
9B3-17	<i>P. laurylsulfatiphila</i>	Well-preserved pine forest
3T4-21	<i>P. baetica</i>	Well-preserved oak forest

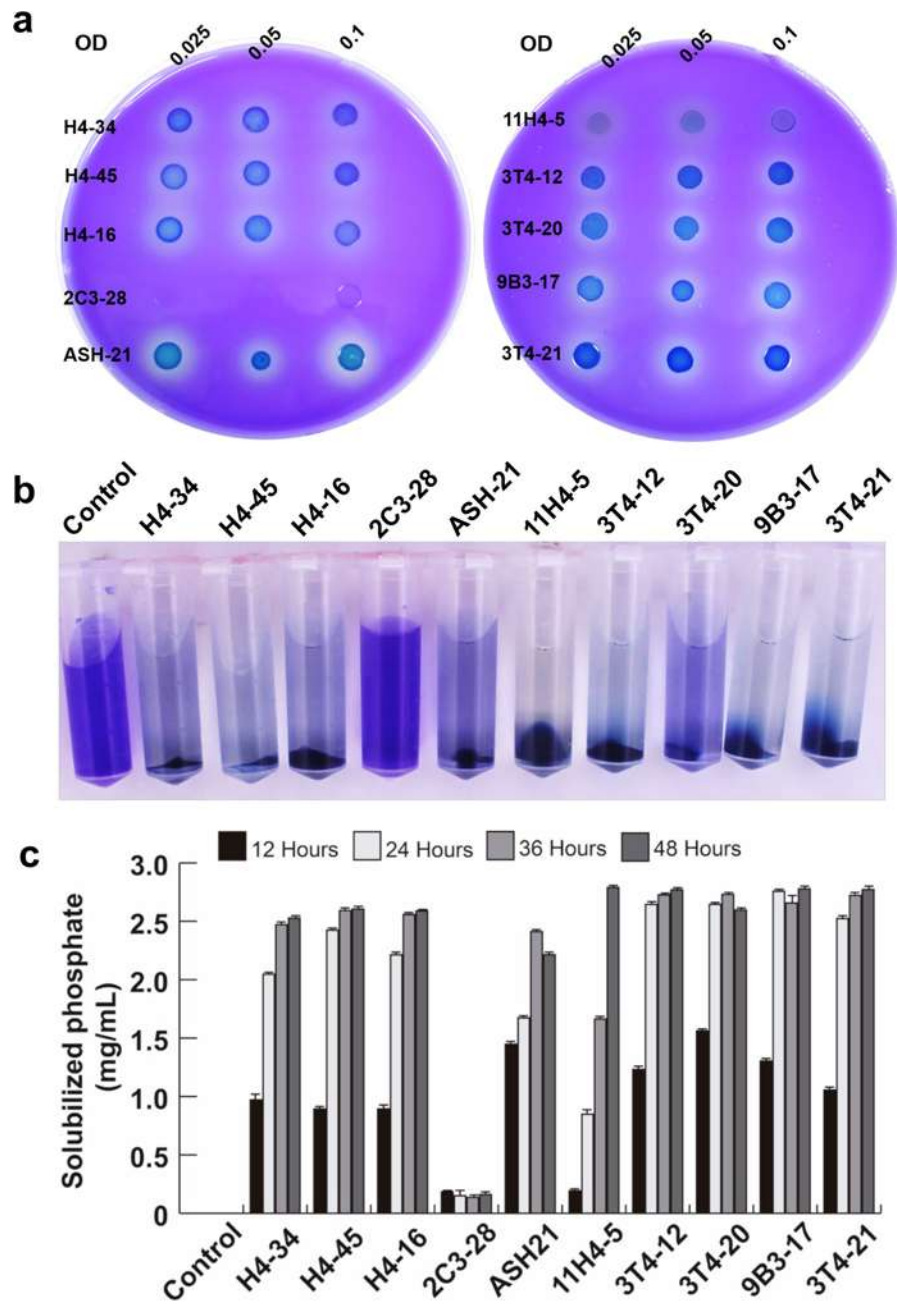


Fig. 1 The effects of bacterial isolates on phosphate solubilization. **a** Bacterial isolates were cultured on PKV-BMP medium supplemented with agar at 30 °C for 48 h. **b** Bacterial isolates were cultured on PKV-BMP liquid medium starting at 0.025 OD₆₀₀ at 30 °C, 180 rpm for 48 h. **c** Solubilized phosphate. The data represent the mean $\pm$ SD ($n=3$). The experiment was repeated twice with similar results

analyses, P_i solubilization started at 12 h and was maximum after 36 h (Fig. 1c). These data evidence the P_i-solubilizing properties of the bacteria isolated from the above-mentioned environments.

Molecular Characterization of Highly Efficient Phosphate-Solubilizing Bacteria

In order to identify the different isolates, including the poorly P_i-solubilizing strain 2C3-28, their 16S rRNA genes were sequenced and compared with public database sequences to establish phylogenetic relationships. The closest homologues for Pi-solubilizing strains were from *Pseudomonas brassicae* (H4-34, H4-45, and H4-16 isolates), *Pseudomonas lurida* (ASH21), *Pseudomonas extremorientalis* (11H4-5), *Pseudomonas chlororaphis* (3T4-12 and 3T4-20), *Pseudomonas laurylsulfatiphila* (9B3-17), and *Pseudomonas baetica* (3T4-21) (Fig. 2a), whereas 2C3-28 was close to *Solibacillus isronensis* (Fig. 2b). From the tree illustration, there is an *S. kalamii* and an *S. silvestris* sequence equally close to *S. isronensis*. It was of interest that all Pi-solubilizing strains belong to the genus *Pseudomonas* (Fig. 2a), suggesting the ubiquity of this trait amongst this group.

Arabidopsis Biomass Production upon Root Contact with Bacterial Streaks

To investigate if the already isolated bacteria could promote plant growth along with their high Pi-solubilizing capability [29], *Arabidopsis* seedlings were germinated and grown for 5 days on agar plates supplemented with 0.2 $\times$ MS agar medium and then transferred over single bacterial streaks from each bacterial species and incubated for six additional days. Notably, seedlings in contact with *P. brassicae* and *P. laurylsulfatiphila* affiliates had 2-to 3.5-fold greater shoot biomass than seedlings not in contact with bacterial streaks, whereas the isolates related to *P. chlororaphis*, *P. lurida*, *P. extremorientalis*, *P. baetica*, and *Solibacillus isronensis* had no significant effect (Fig. 3a). For root biomass production, *P. brassicae*, *P. extremorientalis*, and *P. laurylsulfatiphila* affiliates were inducers, whereas the other isolates were not (Fig. 3b). Thus, an enhanced capability to solubilize Pi did not correlate with plant growth promotion in a medium where nutrient supplements are adequate for plant growth.

Regulation of Root Architecture upon Root Contact with Bacterial Streaks

The spread of bacteria over the root system could explain the different results among strains, and possibly may configure root growth and branching [13, 14]. We assessed root system traits including primary root length, lateral root number, lateral root density, and lateral root length in bacterized seedlings six days after contact with streaks. Six *Pseudomonas* isolates repressed primary root growth by 50–60% (Fig. 4a), while increasing root branching (Fig. 4a,b). *P. baetica*, *P. laurylsulfatiphila*, and *P. brassicae* were the most bioactive species; plants incubated with these reached fourfold to sixfold higher lateral root number and density than uninoculated seedlings (Fig. 4b,c). *S. isronensis* did not affect any of the above-mentioned plant traits and *P. chlororaphis* strongly repressed overall root system architecture development. Figure 5 shows representative images of *Arabidopsis* seedlings grown axenically in the 0.2 $\times$ MS agar medium, and transferred or not over bacterial streaks of *P. brassicae*, *P. lurida*, and *P. chlororaphis* after 6 days of interaction, which highlights the highly contrasting effects of bacterial inoculation.

Induction of Auxin-Responsive Gene Expression by Selected Phosphate-Solubilizing Bacteria

Suppression of primary root growth and enhancement of lateral root formation is a common effect of inoculation with auxin-producing bacteria, for instance *Azospirillum brasilense* and *Micrococcus luteus* [11, 13]. To analyze if the changes in root architecture driven by Pi-solubilizing *Pseudomonas* species could be explained by an enhanced auxin response; *Arabidopsis* seedlings expressing the auxin-inducible reporter gene *DR5::GUS* were inoculated with *P. brassicae*, *P. lurida*, and *P. chlororaphis*, which manifested the most contrasting effects in the plant. Unexpectedly, the strong growth promotion elicited by *P. brassicae* did not correlate with an enhanced histochemical detection of GUS activity (Fig. 6a-d), while seedlings co-cultivated with *P. lurida* and *P. chlororaphis* had a strong induction of the marker over the entire root system (Fig. 6e-h). These results show that although the different bacterial isolates share the property to solubilize phosphate, they clearly differ in modulating auxin-inducible gene expression in the plant host.

Plant Growth Promotion by Selected Phosphate-Solubilizing Bacteria in Medium with Calcium Phosphate

Most agricultural soils are characterized by poor phosphate availability, which can be explained by the strong fixation of this macronutrient with cations. In alkaline-calcareous

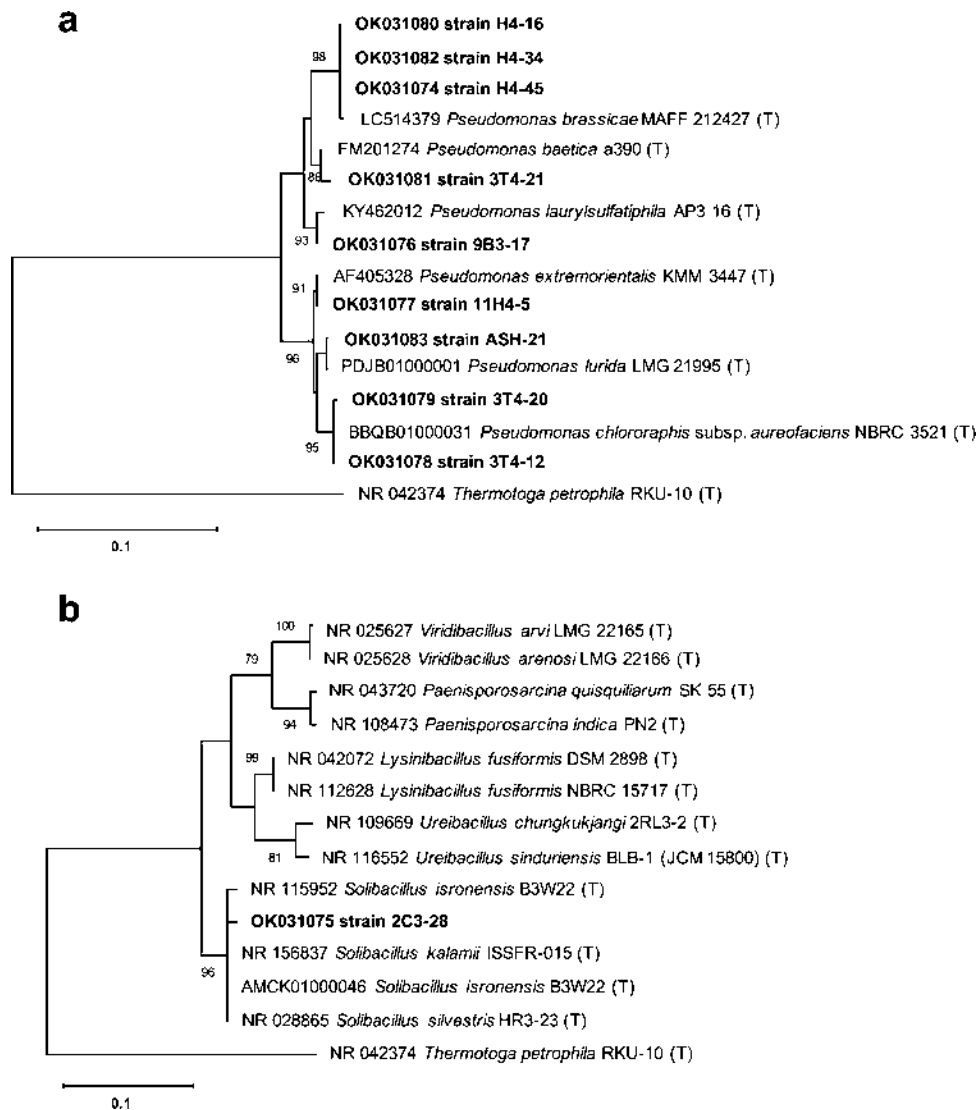
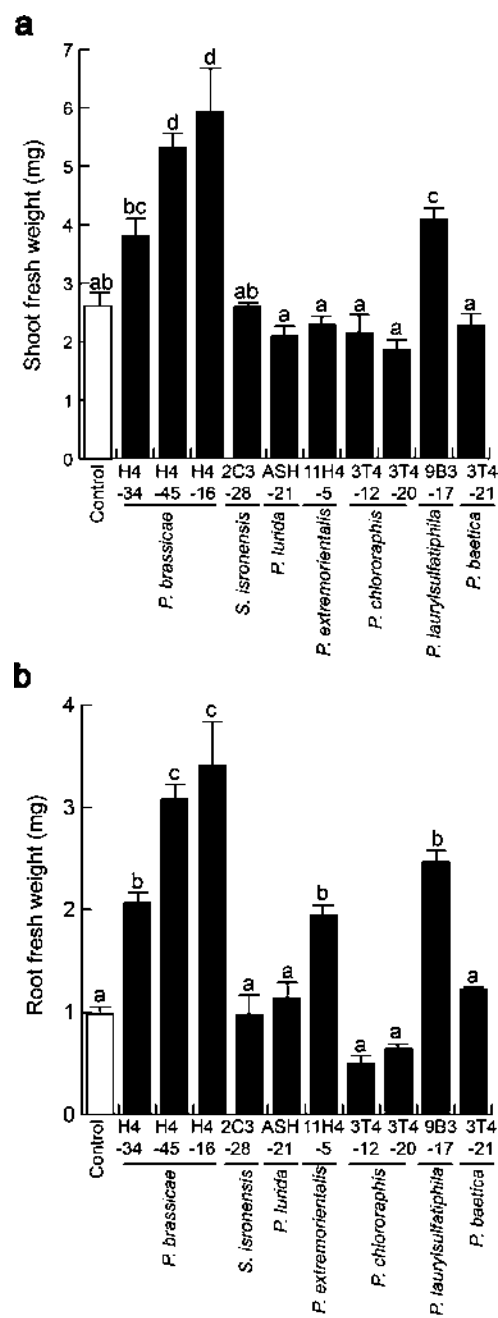


Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences of bacterial strains and additional references were generated using the maximum likelihood method. **a** *Pseudomonas* cluster. **b** *Solibacillus isronensis*. Bootstrap values from 1000 replications are shown as per-

centages at branch points. 16S rRNA sequence of NR 042,374 *Thermotoga petrophila* was used as the outgroup. Scale bar 0.1 substitutions per site

Fig. 3 The effect of phosphate-solubilizing bacteria on *Arabidopsis* seedling biomass production. **a** Shoot fresh weight. **b** Root fresh weight. Data points indicate mean $\pm$ SD ($n=18$). Different letters mean statistically significant differences at $p<0.05$. Tukey post hoc $p<0.05$



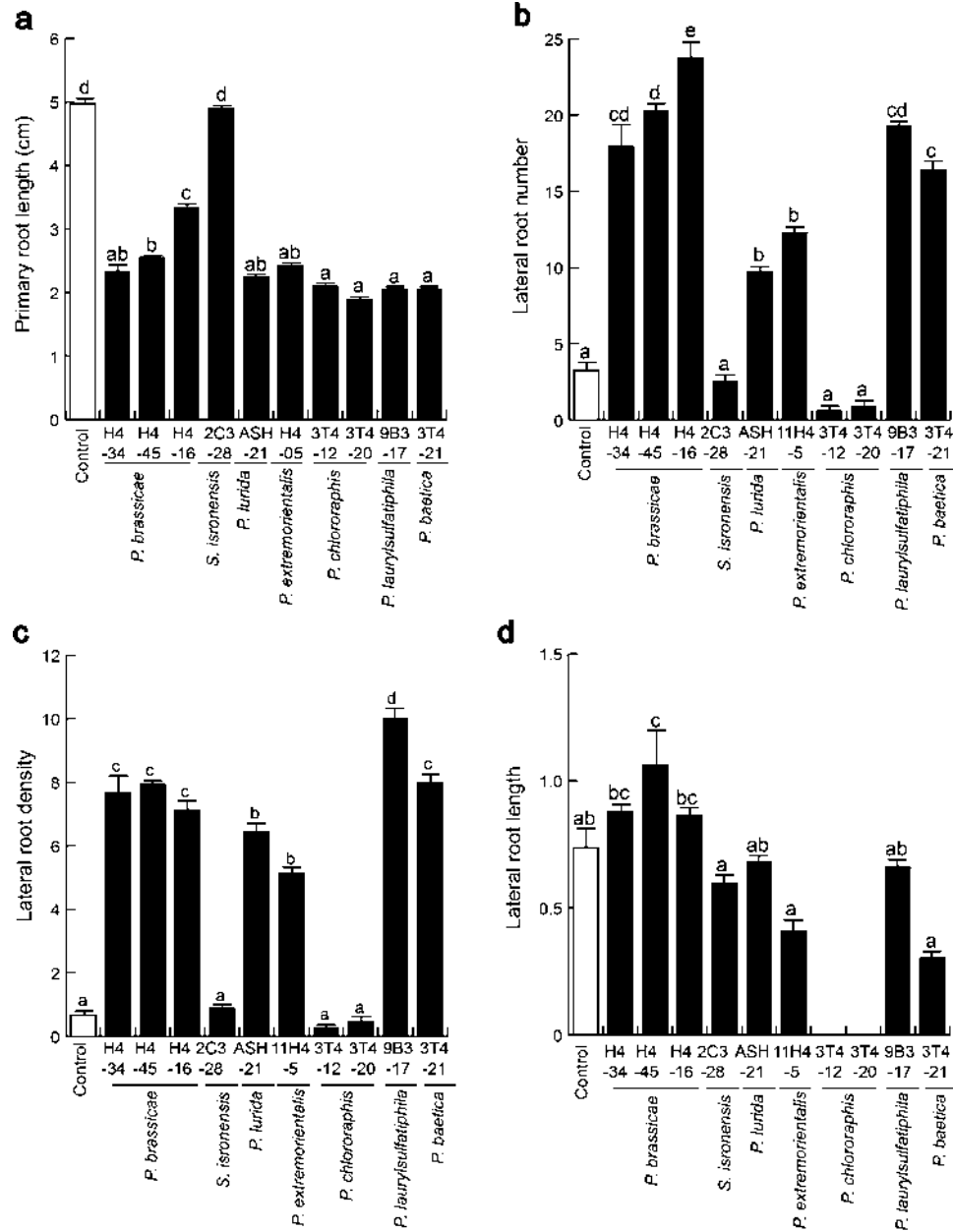


Fig. 4 Root system architecture of bacterized seedlings. Changes in root architecture were registered 6 days after bacterial exposure. **a** Primary root length. **b** Lateral root number. **c** Lateral root density. **d** Lateral root length. Data points indicate mean $\pm$ SD ($n=18$). Different letters mean statistical differences. Tukey post hoc with $p<0.05$

soils, P_i binds calcium to form sparingly soluble minerals [30]. To analyze if the root inoculation with P_i -solubilizing *Pseudomonas* or non P_i -solubilizing *Solibacillus isronensis* could provide an advantage to plants grown in medium supplemented with poorly soluble calcium phosphate as the sole P_i source, the basal MS $0.2\times$ medium was modified via substitution of highly soluble potassium phosphate by low soluble calcium phosphate and growth and development of *Arabidopsis* seedlings was examined. The growth of the un-inoculated seedlings or seedlings inoculated with *S. isronensis* in the calcium phosphate medium was limited and the shoot and root growth was drastically compromised (Fig. 7a,b). By contrast, inoculation with *P. brassicae* or *P. lurida*, but not *P. chlororaphis*, restored shoot and root growth, indicating their potential to help plants to grow and utilize a sparingly available P_i source (Fig. 7c-e). Comparison of primary root growth suggested that both *P. brassicae* and *P. lurida* were equally efficient in supporting P_i usage (Fig. 7f). These data unveil the contrasting potential of P_i -solubilizing *Pseudomonas* as plant growth promoters under stressful conditions.

Discussion

To gain further insights into the correlation of P_i solubilization with other important traits that support plant growth, such as root architecture remodeling, endogenous auxin response and ultimately, growth of plants under sparingly soluble phosphate, we screened a collection of 266 bacterial isolates from diverse environments to select strains with high P_i -solubilizing properties. Our analysis identified nine isolates that were grouped into six different species all belonging to the *Pseudomonas* genus. We also identified a strain close to *Solibacillus isronensis*, a bacterial species with poor P_i -solubilizing capacity, which was used as a control to compare with seedlings grown aseptically.

It was not really surprising that the isolates H4-34, H4-45, H4-16, ASH21, 11H4-5, 3T4-12, 3T4-20, 9B3-17, and 3T4-21, which manifested strong phosphate-solubilizing property when cultured on PKV-BMP media, all corresponded to *Pseudomonas* species, such as *P. brassicae* (H4-34, H4-45, H4-16), *P. lurida* (ASH21), *P. extremorientalis* (11H4-5), *P. chlororaphis* (3T4-12, 3T4-20), *P. laurylsulfatiphila* (9B3-17), and *P. baetica* (3T4-21). Consistent with our screening and 16S rRNA identification, previous reports demonstrated the P_i -solubilizing capability of *Pseudomonas* species and

their probiotic effects when assayed in interactions with plants under varied growth conditions, ranging from growth chambers to the field, using model plants and crops. For instance, *P. putida* (SP21 and SP22) promoted shoot and root development in soybean [31]. *P. corrugata* improved growth of wheat under greenhouse conditions [32], and *P. proteolytica* and *P. azotoformans* increased rosette diameter, leaf area, and biomass of *Arabidopsis* grown in a growth chamber [33]. *Pseudomonas* spp. improved plant growth in the field, under moderate and high P_i levels, thus augmenting the use of fertilizers [34–36]. For endophytic *Pseudomonas*, the P_i -solubilizing properties rely at least in part on gluconic acid (GA) [37].

To gain further insight into the plant probiotic properties of P_i -solubilizing *Pseudomonas* species, the roots of *Arabidopsis* seedlings were placed over bacterial streaks in MS $0.2\times$ medium, which provides readily available nutrients. *P. brassicae* and *P. laurylsulfatiphila* strongly promoted shoot and root growth whereas inoculation with *P. lurida*, *P. extremorientalis*, *P. baetica*, *P. chlororaphis*, or *Solibacillus isronensis* did not enhance shoot or root biomass relative to un-inoculated controls. Moreover, detailed analysis of primary root growth and branching indicated that amongst the newly identified P_i -solubilizing *Pseudomonas* species, all of them repressed root growth by 50–60% while increasing lateral root number and density fourfold to sixfold, with *P. baetica*, *P. laurylsulfatiphila*, and *P. brassicae* being the most bioactive species. Inoculation with *P. brassicae* H4-45 also led to the production of longer lateral roots than in un-inoculated seedlings, suggesting that its beneficial effects promoting root branching may directly improve plant biomass through better exploration of the substrate. Interestingly, *P. chlororaphis* inhibited root system development despite its good P_i -solubilizing properties.

In our plant-bacteria interaction system, the “bacterial streaks” methodology used enabled co-cultivation of many plants with the inoculum at the same time. However, it is not easy to explain how the morphological changes of roots in areas where there is no direct contact with the bacterial streak occur. In this regard, motility systems have been reported for *Pseudomonas* species. In the case of *P. aeruginosa*, a polar flagellum and type 4 pili allow initial attachment of the bacteria, with additional steps required for colonization [38]. Alternatively, spread of diffusible metabolites and/or volatiles from the bacterial streak may induce the development of lateral roots and root hairs above and below the bacterial streaks. To the best of our knowledge, how bacterial motility influences root behaviors has not been investigated and remains as an important aspect that deserves attention.

Several species specific factors may contribute to plant biomass accumulation. *Pseudomonas* is one of the most common and thoroughly investigated bacterial genera, which

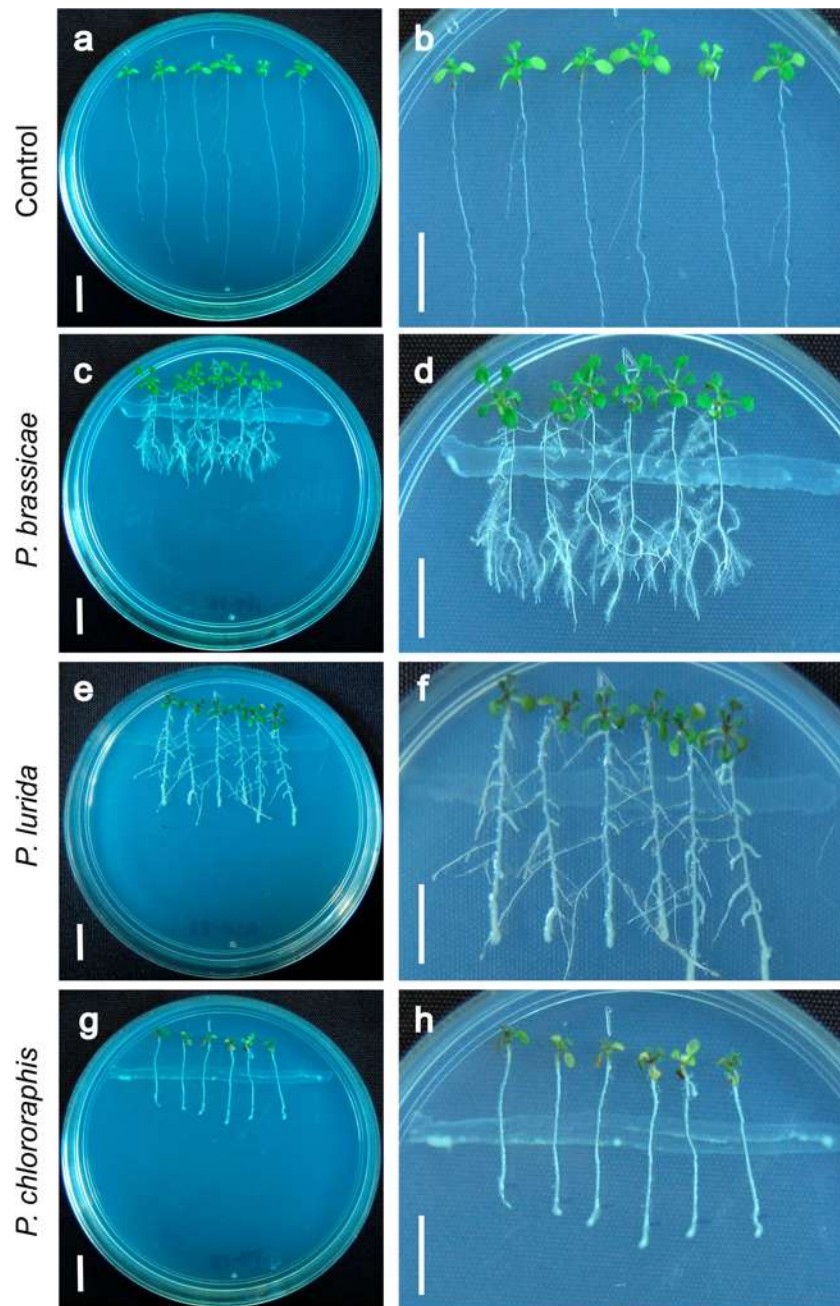
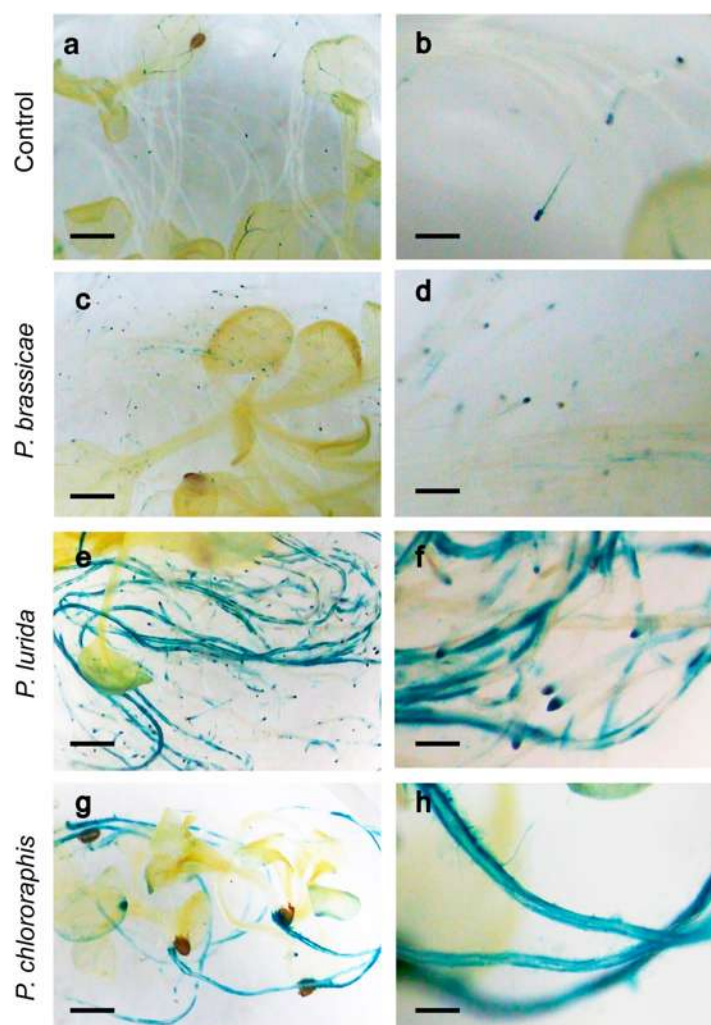


Fig. 5 Phenotypes of *Arabidopsis* seedlings inoculated with selected phosphate-solubilizing *Pseudomonas* species. Representative images demonstrate different biostimulation categories of seedlings 6 days after transfer over bacterial streaks (**a, b**). Non-inoculated seedlings (**c, d**). *P. brassicae* (**e, f**), *P. lurida* (**g, h**), *P. chlororaphis*. Scale bar = 1 cm, $n = 18$. Different letters mean statistical differences. Tukey post hoc with $p < 0.05$

includes both plant growth-promoting (e.g. *P. putida* and *P. fluorescens*) and -pathogenic (e.g., *P. aeruginosa*) species. From our results, *P. baetica*, *P. laurylsulfatiphila*, and

P. brassicae can be regarded as plant growth promoters, whereas *P. chlororaphis* behaved as a growth repressing bacterium, probably due to the production of as yet unidentified metabolites, antibiotics, and/or virulence factors. Very recently, Raio and Puopolo (2021) noted that the *P. fluorescens* complex contains at least eight phylogenetic groups and each of these includes several species sharing ecological and physiological traits [39]. According to the authors, *P. chlororaphis* belongs to a separate group, and these bacteria produce metabolites that are toxic to other organisms, including

Fig. 6 Auxin-responsive *DR5::uidA* expression pattern in shoot and root of *Arabidopsis* seedlings. Seedlings expressing the *DR5::uidA* marker were placed over streaks of selected *Pseudomonas* species (**a, b**). Basal GUS expression in non-inoculated seedlings (**c, d**). The promoting effects by *P. brassicae* showed a similar expression with the control (**e–h**). *P. lurida* and *P. chlororaphis* strongly increased the auxin-inducible expression in the root system. Scale bar = 1 mm, $n = 18$. Different letters mean statistically significant differences. Tukey post hoc with $p < 0.05$



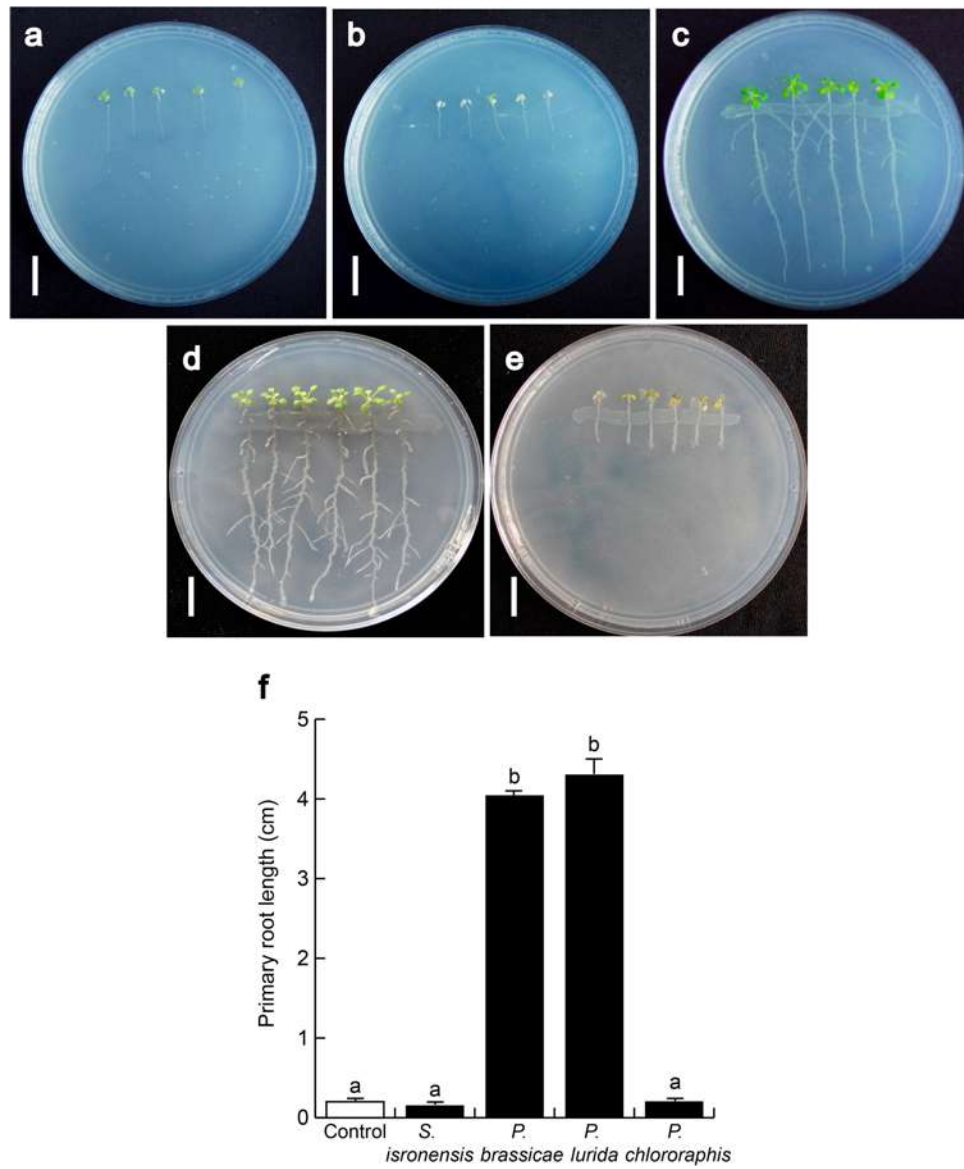


Fig. 7 *P. brassicae* and *P. lurida* improve plant growth in medium supplemented with sparingly soluble phosphate source. The MS 0.2×solid media was modified replacing potassium phosphate with calcium phosphate. **a** Un-inoculated seedlings **b** *S. isronensis*, **c** *P.*

brassicae, **d** *P. lurida*, **e** *P. chlororaphis*, **f** Primary root length. Scale bar=1 cm. Data points indicate mean $\pm$ SD ($n=18$). Different letters mean statistical differences. Tukey post hoc with $p<0.05$

antibiotics such as phenazines, pyrrolnitrin, 2-hexyl, 5-propyl resorcinol, and hydrogen cyanide; siderophores such as pyoverdine and achromobactin; and a complex blend of volatile organic compounds (VOCs) that effectively contribute to the control of several plant pathogens, nematodes, and insects. Although in a field situation the number of possible interactions is much higher, for plants grown in vitro the release of siderophores and VOCs may directly account for repression of plant growth or may behave as auxin signal mimics. Our data, certainly, contribute to the understanding of *P. chlororaphis*-plant interactions.

Alterations in auxin homeostasis have been considered a critical factor in the phytostimulation driven by *Azospirillum brasilense* and *Micrococcus luteus*, which interact with roots and increase root expansion by means of root branching [11, 13]. Comparable effects have been reported for *Pseudomonas putida*, *Pseudomonas fluorescens*, and the plant pathogen *Pseudomonas aeruginosa*, which strongly repress primary root growth through the production of cyclodipeptides that mimic auxin action [9, 10]. This was not the case for *P. chlororaphis*, which despite strongly inducing the auxin-responsive reporter gene *DR5::GUS* failed to enhance the production of more lateral roots. In contrast, *P. brassicae* strongly increased root branching and length of the lateral roots, but could not induce the auxin-responsive reporter gene. The seedlings co-cultivated with *P. lurida* or *P. chlororaphis* had a strong induction of the marker over the entire root system, indicating its participation in the molecular communication established with *Arabidopsis* through an auxinic mechanism. These results show that although different *Pseudomonas* species share the property to solubilize phosphate, the suppression of primary root growth can apparently occur by more than one mechanism.

Phosphate is a critical macronutrient that influences both auxin synthesis or auxin sensitivity with an important role in root architecture responses of plants, drives plant-bacteria interaction, and modulates the composition of root exudates as well as the diversity of the microbiome [40–43]. The root-inhabiting bacteria may contribute to plant mineral nutrient homeostasis by releasing organic acids, protons, and chelating molecules that release P_i from poorly soluble minerals and allow its translocation to photosynthetic and reproductive tissues. To determine if root inoculation with P_i -solubilizing bacteria could provide an advantage to plants grown in medium supplemented with calcium phosphate, the impact of the inoculation of *Arabidopsis* seedlings with *P. brassicae*, *P. lurida*, and *P. chlororaphis* on overall seedling growth and primary root elongation was assessed after 15 days of interaction. *P. brassicae* and *P. lurida* but not *P. chlororaphis* enhanced shoot and root growth, indicating their potential to help plants to grow and utilize a sparingly soluble P_i source. Thus, P_i -solubilizing bacteria may

be interesting candidates as plant growth promoters, from which the three newly identified isolates of *P. brassicae* (H4-34, H4-45, H4-16) represent true plant beneficial bacteria that can assist P_i nutrition and plant growth under different conditions.

Author Contribution J.L.H., R.O.C., and J.L.B. conceived and designed the experiments; J.L.H., E.G.C., K.R.J.V., D.L.S.R., and O.F.R. performed experiments; J.L.H., J.S.L.B., O.F.R., R.O.C., and J.L.B. analyzed the data; R.O.C., J.S.L.B., H.R.C., and J.L.B. provided reagents/materials/analytical tools; J.L.H. and J.L.B. wrote the paper; all authors reviewed and edited the manuscript. R.O.C. and J.L.B. applied for funding.

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Data Availability All data generated or analysed during this study are included in this article and are available upon reasonable request to the corresponding author.

Declarations

Conflict of Interest The authors declare no competing interests.

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