UNIVERSIDAD AUTÓNOMA AGRARIA ANTONIO NARRO

SUBDIRECCIÓN DE POSTGRADO

USO DE PGPR EN *SOLANUM LYCOPERSICUM* PARA CONTROL DE PATÓGENOS Y PROMOCIÓN DE CRECIMIENTO,

RÁPIDA DETECCIÓN DE AUXINAS POR TÉCNICA DE MICROPLACA,

INFLUENCIA DE LA APLICACIÓN DE *Bacillus subtilis* EN PROMOCIÓN DE CRECIMIENTO DE PLANTAS,

Y

EXPRESIÓN DE GENES DE FITOHORMONAS EN *Solanum lycopersicum* TRATADO CON ÁCIDO 3-INDOL ACÉTICO Y *Bacillus subtilis*

Artículos

Que presenta JULIA CECILIA ANGUIANO CABELLO

Como requisito parcial para obtener el Grado: DOCTOR EN CIENCIAS EN PARASITOLOGIA AGRICOLA

Saltillo, Coahuila Diciembre 2016

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Agradecimientos

Al Consejo Nacional de Ciencia y Tecnología (CONACYT) por el apoyo económico a lo largo del postgrado, y por la facilidad proporcionada para acceder a información de interés científico y para el desarrollo de habilidades de investigación.

A la Universidad Autónoma Agraria Antonio Narro por el apoyo en la formación académica, por facilitar el acceso a instalaciones y laboratorios para el desarrollo del proyecto de tesis, y por el apoyo económico para el proyecto, así como para la exposición de trabajos en congresos nacionales e internacionales.

Al Departamento de Parasitología Agrícola por el conocimiento brindado por los maestros investigadores, y su disponibilidad para responder dudas. Además agradezco tanto a maestros como a personal administrativo por el apoyo en trámites y otros requisitos administrativos.

A mis asesores de tesis por el tiempo brindado para el diseño y desarrollo del proyecto. Además del tiempo dedicado a la revisión de resultados y redacción de artículos.

Dedicatoria

A Dios: Por darme la oportunidad de despertar cada día y seguir adelante. Por permitirme tener a tantas personas a mí alrededor que me quieren y me apoyan.

A mis padres: Raymundo Anguiano Muñiz y María Julia Cabello Cortés por la paciencia, consejos, amor y apoyo en todo momento. Por enseñarme que el trabajo y la constancia lo pueden lograr todo.

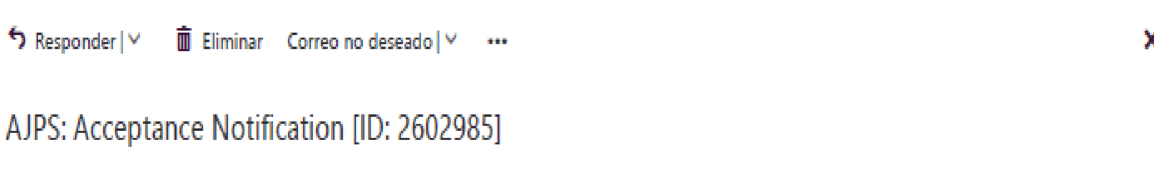
A mis hermanos: Daniel de Jesús Anguiano Cabello y Raymundo Anguiano Cabello por quererme, compartir tantas cosas a mi lado, por ser mis compañeros de vida y motivarme para no dejar de esforzarme cada día.

A mis maestros y asesores: Por todas sus enseñanzas, revisiones de artículos, apoyo y por ponerme retos que me hicieron aprender mucho y madurar.

A mis amigos y compañeros: Por hacer de mi estancia en la Universidad una agradable experiencia, por ayudarme en mis experimentos y por escucharme y compartir conmigo las situaciones de cada día.

A mi esposo. Roberto Arredondo Valdés, por motivarme todos y cada uno de los días a dar lo mejor de mí. Por no permitirme desistir y por todo el optimismo y fe que siempre tuvo en mí. Por amarme, cuidarme y escucharme. Por la paciencia, por hacerme reír y permanecer siempre a mi lado. Por ayudarme en mi tesis.

A mi hija. Cecilia Guadalupe Arredondo Anguiano, por ser mi razón para dar el cien porciento cada día. Por compartirme tanta alegría y por todo el amor que me da.



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Uso de PGPR en *Solanum lycopersicum* para control de patógenos y promoción de crecimiento

Use of PGPR in *Solanum lycopersicum* for pathogen control and growth promotion

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Resumen

El tomate (*Solanum Lycopersicum* L.) es una hortaliza de amplia demanda mundial, por su versatilidad y valor nutritivo; y por lo tanto es necesario incrementar su producción. Los agroquímicos favorecen el rendimiento de las plantas pero su uso indiscriminado ha provocado daño al medio ambiente. Debido a esto surge como alternativa el uso de rizobacterias promotoras de crecimiento de plantas (PGPR), que actúan por mecanismos directos o indirectos. Cada PGPR produce diferentes tipos de metabolitos promotores de crecimiento, e interactúan de distinta forma con el hospedero y entorno. En el presente trabajo, se realiza una revisión sobre el uso de PGPR en tomate y algunos puntos importantes para su selección. Se concluye, que a pesar de que existen múltiples investigaciones, es importante profundizar en el estudio del mecanismo de acción de las PGPR, su interacción con el medio ambiente y los cambios a nivel genético que ocasionan los metabolitos añadidos.

Palabras clave. *Solanum lycopersicum*, promoción de crecimiento, PGPR

Summary

The tomato (*Solanum lycopersicum* L.) is a vegetable worldwide demanded for the versatility and nutritional value; and therefore their production must be increased. Agrochemicals promote the production but their indiscriminate use has caused damage to the environment. Because of this, emerges as an alternative the use of plant growth promoting rhizobacteria (PGPR), which act by direct or indirect mechanisms. Each PGPR produce different types of metabolites that are growth promoters, and interact differently with the host and environment. In this paper, is made a review of the use of PGPR in tomatoes and some important points for their selection. It is concluded that although there are lots of research about PGPR, it is important to deepen the study of the mechanism of action of PGPR, their interaction with the environment and changes at the genetic.

Key words. *Solanum lycopersicum*, growth promotion, PGPR

INTRODUCCIÓN

El tomate (*Solanum Lycopersicum* L.), es la hortaliza más difundida en el mundo y la de mayor valor económico, representando uno de los componentes más frecuentes de la dieta humana[41]. En el mundo son cultivadas más de 5 millones de ha con un producción cercana a los 129 millones de toneladas. Durante el 2008, se produjeron en México 2.26 millones de toneladas de tomate, siendo el principal productor el estado de Sinaloa, cuya producción representó el 35% del total nacional, en segundo lugar Baja California (9%).

Seguidos de Michoacán, San Luis Potosí y Jalisco con 8%, 6% y 5%, respectivamente[70]. Desde el punto de vista nutricional, este vegetal contiene altos niveles de licopeno[52], vitaminas, sales minerales y fibras[17], y además es un antioxidante que reduce el riesgo asociado a distintos tipos de cáncer y enfermedades neurodegenerativas[73]. Por lo anterior, surge la necesidad de desarrollar estrategias para el incremento en la producción de la hortaliza.

Los fertilizantes químicos, incrementan los rendimientos de los cultivos de tomate. Entre los más utilizados se encuentran: Fosfonitrato, sulfato de amonio, superfosfato de calcio triple, nitrato de potasio, fosfato diamónico, calcio, magnesio, hierro, zinc, boro, magnesio y molibdeno[55, 71]. Sin embargo, su uso indiscriminado trae consecuencias al medio ambiente. Bojórquez *et al.*[13]señalaron que los fertilizantes sintéticos no se absorben por completo por las plantas y que el fertilizante no incorporado trae un impacto ambiental adverso, tal como contaminación de mantos acuíferos con NO^{3-}, eutrofización, lluvia ácida y calentamiento global. Debido a esto se hace necesario la búsqueda de nuevas alternativas de fertilización y bioestimulación[8]. Una alternativa es el uso de microorganismos con capacidad para actuar como biofertilizantes, fitoestimuladores y biopesticidas[12] para el fortalecimiento de sistemas de producción sostenible[4].

A las bacterias presentes en el suelo capaces de favorecer el crecimiento de las plantas se les llama “rizobacterias promotoras de crecimiento de plantas”, por sus siglas en inglés PGPR. Dichas PGPR promueven el crecimiento por mecanismos directos o indirectos. Los mecanismos directos se relacionan con la producción de fitohormonas (auxinas, giberilinas, citoquininas, etc). Además pueden afectar la disponibilidad de nutrientes por

la intervención directa en los ciclos biogeoquímicos, en el caso de la fijación biológica de nitrógeno y la solubilización de nutrientes como el fósforo[15]. Indirectamente las PGPR pueden contribuir mediante la inducción de la resistencia sistémica a fitopatógenos, el control biológico de enfermedades, la producción de antibióticos y de sideróforos[62,78].

En tomate son utilizadas PGPR que utilizan tanto mecanismos indirectos como directos para la promoción de crecimiento. Sin embargo, para optimizar su uso es importante conocer la forma en que los PGPR interactúan con otros microorganismos y con las plantas, el mecanismo de acción y las condiciones óptimas a las que producen los metabolitos secundarios. En el presente trabajo se realizará una revisión de las principales PGPR utilizadas en tomate, y algunos aspectos importantes a considerar en la selección de las mismas.

PGPR utilizadas en cultivos de tomate. Los géneros de bacterias *Pseudomonas, Azospirillum*, *Azobacter, Bacillus, Agrobacterium* y *Streptomyces* forman parte de la comunidad microbiana de la rizósfera del tomate[5,39,53,63] y se utilizan para el desarrollo de bioformulados para la promoción de crecimiento e inhibición de patógenos en tomate[12,22,24]. No obstante, el efecto y mecanismos de acción de cada PGPR es distinto y por lo tanto es importante definir el efecto que se desea tener para la selección del PGPR. En la tabla 1 se presentan mecanismos de acción y efectos en *Solanum lycopersicum* por PGPR.

Bacillus spp. Especies de *Bacillus* como *B. subitlis, B. pumillas* y *B. amyloliquefaciens* promueven el crecimiento de plantas de tomate por la producción de metabolitos como

fitohormonas y antibióticos. Tal es el caso de *B. subtilis* que produce auxinas que promueven el crecimiento del tomate e inducen resistencia sistémica contra patógenos como *F. oxysporium*[3,38,52]. El efecto de *Bacillus* spp. se puede ver reflejado en diferentes partes de la planta ya sea tallo[77], raíz[23] o fruto[52]. Dependerá de la concentración de los metabolitos, el método de aplicación o de si se aplica una sola cepa aislada o en consorcios microbianos. La efectividad del uso de consorcios microbianos con *Bacillus* spp. se ha demostrado tanto en campo como en experimentos in vitro[3,25,59].

***Pseudomonas spp*.** *P. fluorescens*, *P. Putida* y *P. rhodesiae* son algunas de las especies que promueven el crecimiento de tomate por mecanismo directo o indirecto. Dichos mecanismos involucran la producción de AIA y otros metabolitos[56,71], así como la activación enzimática. Además pueden ser utilizadas solas o en bioformulados acompañadas de otras PGPR o incluso de extractos vegetales. En cuanto a la aplicación puede ser en semilla o plántula.

***Azobacter* spp.** *Azobacter chroococccum* y *Azobacter brasilense* son utilizadas para la promoción de crecimiento del tomate. El mecanismo de acción involucra la producción de antibióticos, la producción de fitohormonas como el ácido indolacético y la fijación de minerales. Según estudios de Escobar *et al*.[32] cepas de *Azotobacter* spp. en *Lycopersicon esculentum* (tomate) alcanzaron buenos índices de efectividad en volumen radicular y peso de la biomasa seca total, aérea y radicular, esto atribuido a la producción de auxinas y otros mecanismos. Además, es importante considerar que *Azotobacter* puede tener protocooperación y simbiosis con otros microorganismos en consorcios[27].

Cuadro 1. PGPR utilizadas en el control de fitopatógenos y promoción de crecimiento en Solanum lycopersicum.

Microorganismo o consorcio microbiano	Forma de aplicación de tratamientos	Metabolito o mecanismo de promoción de crecimiento	Efecto en la planta	Referencia
Bacillus subtilis	No especifica	Auxinas y antibióticos	Crecimiento del fruto y resistencia sistémica contra *Fusarium oxysporum*	52
Bacillus subtilis	Inoculación de cepa en raíz	Respuesta dependiente e independiente del ácido jasmónico	Promoción de crecimiento de planta y RSI contra *Bemisia tabaco* (mosca blanca)	77
Bacillus subtilis	Inoculación en plántula	Activación de defensa enzimática	Control de *Xanthomonas campestris* pv. Vesicatoria	19
Bacillus subtilis	Inoculación en suelo	Producción de antibióticos. Formación de biofilm en raíz	Control de *Ralstonia solanacearum*	59
Bacillus subtilis	Inoculación en semillas	Activación de defensa enzimática. Inducción en producción de	Largo de planta y raíz, número de hojas. RSI contra	23

		hormonas de crecimiento.	*Alternaria solani* y *Phytophthora infestans*	
Bacillus subtilis	Inoculación en plántula	No especifica	Altura de plantas, crecimiento radicular, número y peso de frutos Contra *Clavibacter michiganensis* subsp. *michiganensis*	66
Bacillus amyloliquefaciens	Inoculación en semilla y plántula	No especifica	Número y calidad de frutos	37
Bacillus amyloliquefaciens	Aplicación en suelo	Producción de antibióticos	RSI contra *Ralstonia solanacearum* y *Oidium neolycopersici*	81
Bacillus megaterium	Rociado en la planta	Regulación de niveles de fitohormonas como etileno y ácido absícico	Peso seco, largo de la planta, biomasa	58
Bacillus pumilus	No especifica	Promoción de actividad enzimática antioxidante	Peso húmedo y peso seco. Reducción de	44

			toxicidad por boro.	
Pseudomonas rhodesiae y Bacillus cereus	Inoculación en suelo, cerca de las raices	No especifica	Altura de brotes, número de hojas, fructificación temprana y biomasa	1
Pseudomonas fluorescens	No especifica	No especifica	Rendimiento de frutos	33
P. fluorescens	Inoculación en el suelo inmediatamente después de sembrar la semilla	Producción de sideróforos	Altura de planta, peso fresco	29
Pseudomonas fluorescens		Activación de defensa enzimática.	RSI[a] contra *Alternaria Solani*	46
Pseudomonas putida	Inoculación en semillas	Ácido indolacético	Desarrollo radicular	36
Azotobacter spp.	Inoculación en suelo	Ácido indolacético, fijación de nitrógeno, solubilización de fosfato	Altura, raíces, biomasa	32
Azorobacter	Inoculación en semilla	No especifica	Altura de plántulas, longitud radicular de plántulas,	31

			rendimiento de frutos	
Azotobacter chroococcum	Inoculación en semilla	No especifica	Altura y longitud radicular	60
Azotobacter sp.	Inoculación en planta	No especifica	Altura de planta y longitud radicular	65
Azospirillum brasilense	Inoculación en semillas	Producción de antibióticos, mayor área de los vasos del xilema, más alta conductividad hidráulica del tallo.	Reducción de estrés de agua por control de *Clavibacter michiganensis* subsp. *michiganensis*	67
Azospirillum brasilense	Inoculación en semillas	No especifica	Altura de planta y longitud radicular	26
Azospirillum brasilense	Inoculación en semilla	Ácido indolacético	Nivel de germinación	48
Streptomyces ahygroscopicus	Aplicación en plántula	No especifica	Control de *Botrytis cinérea*	34
Streptomyces spp.	In vitro	No especifica	Control de enfermedad de “encorchamiento de raíz”	14

[a]RSI-Respuesta sistémica inducida.

Selección de PGPR para promoción de crecimiento de tomate. Para la selección de microorganismos promotores de crecimiento de tomate es necesario definir la parte de la

planta que se desea desarrollar, los metabolitos que producen las cepas a las condiciones del cultivo y las interacciones tanto del microorganismo con la planta como con el resto del entorno (medio ambiente, microbiota, etc). Una vez aplicado el tratamiento es posible evaluar la eficiencia de las PGPR por medio de parámetros agronómicos o métodos moleculares.

Interacción de PGPR con los microorganismos del suelo. La interacción entre una comunidad microbiana puede manifestarse de diferentes modos, ya sea con sinergismos o antagonismos. La posibilidad de que las PGPR nativas afecten la actividad de las PGPR inoculadas es significativamente alta. Ya que ambas pueden simultáneamente solubilizar fósforo, producir auxinas, antibióticos y sideróforos[50]. La interacción entre PGPR y hongos micorrízicos arbusculares (HMA), puede ser selectiva y dependiente de la bacteria y el hongo implicado[10]. Algunas especies de bacterias como *Azotobacter*, *Beijerinckia*, *Clostridium*, *Burkolderias*, y *Azospirillum*, han sido estudiadas en su interacción con HMA[5]. La inoculación de PGPR o HMA pueden promover el crecimiento de las plantas por separado o en colaboración. Según los estudios de Alfonso y Galán[5] aunque la inoculación simple presentó beneficios respecto a las plantas testigo, la coinoculación superó los beneficios individuales de ambos microorganismos (rizobacterias y hongos micorrízicos), desde la fase de semillero hasta la cosecha.

Interacción de PGPR con planta. El modo de acción de las bacterias promotoras de crecimiento no siempre es el mismo, depende de la planta con la que estén interaccionando[75] y de un proceso multigénico influenciado por múltiples factores. Persello-Cartieaux *et al*.[57] desarrollaron un modelo in vitro para describir las interacciones

planta-rizhobacteria. La colonización de las plantas de tomate por rizobacterias está directamente relacionada al crecimiento bacteriano, quimiostaxis, formación de biopelícula e interacciones con exudados de raíz del hospedero[20,76]. Los exudados de la raíz, que pueden actuar como atrayentes químicos e incrementar el crecimiento bacteriano, determinan que microorganismos tendrán éxito en la colonización del hospedero[40]. Las interacciones exudados-bacterias pueden ser químicas, físicas o biológicas[45]. La quimiotaxis bacteriana es el proceso en que la bacteria dirige su movimiento de acuerdo a los químicos en el ambiente y la localización de las condiciones óptimas para su crecimiento y supervivencia[2]. Por su parte, la formación de biofilm además de favorecer la colonización de la rizobacteria protege al hospedero de patógenos[11]. Tan *et al*.[76] determinaron exudados de raíz de tomate, entre ellos ácido oxálico, ácido málico, ácido cítrico, ácido succínico y ácido fumárico que favorecían la quimiotaxis y formación de biopelícula con *Bacillus amyloliquefaciens.*

Metabolitos promotores de crecimiento en tomate. Las auxinas en tomate regulan la promoción de crecimiento en parámetros como longitud de la planta, volumen radicular, floración y maduración de frutos[18]. Una de las principales auxinas, el ácido 3-indolacético (AIA) impacta en el desarrollo radicular de las plantas[9,47]. Sin embargo, los efectos de las auxinas son dependientes de la concentración, que generalmente debe ser mínima para estimular el crecimiento[49]. Las citoquininas son un tipo de hormonas que se cree se sintetizan mayoritariamente en la raíz e intervienen en el desarrollo de hojas y raíces, además de tener un notable y demostrado efecto en el retraso de la senescencia[54]. Los sideróforos son moléculas orgánicas quelatantes que ayudan a la planta a asimilar el hierro inorgánico del suelo y provocan resistencia sistémica en las plantas por la competencia

por hierro[28,42,61]. Otras moléculas orgánicas producidas por las PGPR aumentan la disponibilidad de nutrientes del suelo, como el fósforo[35]. Por su parte, el etileno, que es una hormona vegetal producida bajo estrés y que en altas concentraciones inhibe el crecimiento vegetal, puede verse reducido por acción de las rizobacterias inoculadas[43]. Es importante además considerar la interacción de los metabolitos promotores de crecimiento, ya que diferente relación en la concentración de ellos, traerá diferente efecto en las plantas, tal es el caso del efecto en conjunto de las giberilinas y auxinas en la fructificación del tomate[72].

Parámetros agronómicos utilizados para evaluar PGPR. Los parámetros agronómicos que se utilizan para la selección y evaluación de las PGPR en tomate dependerán de la parte de la planta que se quiera promover[8]. Sánchez *et al.* [71] evaluaron *Enterobacter* sp., *Pseudomonas* sp. y *Bacillus* sp., con los parámetros de Solubilización y mineralización de fósforo, actividad enzimática, síntesis de sideróforos y producción de indoles totales. La biomasa radical es otro parámetro evaluado ya que tiene fuertes repercusiones en la capacidad de las plantas para asimilar los nutrientes del suelo[7]. En cuanto a la asimilación de minerales, las PGRP ayudan a la asimilación de nutrimentos de la planta. Dursun et al.[30] evaluaron el efecto de *Bacillus subtilis*, *Bacillus megaeorum*, *Acinetobacter baumanni* y *Pantoea agglomerans* en el aumento de la concentración de N, P, Mg, Ca, Na, K, Cu, Mn, Fe y Zn en la fruta.

Uso de biología molecular para la selección y evaluación de cepas promotoras de crecimiento. La biología molecular es una herramienta útil en la selección de PGPR y en la evaluación de cambios en el genotipo de las plantas tratadas. El uso de cebadores

permiten amplificar fragmentos de ADN para la identificación de especies, detección de variabilidad genética o identificación de patrones de bandas por especies (fingerprint). Ente las técnicas más utilizadas se encuentran RAPD (*Random amplified polymorphic 10 DNA*), reacción en cadena de la polimerasa (PCR), rep-PCR (*Repetitive sequence based*-PCR)[68,69] ya sea con genes específicos o genes de regiones conservadas como el gen 16S. El gen ribosomal 16S (ADNr 16S) es uno de los más utilizados en estudios de filogenia y taxonomía bacteriana. Es un gen altamente conservado, presentando regiones homólogas a todos los organismos pero también regiones altamente variables, que permiten la clasificación de las bacterias a nivel de género y especie[21,64,69].

El análisis por microarreglos es una técnica recientemente utilizada para la determinación de cambios en la expresión genética de las plantas al interaccionar con microorganismos como *Pseudomonas purtida, Pseudomona fluorescens*, *Pseudomonas thivervalensis; Bacillus* spp.[16,75,79,80]. Observándose cambios notables en la expresión de genes que dan como respuesta la sobreregulación o subregulación de vías y funciones metabólicas. Entre los genes que se ha demostrado cambian sus niveles de expresión por la adición de PGPR se encuentran: genes que involucran metabolismo, señal de transducción y respuesta al estrés, genes de auxinas, genes de producción de etileno, genes realacionados a la patogenicidad[80]. No obstante, en el proceso de selección de PGPR es importante considerar que el aumento en la expresión de algunos genes puede tener como consecuencia la disminución en la expresión de otros genes que también podrían ser de interés. Es el caso observado por Cartieaux *et al*. [16] en el que hubo aumento de genes de inducción de resistencia, pero disminución del proceso de fotosíntesis y con esto reducción del tamaño de la planta.

CONCLUSIONES

El uso de PGPR es una alternativa viable para la promoción de crecimiento de tomate. Sin embargo, para seleccionar la PGPR adecuada es necesario conocer los metabolitos que produce cada rizobacteria, la parte de la planta que se desea promover y las posibles interacciones que tendrán los microorganismos con la microbiota nativa del suelo y con la planta. Una vez seleccionado la PGPR será necesario evaluar los cambios en el fenotipo o genotipo de las plantas tratadas para determinar la efectividad de las rizobacterias aplicadas. Existen multiples investigaciones que pueden orientar hacia la selección de rizobacterias y sus efectos en las plantas, no obstante, aún queda mucho por estudiar en los campos de mecanismos de acción de los metabolitos promotores de crecimiento, en el campo de la genética y biología molecular (expresión de genes y variabilidad genética) y en la interacción entre PGPR y condiciones ambientales (temperatura, humedad, etc.).

Agradecimientos. Este trabajo fue financiado por la Universidad Autónoma Agraria Antonio Narro.

LITERATURA CITADA

1. Adesemoye AO, Obini M, Ugoji, EO. Comparison of plant growth promotion with Pseudomonas aeruginosa and Bacillus subtilis in three vegetables. Brazilian Journal of Microbiology. 2008;*39*(3), 423-426.
2. Adler J, Tso, W.W. Decision-making in bacteria: chemotactic response of Escherichia coli to conflicting stimuli. Science. 1974;184, 1292–1294.

3. Airola GVM. Compostas líquidas con bacterias promotoras de crecimiento en la nutrición de tomate (*Solunum lycopersicum*). Tesis de Maestría en Recursos Naturales y Medio Ambiente. 2010. Instituto Politécnico Nacional.
4. Alarcón A, Ferrera CR. Biofertilizantes: Importancia y utilización en la agricultura. Revista Mexicana de Ciencias Agrícolas. 2012;*26*(2), 191-203.
5. Alfonso, E. T., & Galán, A. L. (2006). Evaluación agrobiológica de la coinoculación micorrizas-rizobacterias en tomate. Agronomía costarricense: Revista de ciencias agrícolas, 30(1), 65-73.
6. Alfonso ET, Leyva AH. Microorganismo Benéficos como Biofertilizantes eficientes para el cultivo del tomate (*Lycopersicum esculentum* Mill). Revista Colombiana de Biotecnología. 2005;2:47-54.
7. Antoun H, Prevost D. Ecology of plant growth promoting rhizobacteria. In: Siddiqui ZA, editors. PGPR: Biocontrol and Biofertilization. Netherlands. Springer. 2007, 1-38 pp.
8. Arteaga M, Garcés N, Guridi F, Pino JA, López A, Menéndez JL, Cartaya O. Evaluacion de las aplicaciones foliares de humus liquido en el cultivo del tomate (*Lycopersicon esculentum* Mill) var. Amalia en condiciones de producción. Cultivos Tropicales. 2006;27(3), 95-102.
9. Ashrafuzzaman M, Islam M, Ismail M, Shahidullah S, Hanafi M. Evaluation of six aromatic rice varieties for yield and yield contributing characters. Int. J. Agric. Biol. 2009;11:616-620.
10. Azcón R. Papel de la simbiosis micorrízica y su interacción con otros microorganismos rizosféricos en el crecimiento vegetal y sostenibilidad agrícola.

In: Ecología, fisiología y biotecnología de la micorriza arbuscular. México, Mundi-Prensa, 2000, p.15.

11. Bais HP, Fall R, Vivanco JM. Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol. 2004;134, 307–319.
12. Berg G. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl. Microbiol. Biotechnol. 2009;84: 11-18.
13. Bojórquez ADA, Gutiérrez CG, Báez JRC, Sánchez MAA, Montoya LG, Pérez EN. Biofertilizantes en el desarrollo agrícola de México. Ra Ximhai. 2010;*6*(1), 51-56.
14. Bubici G, Marsico AD, D'Amico M, Amenduni M, Cirulli M. Evaluation of Streptomyces spp. for the biological control of corky root of tomato and Verticillium wilt of eggplant. Applied soil ecology. 2013;72, 128-134.
15. Camelo M. Vera SP, Bonilla RR. Mecanismos de acción de las rizobacterias promotoras del crecimiento vegetal. Revista Corpoica. Ciencia y Tecnología Agropecuaria. 2011; 12.
16. Cartieaux F, Thibaud MC, Zimmerli L, Lessard, Sarrobert C, David P, Gerbaud A, Robaglia C, Somerville S, Nussaume. Transcriptome analysis of Arabidopsis colonized by a plant-growth promoting rhizobacterium reveals a general effect on disease resistance. The Plant Journal.2003; 36 (2): 177-188.
17. Casanova AS, Gómez O, Laterrol H, Anais G. Manual para la producción protegida de hortalizas. Editorial Agroinfor, MINAG, La Habana, Cuba. 2007. 112 p. ISBN: 959-7111-37-3.

18. Celis L, Gallardo I. Estandarización de métodos de detección para promotores de crecimiento vegetal (ácido indol acético y giberelinas) en cultivos microbianos. Tesis de Licenciatura en Microbiología Agrícola y Veterinaria. 2008. Pontificia Universidad Javeriana.
19. Chandrasekaran M, Chun SC. (2016). Induction of defense-related enzymes in tomato (Solanum lycopersicum) plants treated with *Bacillus subtilis* CBR05 against *Xanthomonas campestris* pv. vesicatoria. Biocontrol Science and Technology. 2016;(just-accepted), 1-21.
20. Chen Y, Yan F, Chai Y, Liu H, Kolter R, Losick R, Guo JH. Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. Environmental microbiology. 2013;15(3), 848-864.
21. Chenoll E, Macián MC, Aznar R. Identification of *Carnobacterium, Lactobacillus*, *Leuconostoc* and *Pediococcus* by rDNA-based techniques. Syst Appl Microbiol. 2003;26:546-556.
22. Choudhary DK, Johri BN. Interactions of Bacillus spp. and plants–with special reference to induced systemic resistance (ISR). Microbiological research. 2009;164(5), 493-513.
23. Chowdappa P, Kumar SM, Lakshmi MJ, Upreti KK. Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3. Biological Control. 2013;*65*(1), 109-117.
24. Clayet-Marcel J, Larcher M, Bartrand H, Rapior S, Pinochet X. Plant growth enhancement by rhizobacteria. Nitrogen assimilation by plant: physiological,

biochemical, and molecular aspects. En: JF Morot-Gaudry, editor. Science publishers Inc., Enfield. 2001.

25. Cordero-Ramírez, JD. Microorganismo de la rizósfera de tomate (*Lycopersicon esculentum* Mill.) como antagonistas de *Fusarium oxysporum* f. sp. radicis-lycopersici. Tesis de Maestría en Recursos Naturales y Medio Ambiente. 2008 Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional Unidad Sinaloa e Instituto Politécnico Nacional.
26. Cortés-Jiménez D, Gómez-Guzmán A, Iturriaga G, Suárez R, Montero Alpírez G, Escalante F. Microorganisms associated to tomato seedlings growing in saline culture act as osmoprotectant. Brazilian Journal of Microbiology. 2014;*45*(2), 613-620.
27. Coyne, M. Microbiología del suelo: un enfoque exploratorio (No. 631.46 C881m). Madrid, ES: Edit. Paraninfo. 2000.
28. Crowley DE. Microbial siderophores in the plant rhizosphere. In: Iron, Nutrition in Plants and Rhizosphere Microorganisms. Barton LL, Abadia J, editores. Springer. Riverside, USA. p.169-198.
29. Domenech J, Reddy MS, Kloepper JW, Ramos, B, Gutierrez-Manero J. Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. BioControl. 2006;*51*(2), 245-258.
30. Dursun A, Ekinci M, Donmez MF. Effects of foliar application of plant growth promoting bacterium on chemical contents, yield and growth of tomato (*Lycopersicon esculentum* L.) and cucumber (*Cucumis sativus* L.). Pak. J. Bot. 2010; 42(5), 3349-3356.

31. Elein TA, Leyva A, Hernández A. Microorganismos benéficos como biofertilizantes eficientes para el cultivo del tomate (*Lycopersicon esculentum*, Mill). Revista colombiana de Biotecnología. 2005;7(2), 47.
32. Escobar C, Horna Y, Carreño C, Mendoza G. Caracterización de cepas nativas de *Azotobacter* spp. y su efecto en el desarrollo de *Lycopersicon esculentum* Mill." tomate" en Lambayeque. Scientia Agropecuaria. 2011;*2*(1), 39-49.
33. Gagné S, Dehbi, L, Le Quéré D, Cayer F, Morin, JL, Lemay R, Fournier N. Increase of greenhouse tomato fruit yields by plant growth-promoting rhizobacteria (PGPR) inoculated into the peat-based growing media. *Soil* Biology and Biochemistry. 1993;*25*(2), 269-272.
34. Ge BB, Cheng Y, Liu Y, Liu BH, Zhang KC. Biological control of Botrytis cinerea on tomato plants using Streptomyces ahygroscopicus strain CK-15. Letters in applied microbiology. 2015;61(6), 596-602.
35. Goldstein AH. Future trends in research on microbial phosphate solubilization: one hundred years of insolubility. In: First International Meeting on Microbial Phosphate Solubilization. Velázquez E, Rodríguez BC, editores. New York, USA. Springer, 2007, p.91-96.
36. Gravel V, Antoun H, Tweddell RJ. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). Soil Biology and Biochemistry. 2007; 39(8), 1968-1977.
37. Gül A, Kidoglu F, Tüzel Y. (2008). Effects of nutrition and *Bacillus amyloliquefaciens* on tomato (*Solanum lycopersicum*, L.) growing in perlite. Spanish Journal of Agricultural Research.2008;6(3), 422-429.

38. Gupta VP, Bochow H, Dolej S, Fischer I. Plant growth-promoting *Bacillus subtilis* strain as potential inducer of systemic resistance in tomato against *Fusarium* wilt. Journal of Plant Diseases and Protection. 2000;145-154.

39. Haas H, Défago G. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 2005; 3: 307-319.

40. Hartmann A, Schmid M, Tuinen DV, Berg G. Plant-driven selection of microbes. Plant Soil. 2008;321, 235–257.

41. Hernández MI, Chailloux M. La nutrición mineral y la biofertilización en el cultivo del tomate (*Lycopersicon esculentum* Mill.). Temas de Ciencia y Tecnología. 2001;15 (3): 11-27.

42. Höfte M, Bakker PA. Competition for iron and induced systemic resistance by siderophores of plant growth promoting rhizobacteria. In Microbial siderophores. Springer Berlin Heidelberg, 2007, p. 121-133.

43. Husen E, Wahyudi AT, Gianto AS. (2011) Soybean response to 1-aminociclopropano-1-carboxylate deaminase-producing *Pseudomonas* under field soil conditions. Amer. J. Agric. Biol. Sci. 1011;6:273-278.

44. Khan A, Ali L, Chaudhary HJ, Munis MFH, Bano A, Masood S. (2016). *Bacillus pumilus* alleviates boron toxicity in tomato (*Lycopersicum esculentum* L.) due to enhanced antioxidant enzymatic activity. *Scientia Horticulturae*. 2016; *200*, 178-185.

45. Lambers H, Mougel C, Jaillard B, Hinsinger P. Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. Plant Soil. 2009; 321, 83–115.

46. Latha P, Anand T, Ragupathi N, Prakasam V, Samiyappan R. Antimicrobial activity of plant extracts and induction of systemic resistance in tomato plants by

mixtures of PGPR strains and Zimmu leaf extract against *Alternaria solani*. Biological Control. 2009; 50(2), 85-93.

47. Luna Martínez, L., Martínez Peniche, R. A., Hernández Iturriaga, M., Arvizu Medrano, S. M., & Pacheco Aguilar, J. R. (2013). Caracterización de rizobacterias aisladas de tomate y su efecto en el crecimiento de tomate y pimiento. *Revista fitotecnia mexicana*, *36*(1), 63-69.
48. Mangmang JS, Deaker R, Rogers G. Early seedling growth response of lettuce, tomato and cucumber to *Azospirillum brasilense* inoculated by soaking and drenching. *Hort. Sci.(Prague)*. 2015;*42*, 37-46.
49. Mantilla M. Evaluación de la acción de un bioinoculante sobre un cultivo de crisantemo (*Chrysanthemun morifolium* var. yoko ono) en periodo de enraizamiento. 2007. Tesis de Licenciatura en Microbiología Agrícola y Veterinaria. Pontificia Universidad Javeriana.
50. Martínez-Viveros O, Jorquera MA, Crowley DE, Gajardo G. Mora ML. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. Journal of soil science and plant nutrition. 2010;10(3), 293-319.
51. Miller EC, Hadley CW, Schawartz, SJ, Erdman JW, Boileau TMW, Clinton SK. Lycopene, tomato products, and prostate cancer prevention. Have we established causality? Pure Appl. Chem. 2002;74(8):1435-1441.
52. Murphy JF, Zehnder GW, Schuster S, Sikora DJ. Polston JE, Kloepper JW. Plant Growth-promoting rhizobacterial mediated protection in tomato against tomato mottle virus. Plant Disease. 2000;84:778-779.
53. Nihorimbere V, Ongena M, Cawoy H, Brostaux Y, Kakana P. Jourdan E, Thonart P. Beneficial effects of *Bacillus subtilis* on field-grown tomato in Burundi:

Reduction of local *Fusarium* disease and growth promotion. Afr. J. Microbiol. Res. 2010;4, 1135-1142.

54. Orrego JAE. Potencial de propagación *in vitro* para el tomate de árbol partenocárpico Cyphomandra betacea Cav.(Sendt). Revista Facultad Nacional de Agronomia Medellin. 2005;*58*(1).
55. Paredes Z. Manual del cultivo de tomate en invernadero. Departamento de Cundinamarca–Colombia Corpoica primera edición, junio, 2009, p.56.
56. Patten CL, Glick R. Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. Appl. Environ. Microbiol. 2002;68, 3795–3801.
57. Persello-Cartieaux F, David P, Sarrobert C, Thibaud MC, Achouak W, Robaglia C, Nussaume L. Utilization of mutants to analyze the interaction between *Arabidopsis thaliana* and its naturally root-associated *Pseudomonas*. Planta. 2001;212(2), 190-198.
58. Porcel R, Zamarreño AM, García-Mina JM, Aroca R. Involvement of plant endogenous ABA in *Bacillus megaterium* PGPR activity in tomato plants. *BMC plant biology*. 2014;*14*(1), 1.
59. Porcel R, Zamarreño AM, García-Mina JM, Aroca R. Involvement of plant endogenous ABA in *Bacillus megaterium* PGPR activity in tomato plants. BMC plant biology. 2014; 14(1), 1.
60. Pulido LE, Medina N, Cabrera A. La biofertilización con rizobacterias y hongos micorrízicos arbusculares en la producción de posturas de tomate (*Lycopersicon esculentum* Mill.) y cebolla (*Allium cepa* L.). I. Crecimiento vegetativo. *Cultivos Tropicales*. 2003;*24*(1), 15-25.

61. Radzki W, Manero FG, Algar E, García JL, García-Villaraco A, Solano BR. Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. Antonie Van Leeuwenhoek. 2013;*104*(3), 321-330.

62. Rao M, Lee H, Mullet J, Davirs K. Jasmonic acid signaling modulates ozone-induced hypersensitive cel death. Plant Cell. 2000;12:1633-1646.

63. Rentería GA. Nutrición de tomate y producción de licopeno. Tesis de Maestría en Ciencias con especialidad de Edafología. 2013. Colegio de Postgraduados.

64. Rodicio M, Mendoza MC. Identificación bacteriana mediante secuenciación del ARNr 16S: fundamento, metodología y aplicaciones en microbiología clínica. Enferm Infecc Microbiol Clin. 2004;22:238-245.

65. Rodríguez BMA. Efecto de rhizobacterias *Azotobacter*, *Acetobacter* y *Azospirillum* sp, sobre plántulas de tomate (*Solanum lycopersicum* L.). Tesis de Licenciatura en Ingeniería de Agronomía en Producción. 2015. Universidad Autónoma Agraria Antonio Narro.

66. Rojas LL. Control biológico del cáncer bacteriano *Clavibacter michiganensis* subsp. *michiganensis* con dos cepas de *Bacillus subtilis* en tomate *Solanum lycopersicum* Mill. variedad Cherry y Saladette. Tesis de Licenciatura en Ingeniería en Agrónomo Parasitólogo. 2014. Universidad Autónoma Agraria Antonio Narro.

67. Romero AM, Vega D, Correa OS. (2014). *Azospirillum brasilense* mitigates water stress imposed by a vascular disease by increasing xylem vessel area and stem hydraulic conductivity in tomato. Applied Soil Ecology. 2014;82, 38-43.

68. Rubio MB, Domínguez S, Monte E, Hermosa R. Comparative study of Trichoderma gene expression in interactions with tomato plants using high-density oligonucleotide microarrays. Microbiology. 2012;158, 119–128.

69. Rubio EJ. 2011. Caracterización molecular y funcional de bacterias del género *Azotobacter* aisladas de suelos de la República Argentina. El rol de las auxinas en la respuesta a la inoculación de trigo. Tesis de Maestría en Producción Vegetal. 2011. Universidad de Buenos Aires.

70. SAGARPA. Monografía del cultivo de tomate. Estudio de gran visión y factibilidad económica y financiera para el desarrollo de infraestructura de almacenamiento y distribución de granos y oleaginosas para el mediano y largo plazo a nivel nacional. SAGARPA. 2010. 256 p.

71. Sánchez LBD, Gómez-Vargas RM, Garrido RMF, Bonilla BRR. Inoculación con bacterias promotoras de crecimiento vegetal en tomate bajo condiciones de invernadero. Revista Mexicana de Ciencias Agrícolas.2012;Vol.3, Núm.7. p. 1401-1415.

72. Serrani JC. Interacción de Giberelinas y Auxinas en la Fructificación del Tomate. Tesis de Doctorado en Ingeniería Agrónomica. 2008. Universidad Politécnica de Valencia.

73. Shinivasan. Safer tomato production techniques. A field guide for soil fertility and pest managment. AVRDC. The World Vegetable Center. Taiwan. 2010.

74. Somers E, Vanderleyden J, Srinivasan M. Rhizosphere bacterial signalling: a love parade Veneta our feet. Crit. Rev. Microbiol. 2004;30, 205–240.

75. Srivastava S, Chaudhry V, Mishra A, Chauhan PS, Rehman A, Yadav A, Nautiyal CS. Gene expression profiling through microarray analysis in *Arabidopsis*

thaliana colonized by *Pseudomonas putida* MTCC5279, a plant growth promoting rhizobacterium. Plant signaling and behavior. 2012;*7*(2), 235-245.

76. Tan S, Yang C, Mei X, Shen S, Raza W, Shen Q, Xu Y. The effect of organic acids from tomato root exudates on rhizosphere colonization of *Bacillus amyloliquefaciens* T-5. Applied soil ecology. 2013;*64*, 15-22.
77. Valenzuela-Soto JH, Estrada-Hernández MG, Ibarra-Laclette E, Délano-Frier, JP. Inoculation of tomato plants (*Solanum lycopersicum*) with growth-promoting *Bacillus subtilis* retards whitefly *Bemisia tabaci* development. *Planta*. 2010;*231*(2), 397-410.
78. Voinnet, O. RNA silencing compared with innate immunity. Nature Rev. Gen. 2005;6:206-220.
79. Wang S, Wu H, Qiao J, Ma L, Liu J, Xia Y, Gao X. 2009. Molecular mechanism of plant growth promotion and induced systemic resistance to tobacco mosaic virus by *Bacillus* spp. J Microbiol Biotechnol. 2009; 19(10), 1250-1258.
80. Wang Y, Ohara Y, Nakayashiki H, Tosa Y, Mayama S. Microarray analysis of the gene expression profile induced by the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5 in *Arabidopsis*. Molecular Plant-Microbe Interactions. 2005;18(5), 385-396.
81. Yamamoto S, Shiraishi S, Kawagoe Y, Mochizuki M, Suzuki S. Impact of *Bacillus amyloliquefaciens* S13-3 on control of bacterial wilt and powdery mildew in tomato. Pest management science. 2015;71(5), 722-727.

Fast detection of auxins by microplate technique

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ABSTRACT

Plant growth promotion indole-3-acetic acid (IAA) is the most abundant natural auxin that plays diverse roles in plant growth, development and plant immunity. Perturbing auxin homeostasis appears to be a common virulence mechanism, as many pathogens can synthesize auxin-like molecules. In other hand, the addition of plant growth promotion rhizobacterias (PGPR) that are able to produce auxins promote plant growth and provide protection against pathogens. Techniques as high performance liquid chromatography (HPLC) and gas chromatography (GC) are used to quantify auxins produced by microorganism and plants at high precision and sensitivity, eventhough those technique are expensive and requires big amount of solvents. For these reason, the aim of the present study was developed a fast microplate technique for auxin detection, in *Bacillus subtilis* strains using salkowski reagent. For auxin quantification were done calibration curves with alcohol, landy medium and water and the R^2 were calculated. The microplate techniques were able to quantify auxin production by *B. subtillis* stains.

Key words. Auxin, microplate, *Bacillus subtilis*

INTRODUCTION

Plant hormones have pivotal roles in the regulation of plant growth, development, and reproduction (Pieterse et al, 2012). Additionally, they emerged as cellular signal molecules with key functions in the regulation of immune responses to microbial pathogens, insect herbivores, and beneficial microbes (Pieterse et al, 2012). Recent studies indicate that hormones such as abscisic acid (ABA), auxin, gibberellic acid (GA), cytokinin (CK), brassinosteroids (BR) and peptide hormones are implicated in plant defense signaling pathways (Bari and Jones, 2009). In other hand, beside the endogenous production of phytohormones, exogenous phytohormones can be provided by plant growth-promoting rhizobacteria (PGPR) (Fernandes, *et al*., 2011; Ahemad and Kibret, 2014). The PGPR produces hormones such as auxins, gibberellins, cytokinins and polyamines (Fernandes *et al*., 2011). Auxin is an important plant hormone that affects almost all aspects of plant growth and development and immunity (Spoel and Dong, 2008). Perturbing auxin homeostasis appears to be a common virulence mechanism, as many pathogens can synthesize auxin-like molecules (Spoel and Dong, 2008). This way, the addition of PRGR producers of auxins promote plant growth and provide protection against pathogens. *Bacillus* sp., *Bacillus subtillis*, *Mycobacterium* sp. and *Pseudomonas putida* are examples of auxin producers (Ahemad and Kibret, 2014; Pandey *et al.*, 2006; Parani & Saha, 2012; Dey *et al*., 2004; Wahyudi *et al*., 2011; Solís *et al*., 2013). The discovery and study of PGPR requires fast and reliable methods for quantifying phytohormones. For auxin and indoles detection are used techniques as high performance liquid chromatography (HPLC) (Fernandez et al., 2011), thin layer chromatography (TLC) (Castillo *et al*., 2007) and spectrophotometric techniques with salkowski reagent (Cubillos *et al*., 2009). Even though these techniques are expensive because of the use of

columns and solvents (HPLC) or are not practical for processing big quantity of samples at the same time (TLC). The aim of this study was to develop a microplate method for quantify auxins, with less amount of reagents, and for processing more samples in fewer time. For auxin quantification were realized calibration curves using 3-indol acetic acid (IAA) dissolved in water, Landy medium or alcohol, and using salkowski reagent. The microplate techniques developed were used to quantify auxins in *Bacillus subtilis* fermentation broths proved previously as auxin producers.

MATERIALS AND METHODS

Bacterial strains and fermentation conditions. BS8 strain was obtained from Cinvestav Irapuato. BS14 and BSN strains were gotten from Phytophatology department from the Antonio Narro Agrarian Autonomous University. Fermentation was realized in landy medium added with tryptophan. Landy medium was prepared as follow: glutamic acid 5.0 g/L, KH_2PO_4 0.5 g/L, K2HPO4 0.5 g/L, $MgSO_4.7\ H_2O$ 0.2 g/L, $MnSO_4.H_2O$ 0.01 g/L, NaCl 0.01 g/L, $FeSO_4\cdot 7$ H2O 0.01 g/L, $CuSO_4.7\ H_2O$ 0.01 g/L, $CaCl_2.2\ H_2O$ 0.015 g/L and tryptophan (final concentration 5mM). A solution of glucose microfiltrated was added to the medium for a final concentration of 1%. A preinoculum was realized by inoculation in 50 mL of landy medium, incubated 48 h at 28 °C. From preinoculum were prepared the fermentation broths at a concentration of $1x10^6$ CFU/mL in Landy medium, and was shaken for 72 h, 120 rpm and 25°C (Awais, 2010). The fermentation broths were centrifugated at 12,500 rpm for 15 min and 15°C to get the supernatant for quantifying auxins (Sayyed *et al*., 2005; Yu *et al*., 2011).

Auxins quantification. Salkowski reagent was prepared as follow: 15 mL of H_2SO_4, 25 mL of distilled water, 0.75 mL of $FeCl_3 \cdot 6H_2O$ (0.5 M) (Gordon and Weber 1951). For calibration curves were prepared stocks of 3-indol acetic acid (IAA) at concentration of 100,000 ppm in ethanol. And work solutions at 16,000 ppm in water, ethanol and or Landy medium (Mata and Méndez-Natera, 2009). For calibration curve were placed in all a row of the microplate 100 µL of the dissolvent (Landy medium, water or ethanol), and then in the first line were added 100 µL of 16,000 ppm work solutions, for get a first point in the calibration curve of 8,000 ppm. It is mixed and 100 µL are taken to the second line (4000 ppm), then mixed and 100 µL are taken to the third line (2000 ppm). This procedure is followed until the line 11 (Mdee, 2009; Kouassi *et al*., 2012; Calvo, 2012). The line 12 is the concentration 0 ppm. 100 µL of salkowski reagent are added to every well, incubated for 30 min and read at 520 nm (Gordon y Weber 1951; Wahyudi et al, 2011; Abdel-Aziz, 2013). Each curve was performed by triplicate. The concentration of IAA in *Bacillus subtilis* strains was evaluated in microplate as follow: 100 µL of cell free supernatant were mixed with 100 µL of salkowski reagent in microplate, incubated for 30 min and read at 520 nm. Absorbance was used to calculate the auxin concentration with the calibrations curves equation.

RESULTS AND DISCUSSION

The purple-pink complexes were observed in the microplate formed by the IAA and the salkowski reagent. R^2 were gotten from calibration curves and indicates the lineality in the evaluated range. From it were get the ranges were its possible interpolate samples to calculate the auxins concentration. At lower concentration range the R^2 was higher than at higher concentration. Each solvent presented different linearity. Since IAA dissolves better in alcohol the lineality with ethanol is higher than in other solvents (Table 1).

Table 1. Solvents, concentration range and R^2 in auxins calibration curves.

Solvent	Concentration range	R^2	Calibration curves equations
Alcohol	0-2000 ppm	0.93	y = 0.0014x + 0.3191
Alcohol	0-31.25 ppm	0.99	y = 0.0107x – 0.0032
Landy medium	0-2000 ppm	0.94	y = 0.0012x + 0.2209
Water	0-4000 ppm	0.80	y = 0.0005x + 0.576

Auxins concentrations in samples were calculated using the calibration curve with ethanol from 0 to 31.25 ppm, which had the higher R^2. The auxins concentration calculated for the *Bacillus subtillis* strains were 2.78 ppm for BS8, 2.59 ppm for BS14 and 18.59 ppm for BSN. The detection of auxins in *Bacillus subtilis* strains probed that they are able to promote plant growth and they can elicit significant reductions in the incidence or severity of various diseases on a diversity of hosts (Kloepper et al, 2004).

Over the years, large efforts have been put in the development of more sensitive and precise methods of analysis and quantification of plant hormone levels in plant tissues and microorganisms. However, although the techniques have evolved, and new methods have been implemented, the sample preparation is still the limiting step of auxin analysis, including extraction, purification and derivatization (Porfirio et al, 2015). The quantification of auxins by salkowski reagent doesn't needs an exhaustive sample preparations. It´s only require the separation of the supernatant by centrifugation after the

fermentation time. Even though, the salkowski reagent is not specific for only one type of auxin. The principal auxin detected by salkowski reagent is the IAA but other auxins can also form complex with the reagent. Glickmann and Dessaux (1995) demonstrated that the production of Salkowski positive compounds, even at low concentrations, was always correlated with production of IAA or indolepyruvic acid (IPyA). For these reason the microplate technique should be complement with other technique as chromatography, if the study needs to quantify a specific auxin.

The developed microplate methods show advantages as analysis of large numbers of samples in a short time with low cost. Even GC-MS is technically the best method to measure IAA, because of high sensitivity and specificity (Kim and Park, 2006; Idris *et al.*, 2007), the high cost for setting and maintenance makes it difficult for daily use in ordinary laboratory (Kim and Park, 2006). The range of lineality of the microplate technique with ethanol and a R^2 of 0.99 is low related to the studies of Szkop in 2013 who quantify by RP-HPCL samples containing indolic compounds at 0.0625–125 ppm, using calibration curves with coefficient of determination (R^2) $\geq$ 0.998, eventhough samples can be diluted for make an approximation of auxin concentration by microplate technique before using another technique. Meanwhile, Kim and Park (2006) established a standard method to quantify IAA based on HPLC, adopting fluorescence detector and Indole-3-propionic acid (IPA) as internal standard and calculated the IAA concentration with the peak area. A microplate method for auxins was realized by Sarwar and Kremer in 1995, were resulted in 95% less sample and reagent volume and it´s faster than the traditional method, since the standard method required each sample by separately. They realized a calibration curve with 6 points from 0 to 40 IAA equivalents (ppm), but it´s not specify the solvent where

it is realized. Alcohol calibration curves with ethanol got a higher R^2 because of the IAA solubility properties. Even though it´s important realize calibration curves with other solvents because most of fermentations broths are colorful and it could interference with the UV spectrometer absorbance. Landy medium, a specific medium enriched with tryptophan for auxin production, presented a different color to the salkowski reagent but is still colorful. For this reason, colorless mediums for auxin production must be developed for colorimetric techniques.

The developed method could be used also to quantify auxins production in plants. However, because hormones production occur in very low amounts in plant extracts, and are very rich in interfering substances, especially secondary metabolites, must undergo several purification steps using unrelated separation mechanisms in order to increase purification efficiency. And specially is important to know which part of the plant contain the highest concentration of auxin. Generally auxins are produced in the meristematic areas of the plant stem although other plant parts, for instance, shoots, roots and leaves may also produce auxins (Ahmed and Hasnain, 2014). Auxin concentration varies depending on the influx and efflux from the tissues, its biosynthesis from tryptophan and formation of IAA conjugates (Tanimoto, 2005). The homeostasis between the endogenous and exogenous auxins contribute in the plant developed and immunology, for these reason the quantification of auxins produced by PGPR is important to select the strain with the right production of phytohormones.

CONCLUSION

The developed method offers an alternative to detect auxin-producing strains. Although the method is not as precise as the chromatographic methods or the traditional methods, they allow to have a previous selection of strains for saving of reagents and time.

REFERENCE

[1] Spoel, S. H., and Dong, X. (2008) Making sense of hormone crosstalk during plant immune responses. Cell Host & Microbe, 3(6), 348-351.

[2] Pieterse, C. M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. (2012) Hormonal modulation of plant immunity. Annual review of cell and developmental biology, 28, 489-521.

[3] Bari, R., and Jones, J. D. (2009) Role of plant hormones in plant defence responses. Plant molecular biology, 69(4), 473-488.

[4] Fernandes G.R., Aparecida N.P., Luque C.T., De Macedo L.E. (2011) Auxin-Producing Bacteria Isolated from the roots of *Cattleya walkeriana*, an endangered Brazilian Orchid, and their role in acclimatization. R. Bras. Ci. Solo, 35:729-737.

[5] Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. Journal of King Saud University-Science, 26(1), 1-20.

[6] Pandey, A., Trivedi, P., Kumar, B., and Palni, L. M. S. (2006) Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in the Indian Central Himalaya. Current microbiology, 53(2), 102-107.Dey et al, 2004.

[7] Parani, K., and Saha, B. K. (2012) Prospects of using phosphate solubilizing *Pseudomonas* as bio fertilizer. Eur J Biol Sci, 4(2), 40-44.

[8] Dey, R., Pal, K. K., Bhatt, D. M., and Chauhan, S. M. (2004) Growth promotion and yield enhancement of peanut (Arachis hypogaea L.) by application of plant growth-promoting rhizobacteria. Microbiological research, 159(4), 371-394.

[9] Wahyudi, A. T., Astuti, R. P., Widyawati, A., Meryandini, A., and Nawangsih, A. A. (2011) Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. J Microbiol Antimicrob, 3(2), 34-40.

[10] Solís, D. R., Pérez, M. C., and Santoyo, G. (2014) Mechanisms for estimating plant growth for bacteria of the genus *Bacillus*. Biological Journal of the Biological science Michoacana University of San Nicolas of Hidalgo, 15(2), 36-41.

[11] Castillo, G., Ortega, G., Carabeo, V., Delgado, G., and Michelena, G. (2007). Qualitative determination of gibberellins and auxins by thin layer chromatography. ICIDCA., 41(1), 12-17.

[12] Cubillos, J. R., Castellanos, D. E., and Argüello, H. (2009) Selection of Plant Growth Promoter Microorganisms (Indole Acetic Acid) from Rizosferic Soil Samples as the First Stage in the Development of a Biofertilizer. Journal Brasileira de Agroecología, 4(2).

[13] Awais, M., Pervez, A., Yaqub, A., and Shah, M. M. (2010) Production of antimicrobial metabolites by *Bacillus subtilis* immobilized in polyacrylamide gel. Pakistan J. Zool, 42(3), 267-275.

[14] Sayyed, R. Z., Badgujar, M. D., Sonawane, H. M., Mhaske, M. M., and Chincholkar, S. B. (2005) Production of microbial iron chelators (siderophores) by fluorescent pseudomonads. Indian Journal of Biotechnology, 4(4), 484-490.

[15] Yu, X., Ai, C., Xin, L., and Zhou, G. (2011) The siderophore-producing bacterium, *Bacillus subtilis* CAS15, has a biocontrol effect on *Fusarium* wilt and promotes the growth of pepper. European Journal of Soil Biology, 47(2), 138-145.

[16] Mata, N. M., and Méndez-Natera, J. R. (2009) Effect of indole-3-acetic acid and naphthaleneacetic acid on the length and width of melon fruit (*Cucumis melo* L.) Edisto 47. Journal Científica UDO Agrícola, 9(3), 530-538.

[17] Mdee, L. K., Masoko, P., and Eloff, J. N. (2009) The activity of extracts of seven common invasive plant species on fungal phytopathogens. South African Journal of Botany, 75(2), 375-379.

[18] Kouassi, K. H. S., Bajji, M., Brostaux, Y., Zhiri, A., Samb, A., Lepoivre, P., and Jijakli, H. (2012) Development and application of a microplate method to evaluate the efficacy of essential oils against *Penicillium italicum* Wehmer, *Penicillium digitatum* Sacc. and *Colletotrichum musea* (Berk. MA Curtis) Arx, three postharvest fungal pathogens of fruits. Biotechnologie, Agronomie, Société et Environnement= Biotechnology, Agronomy, Society and Environment [= BASE], 16(3), 325-336.

[19] Calvo, P., and Zúñiga, D. (2010). Physiological characterization of *Bacillus* spp. Isolated from the potato rhizosphere (*Solanum tuberosum*). Journal Ecologia aplicada,9(1), 31-39.

[20] Gordon, S. A., and Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. Plant Physiology, 26(1), 192.

[21] Abdel-Aziz (2013) Extracellular Metabolites produced by a novel strain, *Bacillus alvei* NRC-14:5. Multiple plant-growth promoting properties. J. Basic Appl. Sci. Res., 3(1)670-682.

[22] Kloepper, J. W., Ryu, C. M., and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by Bacillus spp. Phytopathology, 94(11), 1259-1266.

[23] Porfírio, S., da Silva, M. D. G., Peixe, A., Cabrita, M. J., and Azadi, P. (2016). Current analytical methods for plant auxin quantification–A review. Analytica chimica acta, 902, 8-21.

[24] Glickmann, E., and Dessaux, Y. (1995). A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Applied and environmental microbiology, 61(2), 793-796.

[25] Kim, Y. J., Oh, Y. J., and Park, W. J. (2006) HPLC-based quantification of indole-3-acetic acid in the primary root tip of maize. J Nano Biotech, 3, 40-45.

[26] Idris, E. E., Iglesias, D. J., Talon, M., and Borriss, R. (2007) Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Molecular Plant-Microbe Interactions, 20(6), 619-626.

[27] Szkop, M., and Bielawski, W. (2013) A simple method for simultaneous RP-HPLC determination of indolic compounds related to bacterial biosynthesis of indole-3-acetic acid. Antonie van Leeuwenhoek, 103(3), 683-691.

[28] Sarwar, M., & Kremer, R. J. (1995). Determination of bacterially derived auxins using a microplate method. Letters in applied microbiology, 20(5), 282-285.

[29] Ahmed, A., and Hasnain, S. (2014). Auxins as one of the factors of plant growth improvement by plant growth promoting rhizobacteria. Pol. J. Microbiol, 63, 261-266.

[30] Tanimoto E. (2005). Regulation and root growth by plant hormones roles for auxins and gibberellins. Critical Rev. Plant. Sci. 24: 249–265

Influence of application of *Bacillus subtilis* in plant growth promotion

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ABSTRACT

During this investigation, the aim was to evaluate the efficiency of three *Bacillus subtillis* strains (BS8, BS14 and BEB13) in the growth promotion of vegetal models in bioclimatic chamber conditions. The experiment was carried out with randomized design. Experimental facts evaluated were vegetal model (*Arabidopsis thaliana* and *Solanum lycopersicum*), phenological stage (seeds and seedlings of *Solanum licopersicum*) and the fraction of the fermentation broth added (cell-free supernatant and broth fermentation treatment. In addition were determinated auxins (3-indolacetic acid, tryptamine and 3-indolilacetic acid), jasmonic acid and siderophores in fermentation broths to relate the effect in vegetal model to the concentration of metabolites. Results show that strain promoted better the germination rate when cell free supernatant are added. In seedlings, Bacillus subtilis strains promoted the length of stem and number of leaflets. The growth promotion effect of strains it influence for the concentration of 3-indole-acetic acid, siderophores and jasmonic acid.

Key Words. *Bacillus subtillis*, 3-indolacetic acid, *Solanum lycopersicum*, *Arabidopsis thaliana,* phenological stage, agronomic parameters.

INTRODUCTION

Plants growth promoting rizobacteria (PGPR), estimulate the plant growth by direct and indirect mechanisms. The indirect promotion occurs by production of antibiotics and metabolites that help plants to assimilate nutrients (Ahmad et al. 2008). Direct stimulation occurs by production of phytohomones as auxins, cytokinins and gibberellins, organic

compounds and compounds that increase the vegetal immunity; as jasmonic acid, salicylic acid and phytoalexins (Ahmad et al. 2008; Rojas-Solis et al. 2013). In *Solanum lycopersicum* (tomato) are used PGPR of the genus *Pseudomonas, Azospirillum*, *Azobacter, Bacillus, Agrobacterium* y *Streptomyces* (Alfonso et al. 2005; Haas y Défago 2005; Nihorimbere 2010; Rentería 2013), and can be used alone or in bioformulates (Berg 2009; Choudary y Johri 2008). In the *Bacillus* genus is included *Bacillus subtilis*, and is used for growth promotion of tomato and biocontrol of pathogens as *Fusarium oxysporum* (Yu et al. 2011). Auxins, cytokinins, siderophores and antibiotic are examples of metabolites produced by *Bacillus subtilis*. Cytokinins can be produced endogenously by the plant, but the cytokinins exogenous addition increase the process of growth (Arkhipova et al. 2005). Cytokinins functions are induction of amilase and protease activity, and auxin synthesis (Mantilla 2007). Meanwhile, siderophores chelate the environmental iron and thus make it more digestible for plants (Abdel-Aziz 2013). Some siderophores are considered as antibiotics because they limited the iron for pathogens growth (Aguado-Santacruz et al. 2012). The 3-indolic acetic acid (AIA), one of the most important auxins, control process as cell division, vascular tissue differentiation, formation of apical domain, and organ development (Blakeslee et al. 2005; Tsavkelova et al. 2006). About antibiotics, *Bacillus subtilis* is able to produce more than a dozen of antibiotics with a great diversity of chemical structures. *S*urfactine, iturine A and bacillibactine are examples of antibiotic produced by *Bacillus subtilis* and are able to control the growth of tomato pathongens. Even though, the effect of *Bacillus subtilis* will depend of the interaction of the microorganism with the environment, host (plant) and native microbiote of soil. Also depends on the balance between auxins and other phytohormones (Buensanteai, et al. 2008). Tryptophan, jasmonic acid and siderophores in

the culture medium can improve the production of auxins (Bharucha, 2013). As such, many other factors could influence the efficient of *Bacillus subtilis*. The aim of this study was to evaluate the influence of the method of application of *Bacillus subtilis* in plant growth promotion, using *Solanum lycopersicum* and *Arabidopsis thaliana* as vegetal models. The hypothesis states that each strain will have a different effect on agronomic parameters depending on the application method.

MATERIALS AND METHODS

Strains and seeds obtaining

Three strains of *Bacillus subtilis* were evaluated (BEB-13, BS14, and BS8). BEB-13 strain was provided by the Research Center and Advanced Studies of IPN (CINVESTAV), and, BS8 and BS14 by the Department of molecular parasitology of the Antonio Narro Agrarian Autonomous University. Strains were activated in TGE (tryptone glucose yeast extract) medium at 28°C (Calvo y Zuñiga 2010).

***Bacillus subtilis* fermentation broth**

Fermentations were realized in landy medium added with tryptophan. Landy medium was prepared as follow: glutamic acid 5.0 gL^{-1}, KH_2PO_4 0.5 gL^{-1}, K_2HPO_4 0.5 gL^{-1}, $MgSO_4 \cdot 7 H_2O$ 0.2 gL^{-1}, $MnSO_4 \cdot H_2O$ 0.01 gL^{-1}, NaCl 0.01 gL^{-1}, $FeSO_4 \cdot 7 H_2O$ 0.01 g/L, $CuSO_4 \cdot 7 H_2O$ 0.01 g/L, $CaCl_2 \cdot 2 H_2O$ 0.015 g/L and tryptophan (final concentration 5mM). A solution of glucose microfiltrated was added to the medium for a final concentration of 1%. A preinoculum was realized by inoculation in 50 mL of landy medium, incubated 48 h at 28 °C. From preinoculum were prepared the fermentation broths at a concentration of

$1x10^6$ CFU/mL in Landy medium, and was shaked for 72 h, 120 rpm and 25°C (Awais 2010).

Siderophores detection for CAS medium

For siderophore detection in fermentation broth was used the Brian et al (2011) technique. The technique is based on the use of chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators. The protection when are using these reagents is need because they are moderately harmful to health. The CAS / HDTMA complex is linked tightly to ferric iron and produces blue color. When a strong chelator as a siderophore removes the iron from complex, the color changes from blue to orange (Abdel-Aziz 2013).

Quantification of auxins and jasmonic acid by HPLC

For quantification of AIA, tryptamine, 3-indolilacetonitrilo, and jasmonic acid from fermentations broths of *Bacillus subtilis* was used a HPLC coupled with UV detector. Calibration curves were conducted over a range of 1 to 1000 ppm in mobile phase (methanol-water-acetic acid in ratio 60: 40: 1). For samples, 50 mL of fermentation supernatant were adjusted to pH 2.8 with HCl 1M and extracted 3 times with 50 mL of ethyl acetate. The organic phases were combined and was added anhydrous sodium sulfate to remove moisture (Castillo, 2005). The solvent was removed with rotary evaporator at 60 °C. Finally, the residue was resuspended with 2 mL of mobile phase (methanol-water-acetic acid in ratio 60:40:1) and microfiltered to place in HPLC vials. Chromatographic separation was performed on a reversed phase column C18, using as mobile phase methanol-water-acetic acid (60:40:1), previously degassed and a flow rate of 0.7 mL/min.

Detection was realized at 296 nm with UV detector by interpolating on the calibration curve (Castillo et al. 2005).

Cell-free supernatant treatments in seed

Cell-free supernatants were gotten from *Bacillus subtilis* fermentation broths (BS8, BS14, and BEB-13) by centrifugation at 12,000 rpm, 10 min and 4°C and were filtered with 0.20µm microfilter. For treatments, petri dishes with murasige and skoog medium (MS) were mixed with 1 mL of supernadant before solidification. Petri dishes with 1 mL of landy medium were used as chemical control and petri dishes only with MS as absolute control. Subsequently, *Arabidopsis thaliana* and *Solanum lycopersicum* seeds were added to petri dishes. Were realized nine replicates per treatment. The boxes were placed vertically in bioclimatic chamber at 25 ° C with a photoperiod of 16/8 h light-dark. The germination percentage of seeds was evaluated after 7 days of treatment application (Lakshmanan 2013).

Fermentation treatments in seeds

Solanum lycopersicum and *Arabidopsis thaliana* seeds were immersed 30 minutes in fermentation broths of *Bacillus subtilis*. Subsequently, seeds were placed in petri dishes with MS medium. As chemical control seeds were immersed in landy medium. For absolute control, seeds without treatment were placed in MS petri dishes. Were realize 9 replicates per treatment. Petri dishes were placed vertically in a bioclimatic chamber at 25 ° C with a photoperiod of 16/8 h light - dark (Gomez-Luna et al. 2012). The germination percentage of seeds was evaluated after 7 days of treatment application.

Fermentation treatments in seedlings

Solanum lycopersicum seeds were germinated in peat moss substrate. 21 days germinated seedlings were transplanted to containers with peat moss, perlite and vermiculite in a rate 3:2:1. For treatments, 3 mL of fermentation broths (BS8, BEB-13, and BS14) were added near the root with syringes. Seedlings added with 3mL of landy medium were used as chemical control (CQ) and seedlings without treatment as absolute control (CA). Each treatment was performed with 7 repetitions (Mena-Violante 2009). Stem length and number of leaflets were evaluated daily by 60 days after treatments application. At the day 60 were evaluated: seedlings wet weight, root wet weight, seedlings dry weight, root dry weight, stem plus leaves dry weight, seedlings humidity percentage, root humidity percentage and root length (Gómez-Luna et al 2012; Mena-Violante et al. 2009).

Stadistical analysis

The experiment was established under an ANOVA completely randomized design with a significance level of 0.05 and comparison of Tukey.

RESULTS

Growth promoter metabolites detected in *Bacillus subtilis* fermentations

Siderophores, auxins and jasmonic acid were detected in *Bacillus subtillis*. CAS media demonstrated that *Bacillus subtilis* strains produced siderophores under the conditions in which the assay was performed. Siderophores help plants to assimilate iron and reduce the pathogens incidence. Meanwhile, auxins as IAA, tryptamine and 3-indolilacetonitrile were detected in all fermentation broths at different concentrations. The BS14 strain had the highest concentrations of AIA (147.80 ± 3.03 ppm) and 3-inlolilacetonitrile (352.64 ±

19.37 ppm), and BEB-13 the highest concentration of tryptamine (605.54 ± 39.60 ppm). AIA concentration in evaluated strains was higher compared with previous studies (Wahyudi et al. 2011; Luna et al. 2013). Jasmonic acid was detected in BS8 fermentation broth at a concentration of 54.48 ppm. Table 1 shows the concentrations of metabolites detected in each strain. Since concentrations of metabolites in each strain was different, the effect in plants was expected different. Precursors of AIA (tryptamine and 3-indolilacetonitrile) are indicators of the potential of strains to produce more phytohormones under specific fermentation conditions. Jasmonic acid was evaluated since it promote systemic resistance.

Table 1 Concentrations of AIA, tryptamine, 3-indolilacetonitrile and jasmonic acid detected in *Bacillus subtilis* fermentation broths by HPLC spectroscopy. Expressed in

	AIA	**Triptamine**	**3-indolilacetonitrile**	**Jasmonic acid**
BS8	129.53 ±3.23	387.64 ±29.84	**352.64 ±19.37**	**54.48**
BS14	**147.80 ±3.03**	136.74 ±50.20	194.33 ±8.73	0.00
BEB-13	113.39 ±6.8	**605.54 ±39.60**	118.04 ±4.74	0.00

Effect of *Bacillus subtilis* in seed of *Arabidopsis thaliana* and *Solanum lycopersicum*

Solanum lycopersicum seeds treated with *Bacillus subtillis* fermentation broth or cell-free supernatant didn´t geminated after seven days of treatment application. In *Arabidopsis thaliana* seeds treated with supernatant the percentages of seeds germination were 44 % for BS14, 11% for BS8 and 0% BEB-13, chemical control (CQ) 22% and absolute control (CA) 11%. In *Arabiospsis thaliana* seeds treated with fermentation broth the germination

percentages gotten were BS8 77%, BS14 44%, BEB-13 0%, CQ 22% and CA 33%. Results showed that different results are obtained if the cell-free supernatant or fermentation broth is applied. BS14 had the highest percentage of germination when supernatants were applied, and BS8 when was applied the fermentation broth. Seeds treated with BEB-13 didn´t germinated, this may be related to an excessive production of secondary metabolites that inhibit the seed germination. After fifteen days of treatments application, chemical and absolute controls got contaminated, but not those treated with *Bacillus subitlis*. This is related to the production of siderophores by the *B. subtillis* strains, and the possible production of antibiotics.

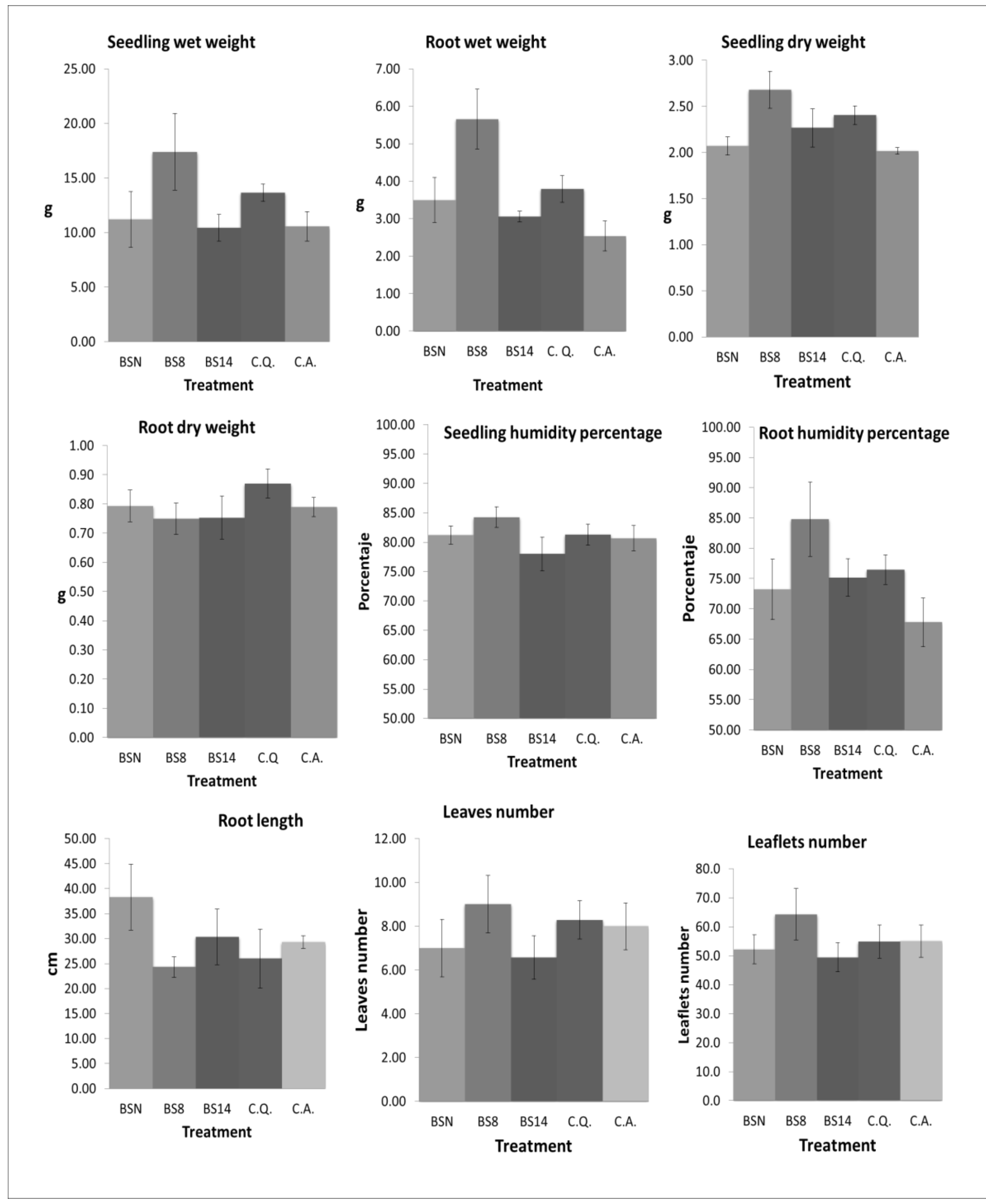
Effect of *Bacillus subtilis* fermentation broth in seedlings of *Solanum lycopersicum*

Stem length measures taken in the day 60 after treatments application, show that *Bacillus subtilis* strains were able to promote length. Respect to absolute control, BS8 increased stem length 45%, BS14 33%, BEB-13 17% and chemical control 9 %. There was realized a growth curve of treated seedlings along 60 days, where was observed that in every growth stage a different *Bacillus subtilis* strain had the best promotion. This mean that depending on the phenological stage of the plant, the effects of each treatment will be different. Therefore, daily measurements were statistically evaluated, along 60 days, for knowing the promotion effect of treatments along the whole experiment and not only at the day 60. Statistical analysis showed BS8 (a) as the one with higher promotion of stem length, followed by BS14 (b) and BEB-13 (c) and controls (c). The curve of growth promotion, by the 60 days, allowed to identify the best treatment in each plant stage, and to identify the days when another dose of *Bacillus subtilis* should be apply. For the parameter of leaflets number, statistical analysis were performed considering daily

measurements, along 60 days. BS8 treatments (A) showed the highest number of leaflets, followed by those treated with BS14 (B), BEB-13 (BC), chemical control (BC), and finally the absolute control (C). The increase in leaflets number are related to the presence of auxins in fermentation broths.

Root wet weight, seedlings dry weight, root dry weight, seedlings humidity percentage, root humidity percentage and root length didn´t show statistical difference between treatments. However, there were observed differences in the type of roots. Some *Bacillus subtilis* strains promoted primary roots and others the secondary roots. For seedling wet weight (leaves plus stem and root) the best treatment was BS8 (a), followed by chemical control (ab), BEB-13 (ab), the absolute control (b) and finally the BS14 (b) . For stem plus leaves dry weight the best treatment was BS8 (a), followed by BS14 (ab), BEB-13 (ab) and chemical control (ab), and finally the absolute control (b). The effect of each strain is related with the kind and concentration of promoting metabolites, and with the interaction with other metabolites. Instead BS14 had the highest concentration of AIA, BS8 had the higher promotion in seedling wet weight. Probably, BS8 produces other kind of phytohormones as cytokinins. Meanwhile, BS14, which had the highest concentration of AIA, had the lowest promotion in the wet weight. BEB-13 strain, which had the lowest concentration of AIA, promoted root length but provided a short stem. Meanwhile, BS14 which had the highest concentration of AIA, promoted the stem length but got a short root. Finally, BS8 presented lot of secondary roots. Figure 1 shows the agronomic parameters evaluated in seedlings treated by *Bacillus subtillis* strains.

Figure 1. Agronomic parameters evaluated in seedlings treated by *Bacillus subtillis* strains (BEB-13 (BSN), BS8, and BS14), chemical control (C.Q.) and absolute control (C.A.).



DISCUSSIONS AND CONCLUSION

Bacillus subtilis is a PGPR that promotes growth for mechanism as phytohormones productions. Despite, the effect depend of several facts. Results of the present study showed that the concentration of each detected metabolite, the vegetal model, the application of cell-free supernatant or fermentations broth and the phenological stage of the vegetal model influenced in the efficient of *Bacillus subtilis* strains as growth promoters. The concentration of metabolites quantified in *Bacillus subtilis* determined the effect in agronomic parameters. Some evaluated parameters were increased and other decreased when *Bacillus subtilis* were applied, since metabolites act by antagonism or synergism with other metabolites. In previous studies have been associated accumulation of cytokinins to the increased of plants weight (30%), coupled with high levels of other phytohormones such as AIA and abscisic acid (ABA) (Arkhipova et al. 2005; Rojas- Solis et al. 2013). On other hand, there was observed that the strain which had the highest concentration of AIA, had the lowest promotion in the wet weight, this is because the AIA excess comes to have a negative effect on growth promotion. Jordan and Cassareto (2006) indicate that high concentration of AIA promotes stem elongation but inhibits root growth, while low concentration of AIA induces root growth. The quantification of precursors of AIA proved the potential of strains to produce AIA, when fermentations broths were added to seedlings. Finally, the presence of siderophores avoided the contamination of seeds with pathogens. Siderophores increase iron assimilation and limit the pathogens growth (Tejera-Hernandez et al. 2011). In previous studies, pathogens such as *Phytophthora capsici* (Woo and Kim. 2008), *Fusarium oxysporum* (Cazorla et al. 2007; Yu et al. 2011), and *Podosphaera fusca* (Jung et al. 2006) have been controlled through siderophore production. Also, the plant model used was critical in the effect of *Bacillus*

subtilis. In seeds, the germination rate was promoted when *Bacillus subtilis* were added to *Arabidopsis thaliana* but not when were added to *Solanum lycopersicum*. This mean that in *Solanum lycopersicum* the concentration of metabolites weren´t appropriate for release the seed dormancy. It has been shown that host-rizobacteria interactions favor the promoting effect of *Bacillus* spp. (Martínez-Viveros et al. 2010). Therefore, the interaction between *Bacillus subtilis* and *Solanum lycopersicum* wasn't favorable either by the behavior of the microorganism or by the inappropriate production of metabolites. The effect of *Bacillus subtilis*, in *Arabidopsis thaliana* was different when cell-free supernatant or fermentation broth were added. Some strains promoted the germination rate when supernatant was added and others when the fermentation broth was added. Liu et al in 2013, noted that dormancy or seed dormancy is reduced by the presence of auxin through the stimulation of abscisic acid signaling. The strain with higher concentration of AIA had the better promotion when supernatant was added. Meanwhile, the strain with a middle concentration of AIA and the higher concentration of tryptamine had the better promotion when fermentation broth was added, this was related to a constant production of AIA around the seed by biofilm formation (Bais et al. 2004). Growth promotion by *Bacillus subtilis* also was influenced by the phenological stage of plant. The BEB-13 strain, for example, inhibited the germination of seed but promoted the growth of seedlings. This was related to the phytohormones need in each phenological stage. *Bacillus subtilis* promoted the stem length and leaflets through 60 days without a daily application since the microorganisms were able to produce auxins constantly. Previous studies show that auxins influence in the intensity of promoting cell division, stem elongation and root length. Also auxins delay abscission organs (leaves, flowers and young fruit) by inhibiting hormone ethylene (Van Doorn and Stead 1997). In other hand,

there were realized stem length and leaflets curves through 60 days and was observed that some strains had a better promotion effect in specific days. Therefore, it´s important to define the phenological stage that want to be promoted. This way was conclude that the choice of the method for *Bacillus subtilis* application is key in the growth promotion in *Arabidopsis thaliana*, and probably in others plants.

ACKNOWLEDGMENTS

This research was financial by the Antonio Narro Autonomous Agrarian University.

REFERENCE

1. Abdel-Aziz. 2013. Extracellular Metabolites produced by a novel strain, Bacillus alvei NRC-14:5. Multiple plant-growth promoting properties [online]. J. Basic Appl. Sci. Res. **3**(1)670-682. DOI: 10.7537/marslsj080411.113
2. Aguado-Santacruz, G.A., Moreno-Gómez, B., Jiménez-Francisco, B., García-Moya, E., and Preciado-Ortiz, R.E. 2012. Impacto de los sideróforos microbianos y fitosidéforos en la asimilación de hierro por las plantas: una síntesis [online]. Revista fitotecnia mexicana. **35**(1): 9-21. Available <http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0187-73802012000100004&lng=es&nrm=iso>. ISSN 0187-7380.
3. Ahmad, F., Ahmad, I., and Khan, M.S. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities [online]. Microbiological Research **163**: 173-81. DOI:10.1016/j.micres.2006.04.001
4. Alfonso, E.T., Leyva, A.H. 2005. Microorganismo Benéficos como Biofertilizantes eficientes para el cultivo del tomate (*Lycopersicum esculentum*

Mill) [online]. Revista Colombiana de Biotecnología. **2**: 47-54. Available http://www.revistas.unal.edu.co/index.php/biotecnologia/article/view/498/892

5. Arkhipova T.N., Veselov S.U., Melentiev A.I., Martynenko E.V., Kudoyarova G.R. 2005. Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. [online]. Plant and Soil. **272**: 201-209. DOI: 10.1007/s11104-004-5047-x
6. Awais, M., Pervez, A., Yaqub, A., and Shah, M.M. 2010. Production of antimicrobial metabolites by *Bacillus subtilis* immobilized in polyacrylamide gel. [online]. Pakistan J. Zool. **42**(3): 267-275.
7. Bais H.P., Fall R., and Vivanco J.M. 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production [online]. Plant Physiol. **134**: 307–319. DOI: 10.1104/pp.103.028712
8. Berg G. 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture [online]. Appl. Microbiol. Biotechnol. **84**: 11-18. DOI:10.1007/s00253-009-2092-7
9. Bharucha U., and Patel K. 2013. Optimization of Indole Acetic Acid Production by *Pseudomonas putida* UB1 and its Effect as Plant Growth-Promoting Rhizobacteria on Mustard (*Brassica nigra*) [online]. Agric Res. **2**(3): 215–221. DOI: 10.1007/s40003-013-0065-7.
10. Blakeslee, J.J., Peer, W.A., and Murphy, A.S. 2005. MDR/PGP auxin transport proteins and endocytic cycling. In Plant Endocytosis. *Edited by* Samaj, J., Baluska, F., Menzel D. Berlin Heidelberg. pp. 159-176. DOI: 10.1007/7089_010

11. Brian, C., Daniel, H. and Aeron, M. 2011. Use of blue agar CAS assay for siderophore detection [online]. J. Microbiol. Biol. Education. **12**. DOI: 10.1128/jmbe.v12i1.249

12. Buensanteai, N., Yuen, G.Y., Prathuangwong S. 2008. The biocontrol bacterium *Bacillus amyloliquefaciens* KPS46 produces auxin, surfactin and extracellular proteins for enhanced growth of soybean plant [online]. Thai J. Agric. Sci. **41**(3-4): 101-116. DOI: 10.1007/s40003-013-0065-7

13. Calvo, P., and Zúñiga, D. 2010. Caracterización fisiológica de cepas de *Bacillus* spp. aisladas de la rizósfera de papa (*Solanum tuberosum*) [online]. Ecologia aplicada, **9**(1): 31-39. Available http://www.lamolina.edu.pe/ecolapl/articulo_4_no_1_vol_9.htm

14. Castillo, G., Altuna, B., Michelena, G., Bravo, J. S., & Echeverría, M. A. 2005. Cuantificación del contenido de ácido indolacético (AIA) en un caldo de fermentación microbiana [online]. Anales de Biología **27**: 137-142. Available: http://www.um.es/analesdebiologia/numeros/27/PDF/16-CUANTIFICACION.pdf

15. Cazorla, F. M., Romero, D., Pérez-García, A., Lugtenberg, B. J. J., Vicente, A. D., and Bloemberg, G. 2007. Isolation and characterization of antagonistic Bacillus subtilis strains from the avocado rhizoplane displaying biocontrol activity [online]. Journal of Applied Microbiology, **103**(5): 1950-1959. DOI: 10.1111/j.1365-2672.2007.03433.x

16. Choudhary, D.K., Johri, B.N. 2009. Interactions of *Bacillus* spp. and plants–with special reference to induced systemic resistance (ISR) [online]. Microbiological research. **164**(5): 493-513. DOI: 10.1016/j.micres.2008.08.007

17. Gómez-Luna, B. E., Hernández-Morales, A., Herrera-Méndez, C. H., Arroyo-Figueroa, G., Vargas-Rodríguez, L., and Olalde-Portugal, V. 2012. Aislamiento de bacterias promotoras del crecimiento de la rizósfera de plantas de guayaba (*Psidium guajava*). Ra Ximhai, **8**(3): 97-102.
18. Haas, D., and Défago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads [online]. Nature Reviews Microbiology, **3**(4): 307-319. DOI: doi:10.1038/nrmicro1129
19. Jordan, M., Casaretto, J. 2006. Hormonas y Reguladores del crecimiento: Auxinas, Giberilinas y Citocininas. Capítulo XV. In Fisiología Vegetal. *Edited by* F.A. Squeo and L. Cardemil. Ediciones Universidad de la Serena, Chile. 15;xx-xx. Available http://listas.exa.unne.edu.ar/biologia/fisiologia.vegetal/Auxinasgiberelinasycitocininas.pdf
20. Jung, H. K., Kim, J. R., Woo, S. M., and Kim, S. D. 2006. An auxin producing plant growth promoting rhizobacterium *Bacillus subtilis* AH18 which has siderophore-producing biocontrol activity [online]. Microbiology and Biotechnology Letters, **34**(2): 94-100. Available http://www.koreascience.or.kr/article/ArticleFullRecord.jsp?cn=MSMSBV_2006_v34n2_94
21. Lakshmanan, V., Castaneda, R., Rudrappa, T., and Bais, H. P. 2013. Root transcriptome analysis of Arabidopsis thaliana exposed to beneficial Bacillus subtilis FB17 rhizobacteria revealed genes for bacterial recruitment and plant defense independent of malate efflux [online]. Planta. **238**(4): 657-668. DOI: 10.1007/s00425-013-1920-2

22. Luna Martínez, L., Martínez Peniche, R. A., Hernández Iturriaga, M., Arvizu Medrano, S. M., and Pacheco Aguilar, J. R. 2013. Caracterización de rizobacterias aisladas de tomate y su efecto en el crecimiento de tomate y pimiento [online]. Revista fitotecnia mexicana. **36**(1): 63-69. Available http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0187-73802013000100007

23. Mantilla, M.E. 2007. Evaluación de la acción de un bioinoculante sobre un cultivo de crisantemo (*Chrysanthemum morifolium* var. Yoko ono) en período de enraizamiento. Thesis, Javeriana Pontifical University, Bogota. Available http://www.javeriana.edu.co/biblos/tesis/ciencias/tesis35.pdf

24. Mena-Violante, H. G., Cruz-Hernández, A., Paredes-López, O., Gómez-Lim M.A. and Olalde-Portugal V. 2009. Fruit texture related changes and enhanced shelf-life through tomato root inoculation with *Bacillus subtilis* BEB-13BS [online]. Agrociencia **43**(6): 559-567. Available http://scielo.unam.mx/pdf/agro/v43n6/v43n6a1.pdf

25. Nihorimbere, V., Ongena, M., Cawoy, H., Brostaux, Y., Kakana. 2010. P. Jourdan E, Thonart P. Beneficial effects of *Bacillus subtilis* on field-grown tomato in Burundi: Reduction of local *Fusarium* disease and growth promotion [online]. Afr. J. Microbiol. Res. **4**: 1135-1142. Available http://www.academicjournals.org/journal/AJMR/article-full-text-pdf/9AD97F013412

26. Persello-Cartieaux, F., David, P., Sarrobert, C., Thibaud, M.C., Achouak, W., Robaglia, C., and Nussaume L. 2001. Utilization of mutants to analyze the

interaction between *Arabidopsis thaliana* and its naturally root-associated *Pseudomonas* [online]. Planta. **212**(2), 190-198. DOI: 10.1007/s004250000384

27. Rentería, G.A. Nutrición de tomate y producción de licopeno. Thesis. Graduate College, Mexico. Available http://hdl.handle.net/10521/2050
28. Rojas-Solís D., Contreras-Pérez M., Santoyo G. 2013. Mecanismos de estimulación del crecimiento vegetal en bacterias del género *Bacillus* [online]. Biológicas. **15**(2):36-41. Vol.15, No.2. Available http://www.biologicas.umich.mx/index.php/biologicas/article/view/166
29. Srivastava, S., Chaudhry, V., Mishra, A., Chauhan, P.S., Rehman, A., Yadav, A. and Nautiyal, C.S. 2012. Gene expression profiling through microarray analysis in *Arabidopsis thaliana* colonized by *Pseudomonas putida* MTCC5279, a plant growth promoting rhizobacterium [online]. Plant signaling and behavior. **7**(2): 235-245. DOI:10.4161/psb.18957
30. Tejera-Hernández, B., Rojas-Badía, M. M., and Heydrich-Pérez, M. 2011. Potencialidades del género *Bacillus* en la promoción del crecimiento vegetal y el control biológico de hongos fitopatógenos Revista CENIC [online]. Ciencias Biológicas. **42**(3): 131-138. ISSN (Versión impresa): 0253-5688.
31. Tsavkelova, E.A., Klimova, S.Y., Cherdyntseva, T.A., and Netrusov, A.I. 2006. Microbial producers of plant growth stimulators and their practical use: a review [online]. Applied Biochemistry and Microbiology. **42**(2): 117-126. DOI: 10.1134/S0003683806020013
32. Van Doorn, W.G., and Stead, A.D. 1997. Abscission of flowers and floral parts [online]. Journal of Experimental Botany. **48**(4): 821-837.DOI: 10.1093/jxb/48.4.821

33. Wahyudi, A.T., Astuti, R.P., Widyawati, A., Mery, A., and Nawangsih, A.A. 2011. Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria [online]. Journal of Microbiology and Antimicrobials. **3**(2): 34-40. Available http://www.academicjournals.org/journal/JMA/article-abstract/148DAF29759

34. Woo, S.M., and Kim, S.D. 2008. Structural Identification of Siderophore∧ AH18 from *Bacillus subtilis* AH18, a Biocontrol agent of Phytophthora Blight Disease in Red-pepper [online]. Microbiology and Biotechnology Letters. **36**(4): 326-335. Available http://www.koreascience.or.kr/article/ArticleFullRecord.jsp?cn=MSMSBV_2008_v36n4_326

35. Yu, X., Ai, C., Xin, L., and Zhou, G. 2011. The siderophore-producing bacterium, *Bacillus subtilis* CAS15, has a biocontrol effect on *Fusarium* wilt and promotes the growth of pepper [online]. European Journal of Soil Biology, **47**(2): 138-145. DOI: 10.1016/j.ejsobi.2010.11.001

Phytohormones gene expression in *Solanum lycopersicum* treated by 3-indole acetic acid and *Bacillus subtilis*

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Abstract. In plants, the phytohormones gene expression regulate growth and development. Phytohormones can be produced endogenously by plant or can be added exogenously. The objective of this study was to determine the phytohormones gene expression in *Solanum lycopersicum* treated by 3-indole acetic acid and three strain of *Bacillus subtilis* (proved as auxins producers). The evaluated genes were hormone response genes or involved in the synthesis of hormones (cytokinins, auxins and giberilins). Gene expression were evaluated at 12 and 24 h after treatments were applied. Semi-quatitative RT-PCR reveals that auxin addition up-regulated or down-regulated genes related with other phytohormones as cytokinins and gibberillins. And the over-expression of some hormones genes had as result the down-regulation of other hormones.

Key words

Auxin, gene expression, *Bacillus subtillis*

INTRODUCTION

Auxin induces the expression of a number of growth-associated genes including enzymes of other phytohormones as gibberillins and cytokinins. Auxins regulate diverse cellular and developmental responses in plants, including cell division, expansion and differentiation, patterning of embryos, vasculature and other tissues, and distribution of growth between primary and lateral root and shoot meristems (Reed, 2001), even if auxins are produced endogenous by plants or added exogenous. In addition, resent studies had showed that auxins and other hormones affect at genetic level and influence in acquired resistance by priming plants. The perception and signaling of the plant hormone auxin rely on the cooperative action of several components, among which auxin/indole-3-acetic acid (Aux/IAA) proteins play a pivotal role (Audran-Delalande et al, 2012). Aux/IAA genes were first isolated as members of a family of genes that were rapidly induced in response to auxin (indole-3-acetic acid, IAA) (Reed, 2001). Aux/IAAs are described as short-lived and nuclear-localized proteins (Hagen and Guilfoyle 2002, Liscum and Reed 2002) Audran-Delalande et al, 2012, and biochemical and genetic studies indicated that they generally function as transcriptional repressors of auxin-regulated genes (Tiwari et al. 2001, Tiwari et al. 2004). Aux/IAA proteins were shown to be a direct target of the auxin transport inhibitor response1 (TIR1) and of its paralogs AUXIN RECEPTOR F-BOX/AFB1 and AFB3F-box receptors (AFBs) (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Tan et al. 2007). Audran-Delalande et al, 2012 Canonical Aux/IAA proteins share four conserved amino acid sequence motifs known as domains I, II, III and IV, although several proteins lacking one or more of these domains are also included in the family (Reed 2001). Bioactive gibberellins (GAs) regulate distinct developmental processes during the entire life cycle of a higher plant, including growth

and flowering (Davies, 2004; Fleet and Sun, 2005; Mutasa-Go¨ttgens and Hedden, 2009). GAs are formed via complex biosynthetic pathways, the final part of which is catalysed by 2-oxoglutarate-dependent dioxygenases, referred to as GA-oxidases (Lange et al, 2012).

Gibberellin (GA) biosynthesis is necessary for normal plant development, with later GA biosynthetic stages being governed by multigene families. Arabidopsis thaliana contains five GA 20-oxidase (GA20ox) genes (Plackett et al, 2012; Lange et al, 2012). In Arabidopsis, five genes encode 69 GA20ox enzymes. In Col-0 background, GA20ox1, -2, and -3 are the dominant forms with an important role in growth and fertility while GA20ox4 and 5 have minor roles (Plackett et al., 2012; Nam et al, 2016). Auxin induces the expression of a number of growth-associated genes including gibberellin biosynthesis enzymes (Frigerio et al, 2006; Jaillais and Chory, 2010; Nam et al, 2016).

Cytokinins are abundant in proliferating tissues, such as root and shoot apical meristems, young leaves, and immature seeds (Letham, 1994). Sites of cytokinin biosynthesis are not known well. Tissues with high levels of cytokinins are generally considered to be the sites of cytokinin biosynthesis (Miyawaki et al, 2003). In many plant species the cytokinins biosynthesis is catalysed by adenosine phosphate isopentenyltransferase (IPT), producing isopentenyladenine (iP) nucleotides as CK precursors (Kakimoto, 2001; Takei et al., 2001; Sakamoto et al., 2006). In *Arabidopsis thaliana*, the iP-nucleotides are converted into trans-zeatin (tZ) nucleotides by the cytochrome P450 monooxygenases, CYP735A1 and CYP735A2 (Takei et al., 2004). In tomato, Matsuo et al (2012) isolated and designed two CYP735A-like cDNA sequences, SlCYP735A1 and SlCYP735A2. SlCYP735A1 was mainly expressed in roots, flowers, and young fruits and SlCYP735A2 was expressed in leaves, roots, and young fruits.

The present study investigated the effect of IAA and *Bacillus subtilis* strains (IAA producers) in phytohormones gene expression in *Solanum lycopersicum* seedlings. It has been prove in previous studies that the exogenous addition of phytohormones can affect the production of other phytohomones that try to maintain the homeostasis for the plant growth and development.

METHODOLOGY

Strains and seedlings obtaining. Three strains of *Bacillus subtilis* were evaluated (BEB-13, BS14, and BS8). BEB-13 strain was provided by the Research Center and Advanced Studies of IPN (CINVESTAV), and, BS8 and BS14 by the Department of molecular parasitology of the Antonio Narro Agrarian Autonomous University. Strains were activated in TGE (tryptone glucose yeast extract) medium at 28°C (Calvo y Zuñiga 2010). *Solanum lycopersicum* seeds were germinated in peat moss substrate. 21 days germinated seedlings were transplanted to containers with peat moss, perlite and vermiculite in a rate 3:2:1.

***Bacillus subtilis* fermentation broth.** Fermentations were realized in landy medium added with tryptophan. Landy medium was prepared as follow: glutamic acid 5.0 gL^{-1}, KH_2PO_4 0.5 gL^{-1}, K_2HPO_4 0.5 gL^{-1}, $MgSO_4 \cdot 7 H_2O$ 0.2 gL^{-1}, $MnSO_4 \cdot H_2O$ 0.01 gL^{-1}, NaCl 0.01 gL^{-1}, $FeSO_4 \cdot 7 H_2O$ 0.01 g/L, $CuSO_4 \cdot 7 H_2O$ 0.01 g/L, $CaCl_2 \cdot 2 H_2O$ 0.015 g/L and tryptophan (final concentration 5mM). A solution of glucose microfiltrated was added to the medium for a final concentration of 1%. A preinoculum was realized by inoculation in 50 mL of landy medium, incubated 48 h at 28 °C. From preinoculum were prepared the

fermentation broths at a concentration of 1x10^6 CFU/mL in Landy medium, and were shaken for 72 h, 120 rpm and 25°C (Awais 2010).

IAA solutions. A Stock of IAA at 10,000 ppm was prepared in ethanol. Later, from the stock were prepared the IAA solutions at 50 ppm and 100 ppm with water.

Treatments application to seedlings. Six treatments were evaluated in *Solanum lycopersicum* seedlings: three *Bacillus subtillis* fermentation broths (BEB-13, BS8, BS14), AIA at 50 ppm, AIA at 200 ppm, and seedlings without treatment as absolute control (CA). From each treatment were added 3 mL near the root with syringes. Treatments were performed with two replicates. After 12 h of treatments applications were taken follicles from the apical zone for RNA isolation. In the same way the experiment was performed for evaluation at 24 h.

RNA isolation and Semiquantitative retrotranscriptase PCR (RT-PCR). Total RNA was extracted from apical follicles according to a modified version of the TRIZOL® method (Life Technologies, Grand Island, NY). The ARN quality was observed with a 1 % agarose gel in DEPC wather. Later the ARN was quantify with UV spectrophotometer at 260/280 nm. After, the obtention of cDNA and the PCR were realized with a MyTaq™ One-Step RT-PCR Kit (Bioline) and the manufacturers' protocol was followed. For RT-PCR were used two genes related to auxins: AUXIAA and GH3 (PBS), one gene related to gibberilins (Ga20ox4) and one gene related to cytokinins (SICYP735A1). Also an endogenous gene (G3F) of *Solanum lycopersicum* was evaluated. Sequenses of primers were designed with primer blast of NCBI and was calculated the annealing temperature.

The expected molecular weight were for endogenous gene 190pb; GH3, 177 pb; AUXIAA, 130 pb; GA20ox4, 895 pb and for SICYP7 291 pb, The expected bands were observed in 1% agarose gel by electrophoresis. Relative fold differences were calculated based the Imagen J program.

RESULTS AND DISCUSSION

Results show that the addition of exogenous IAA affect the expression of genes related with phytohormones (Table 1), not only of IAA. The time when ARN was extracted from treated plants also influenced the gene expression. For example the gene of cytokinins presented a different expression at 12 and 24 h. In other hand in the auxins genes, GH3 was down expressed and AUXIAA over expressed at 12 h.

Table 1. Expression of genes in *Solanum lycopersicum* treated by IAA and *Bacillus subtilis*

	12h				**24h**			
	Auxins		**Giberillins**	**Cytokinins**	**Auxins**		**Giberillins**	**Cytokinins**
Treatments	**GH3**	**AUXIAA**	**GA20ox4**	**SlCYP735A1**	**GH3**	**AUXIAA**	**GA20ox4**	**SlCYP735A1**
BS8	0.32	**1.07**	0.00	**1.54**	0.61	0.41	0.20	0.58
BS14	0.51	**1.13**	0.00	**3.90**	0.95	0.57	0.31	0.94
BSN	0.35	0.62	0.00	**2.22**	0.68	0.43	0.03	0.00
AIA 50 ppm	0.35	**3.76**	0.00	**3.70**	0.92	0.54	0.08	0.91
AIA 200 ppm	0.31	0.77	0.00	**1.27**	**1.07**	0.97	0.09	**1.03**
Control	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Gene AUXIAA in *Solanum lycopersicum* treated with BS8, BS14, and with IAA at 50 ppm was overexpressed at 12 h after treatment but down-expressed at 24 h. Auxin/indoleacetic acid (Aux/IAA) genes, encoding short-lived nuclear proteins, are key

regulators in the auxin transduction pathway (Wu et al, 2012). In other hand IAA at 50 ppm increased triplicate the expression of AUXIAA at 12 hours, meanwhile IAA at 200 ppm decrease the gene expression. In contrast at 24 h the treatment of IAA at 200 ppm was the one with the higher expression of AUXIAA. This showed that when the concentration of IAA is low the gene got activated at the first hours and then is depressed, but when the IAA concentration is high first the gene is depressed and at 24 h the gene expression increase. The response to auxins depends to the concentration of auxins (Paponov et al, 2008). This is related to the homeostasis process. Abel and Theologis (1996) reported tan the auxins addition provokes the rapid transitory accumulation of the genes SAURs (por small auxin upregulated RNAs), GH3 and Aux/IAA.

The gene GH3 was down expressed with all the treatments at 12 h, and only IAA at 200 ppm overexpressed GH3 at 24h. To maintain IAA homeostasis in plants, some members of the GH3 gene family may mediate the conjugation of amino acids to IAA (Zhang et al, 2011). Accumulated evidence suggests that some members of the GH3 gene family may be involved in modulating the level of free IAA, jasmonic acid (JA), and salicylic acid (SA) via amino acid conjugation and light signaling. In Arabidopsis thaliana, 20 members of the GH3 gene family have been characterized and subdivided into three groups, namely I (jasmonate-conjugating), II (auxin- and salicylic acid-conjugating), and III (benzoate-conjugating) (Chen et al. 2010). The higher concentration of IAA activated the GH3 at 24 h to avoid the damage of the plant by IAA excess.

Gibberillins gene were down expressed in all the treatments. In contrast, at 12 hours cytokinins gene was over expressed with all treatments. Phytohormones such as

gibberllins and cytokinins play an important role in the regulation and development of plants. However, they sometimes have antagonistic effects in some developmental processes (Weiss and Ori, 2007). Therefore, at certain stages of plant growth, the expression of some genes may be suppressed while others are overexpressed. Similarly, the addition of exogenous phytohormones may alter the production of the same type of added hormones or even of another class of hormones (Ariizumi et al, 2013). Previous studies in Arabidopsis have shown that giberillins signaling inhibits cytokinins. For its part, in the present study gibberillins were down expressed and citikins over expressed as a consequence of auxins addition. Studies by Fleishon et al. (2011) evaluated the balance between giberillins and citokinis by adding exogenous hormones. In that study the phytohormones showed antagonistic effects on molecular processes among them: Giberillins inhibited the induction of first response genes of cytokinis, of the type TRRS (Tomato Response Regulators). In contrast, the exogenous application of gibberillins does not repress the regulation of endogenous citokinins (Genes DELLA) (Ariizumi et al, 2013).

CONCLUSION

The addition of exogenous phytohormones induces plant response in the production of phytohormones. The treatments modified the expression of genes related to phytohormones (increasing or decreasing expression). The AUXIAA gene (auxins) and the gene related to the synthesis of cytokinins showed the greatest increase in expression with treatments applied at 12h. Gene overexpression was greater at 12 h than at 24 h after application of treatments. Overexpression of cytokinin genes may be related to decreased gene expression of gibberilins.

REFERENCES

1. Abel S.A. y Theologis. (1996). Early genes and auxin action. Plant Physiology 111: 9–17. AOYAMA T & A OKA. 2003. Cytokinin signal transduction in plant cells. J Plant Research 116: 221- 231.
2. Ariizumi, T., Hauvermale, A. L., Nelson, S. K., Hanada, A., Yamaguchi, S., & Steber, C. M. (2013). Lifting DELLA repression of Arabidopsis seed germination by nonproteolytic gibberellin signaling. Plant physiology, 162(4), 2125-2139.
3. Audran-Delalande, C., Bassa, C., Mila, I., Regad, F., Zouine, M., & Bouzayen, M. (2012). Genome-wide identification, functional analysis and expression profiling of the Aux/IAA gene family in tomato. *Plant and Cell Physiology*, *53*(4), 659-672.
4. Chen Q, Westfall CS, Hicks LM, Wan S, Jez JM (2010) Kinetic basis for the conjugation o auxin by a GH3 family indole-acetic acidamido synthetase. J Biol Chem 39:29780–29786.
5. Davies PJ. 2004. Plant hormones: biosynthesis, signal transduction, action! Dordrecht, The Netherlands: Kluwer Academic Publishers.
6. Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005a) The F-box protein is an auxin receptor. Nature. 435: 441–445.
7. Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L. et al. (2005b) Plant development is regulated by a family of auxin receptor F box proteins. Dev. Cell 9: 109–119.
8. Fleet CM, Sun TP. 2005. A DELLA cate balance: the role of gibberellin in plant morphogenesis. Current Opinion in Plant Biology 8, 77–85.
9. Fleishon, S., Shani, E., Ori, N., & Weiss, D. (2011). Negative reciprocal interactions between gibberellin and cytokinin in tomato. New Phytologist, 190(3), 609-617.
10. Frigerio, M. *et al*. Transcriptional regulation of gibberellin metabolism genes by auxin signaling in *Arabidopsis. Plant Physiol.* 142, 553–563 (2006).
11. Hagen, G. and Guilfoyle, T. (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol. Biol. 49: 373–385.
12. Jaillais, Y., & Chory, J. (2010). Unraveling the paradoxes of plant hormone signaling integration. *Nature structural & molecular biology*, *17*(6), 642-645.

13. Kakimoto T. 2001. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. Plant and Cell Physiology 42, 677–685.
14. Kepinski, S. and Leyser, O. (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435: 446–451.
15. Lange, M. J. P., Knop, N., & Lange, T. (2012). Stamen-derived bioactive gibberellin is essential for male flower development of Cucurbita maxima L. *Journal of Experimental Botany*, err448.
16. Letham, D.S. (1994) Cytokinins as phytohormones – sites of biosynthesis, translocation, and function of translocated cytokinin. In *Cytokinins: Chemistry, Activity, and Function* (Mok, D.W.S. and Mok, M.C., eds). Boca Raton, FL. USA: CRC Press, pp. 57–80.
17. Liscum, E. and Reed, J.W. (2002) Genetics of Aux/IAA and ARF action in plant growth and development. Plant Mol. Biol. 49: 387–400.
18. Matsuo, S., Kikuchi, K., Fukuda, M., Honda, I., & Imanishi, S. (2012). Roles and regulation of cytokinins in tomato fruit development. *Journal of experimental botany*.
19. Miyawaki, K., Matsumoto-Kitano, M., & Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. *The Plant Journal*, *37*(1), 128-138.
20. Mutasa-Gottgens E, Hedden P. 2009. Gibberellin as a factor in floral regulatory networks. Journal of Experimental Botany 60, 1979–1989.
21. Nam, Y. J., Herman, D., Blomme, J., Chae, E., Kojima, M., Coppens, F., ... & Weigel, D. (2016). Natural Variation of Molecular and Morphological Gibberellin Responses. *Plant Physiology*, pp-01626.
22. Paponov, I. A., Paponov, M., Teale, W., Menges, M., Chakrabortee, S., Murray, J. A., & Palme, K. (2008). Comprehensive transcriptome analysis of auxin responses in Arabidopsis. Molecular Plant, 1(2), 321-337.
23. Plackett, A. R., Powers, S. J., Fernandez-Garcia, N., Urbanova, T., Takebayashi, Y., Seo, M.& Phillips, A. L. (2012). Analysis of the developmental roles of the

Arabidopsis gibberellin 20-oxidases demonstrates that GA20ox1,-2, and-3 are the dominant paralogs. *The Plant Cell*, *24*(3), 941-960.

24. Reed, J. W. (2001). Roles and activities of Aux/IAA proteins in Arabidopsis. *Trends in plant science*, *6*(9), 420-425.
25. Sakamoto T, Sakakibara H, Kojima M, Yamamoto Y, Nagasaki H, Inukai Y, Sato Y, Matsuoka M. 2006. Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. Plant Physiology 142, 54–62.
26. Takei K, Sakakibara H, Sugiyama T. 2001. Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. Journal of Biological Chemistry 276, 26405–26410.
27. Takei K, Yamaya T, Sakakibara H. 2004. Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans–Zeatin. Journal of Biological Chemistry 279, 41866–41872.
28. Tan, X., Calderon-Villalobos, L.I.A., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M. et al. (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446: 640–645.
29. Tiwari, S.B., Hagen, G. and Guilfoyle, T.J. (2004) Aux/IAA proteins contain a potent transcriptional repression domain. Plant Cell 16: 533–543.
30. Tiwari, S.B., Wang, X.J., Hagen, G. and Guilfoyle, T.J. (2001) AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. Plant Cell 13: 2809–2822.
31. Weiss, D., & Ori, N. (2007). Mechanisms of cross talk between gibberellin and other hormones. Plant physiology, 144(3), 1240-1246.
32. Wu, J., Peng, Z., Liu, S., He, Y., Cheng, L., Kong, F., ... & Lu, G. (2012). Genome-wide analysis of Aux/IAA gene family in Solanaceae species using tomato as a model. *Molecular Genetics and Genomics*, *287*(4), 295-311.
33. Zhang, R., Wang, Y., Wang, C., Wei, Z., Xia, D., Wang, Y., ... & Yang, C. (2011). Time-course analysis of levels of indole-3-acetic acid and expression of auxin-responsive GH3 genes in Betula platyphylla. *Plant Molecular Biology Reporter*, *29*(4), 898-905.