EFECTO DE MICROORGANISMOS RIZOSFÉRICOS AUTÓCTONOS (BACTERIAS Y HONGOS MICORRÍZICO ARBUSCULARES) SOBRE LA TOLERANCIA DE LAS PLANTAS AL DÉFICIT HÍDRICO EN ZONAS SEMIÁRIDAS: MECANISMOS IMPLICADOS

ELISABETH ARMADA RODRÍGUEZ

TESIS DOCTORAL

2015

Editorial: Universidad de Granada. Tesis Doctorales
Autora: Elisabeth Armada Rodríguez
ISBN: 978-84-9125-325-9
URI: http://hdl.handle.net/10481/41123

UNIVERSIDAD DE GRANADA

FACULTAD DE CIENCIAS

Programa de Doctorado en Biología Fundamental y de Sistemas

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

ESTACIÓN EXPERIMENTAL DEL ZAIDÍN

Departamento de Microbiología del Suelo y Sistemas Simbióticos

Memoria presentada para optar al grado de Doctor

Fdo: **Elisabeth Armada Rodríguez**

Licenciada en Biología

VºBº del director de la Tesis Doctoral

Fdo.: **Rosario Ázcón González de Aguilar**

Profesora de Investigación del CSIC

Granada

Julio 2015

Este trabajo de Tesis Doctoral ha sido realizado en el grupo de investigación "Micorrizas" del departamento de Microbiología del Suelo y Sistemas Simbióticos, de la Estación Experimental del Zaidín (EEZ-CSIC, Granada).

Dicho trabajo de investigación fue financiado mediante una beca predoctoral concedida por el Ministerio de Ciencia e Innovación asociada al proyecto, AGL 2009-12530-C02-02 titulado "Aplicación de hongos micorrízico arbusculares (AM) y otros microorganismos beneficiosos, como estrategia para incrementar la disponibilidad de P y la eficiencia del uso del agua en la revegetación de zonas semiáridas mediterráneas".

Junto a una beca de movilidad para Estancias Breves, Ministerio de Economía y Competitividad, realizada en el departamento de Ecología Microbiana del Instituto Holandés de Ecología (NIOO-KNAW, Wageningen), bajo la supervisión de la Dra. Amudena Medina.

La doctoranda Elisabeth Armada Rodríguez y la directora de la tesis Rosario Azcón González de Aguilar. Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de la directora de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada, a 30 de Julio de 2015

Directora de la Tesis Doctoranda

Fdo.: Rosario Azcón González de Aguilar Fdo.: Elisabeth Armada Rodríguez

Agradecimientos

Me gustaría escribir unas frases para expresar mi gratitud a todas aquellas personas que me han aportado muchísimo tanto personal como profesionalmente en esta etapa de mi vida.

Ante todo mi gran agradecimiento a mi directora de tesis Rosario Azcón González de Aguilar por confiar en mí desde un principio, y darme la oportunidad de participar en este proyecto del cual me considero privilegiada por ello, además, de contagiarme de su entusiasmo de seguir aprendiendo y de su gran vitalidad.

A mis padres y hermano por todo el apoyo que me han dado durante todo este tiempo, y su esfuerzo para que haya realizado parte de mis sueños, y que de alguna manera quisiera agradecérselo, muchísimas gracias de corazón.

A José Miguel Barea por sus consejos y enseñanzas que me han aportado bastante, y no puedo olvidarme de sus anécdotas y chistes que nos alegran el día. A Juanma y Ricardo, por su compresión y su predisposición a ayudarme en esos momentos de dudas.

Además he tenido la gran suerte de trabajar con Olga la cual es mi compi y por supuesto gran amiga, le agradezco toda su ayuda durante este tiempo y apoyo en esos momentos de incertidumbre, pero que con tenacidad al final ambas lo hemos solucionado, y ya sabes, que en parte también es tu tesis.

A Paqui que le estoy verdaderamente agradecida por todo, y que siempre que la he necesitado a estado ahí.

A mis compañeros de laboratorio por todo esos momentos de risas (Sonia, José, José Luis, Pablo, Rosa) y a todos aquellos compañeros/as que estuvieron un cierto tiempo en el laboratorio, y que han formado parte de este trabajo y por lo que quiero agradecerles su aportación y su amistad: Inma, Pablo y Nacho (Granada); Gabriela (Argentina); Nidia (Colombia); Mª Ángeles, Lizette y Arturo (México); Cynthia y Paola (Chile); Rabaa (Túnez). Dar las gracias a Jesús por su colaboración y enseñanzas.

Y a mis chicos/as de máster que son verdaderamente genios (Tania, Martín, Maxi, Miguel Ángel y Gabriel).

Por supuesto a la gran pandilla, que fue un placer conocerlos y gracias por esos momentos de reuniones y risotadas (Bea, Ana, Migue, Bine, Iván, …) y a las tres compañeras de año (Eli, Sara y Rocio). A Mónica, agradecerle todo su apoyo y consejos, y que ha sido un gran placer el haberla conocido y además el haber trabajado con ella.

Al grupo de biólogos y externos (Inma, Gloria, Alex, David, Fran, Ángel,…) por esos momentos de reuniones y escapadas tras largo tiempo sin vernos, y de Bea que he tenido la gran suerte de coincidir además en estos años de tesis, y que hemos compartido momentos más buenos que malos y de la cual he aprendido mucho, gracias, y no olvidaré ese recibimiento tan a tu estilo.

Agradecerle a Almudena por su apoyo y compresión en mi periodo de estancia en el centro de investigación NIOO-KNAW en Wageningen, como después, y a mis compañeros y amigos que fue un gran placer el conocerlos (Sandra, Alicia, Manu, Mónica, Gauthan, Bárbara, Monika,…) y disfrutar de tan buenos momentos.

A los demás miembros del departamento, que siempre habéis tenido la amabilidad de colaborar y de darme vuestro apoyo y compresión. Gracias.

Por supuesto a Nico y Ruper, que sois verdaderamente esenciales y que ha sido una suerte el haberos conocido, y agradeceros vuestro afecto hacia mí, que es recíproco.

De nuevo, gracias a cada uno de vosotros y a los que no he mencionado pero sabéis que formaís parte de ello, por hacer posible este trabajo del cual estoy muy orgullosa y satisfecha, y el de haber compartido experiencias y situaciones que sería imposible de escribirlas todas.

Bueno, pues ya ha llegado el momento en parte de despedirnos pero que mejor frase que con un

"hasta pronto"

RESUMEN.

Efecto de microorganismos rizosféricos autóctonos (bacterias y hongos micorrízico arbusculares) sobre la tolerancia de las plantas al déficit hídrico en zonas semiáridas: Mecanismos implicados.

Elisabeth Armada Rodríguez

Introducción.

El cambio climático global está ocurriendo y sus efectos negativos aumentarán en los próximos años, por ello tales dificultades se imponen de manera importante en el desarrollo de cultivos de muchas zonas del mundo. Estas dificultades serán especialmente acentuadas en las zonas agrícolas de carácter semiárido actualmente (Denby & Gehring, 2005). El estrés por sequía afecta a las relaciones planta-agua, así como, las respuestas fisiológicas específicas y no específicas (Beck et al., 2007), causando un efecto perjudicial importante en el crecimiento de la planta y la nutrición y por lo tanto, en el desarrollo y producción de cultivos limitantes. De hecho, la sequía se considera como la causa principal de la disminución de la productividad de los cultivos en todo el mundo (Vinocur & Altman, 2005).

En las zonas semiáridas mediterráneas del sureste de España, las escasas e irregulares precipitaciones, y un largo y seco periodo de verano han contribuido drásticamente a la aceleración de los procesos de degradación del suelo. Los cambios ambientales como consecuencia de la pérdida de las comunidades naturales de plantas vienen precedidos por la degeneración de las propiedades físicas y químicas del suelo, además de por una pérdida o reducción de la actividad microbiana.

Varias estrategias se han sugerido para superar los efectos negativos de la sequía (Warren, 1998). Las estrategias más exploradas han sido el cultivo de variedades tolerantes y el uso de la ingeniería genética. Sin embargo, una estrategia alternativa es inducir la tolerancia al estrés de sequía, mediante el uso de microorganismos beneficiosos como hongos micorrízicos arbusculares (MA) y rizobacterias promotoras del crecimiento vegetal (PGPR). Las plantas suelen interactuar con los microorganismos del suelo, y ello hace que sean más eficientes ante las limitaciones ambientales como la sequía.

El funcionamiento y la estabilidad de los ecosistemas terrestres dependen en gran medida de la diversidad y composición de especies de su cubierta vegetal. Por lo que actualmente se acepta que la diversidad y actividad de la microbiota edáfica es la base de uno de los mecanismos que más contribuyen a la conservación del suelo, al desarrollo y mantenimiento de la cubierta vegetal y por consiguiente, a la estabilidad y funcionamiento del ecosistema.

Este trabajo se enfoca dentro de un proyecto que se basa en la recuperación de zonas semiáridas y degradadas del sureste peninsular de España, localizado en el Parque Ecológico "Vicente Blanes", Molina de Segura, provincia de Murcia, mediante la utilización de microorganismos beneficiosos para fomentar la disponibilidad de nutrientes de las plantas y la tolerancia al déficit hídrico.

El objetivo principal de la tesis doctoral es el conocimiento del funcionamiento de los microorganismos autóctonos (bacterias y hongos formadores de micorriza arbusculares), que proporcionan un gran beneficio sobre el desarrollo vegetal en dichas zonas desertificadas.

Para lograr dicho objetivo, se realizaron los siguientes objetivos específicos que se presentan en diferentes capítulos que conforman este trabajo de investigación.

- Desarrollar tecnologías que faciliten la recuperación de la cubierta vegetal en zonas semiáridas, mediante la selección de consorcios de microorganismos promotores del crecimiento, que mejoren la nutrición y la eficiencia en el uso del agua en condiciones de estrés hídrico severo y prolongado.
- Determinar el carácter generalista o específico de la actividad PGPR (plant growth-promoting rhizobacteria) y las habilidades de tolerancia al estrés osmótico de los inóculos seleccionados, así como las posibles sinergias derivadas de la interacción de diversos microorganismos beneficiosos.
- Determinación de los cambios en la biodiversidad microbiana en suelos rizosféricos, correspondientes a las diferentes especies vegetales y tras su inoculación microbiana.
- Efecto comparativo entre los microorganismos beneficiosos con los fertilizantes ante la tolerancia al estrés hídrico en planta.
- Validar los beneficios del uso de microorganismos autóctonos de un área Mediterránea degradada, en plantas de importancia agronómica como el maíz.

CAPÍTULO 1.

Aislamiento y caracterización de rizobacterias promotoras del crecimiento vegetal de zonas semiáridas del sureste peninsular de España.

El objetivo de este estudio fue el aislamiento y caracterización de rizobacterias autóctonas adaptadas a ambientes semiáridos y su evaluación basado en las habilidades de promoción de crecimiento en la especie vegetal *Lactuca sativa* bajo condiciones de sequia.

Se estudio los mecanismos empleados por dichas especies de rizobacterias aisladas, para soportar tales condiciones de estrés hídrico, mediante su cultivo en *in vitro* con altos niveles de polietilenglicol (PEG) que asemejan a un estrés osmótico. Sus habilidades PGPR tal como la solubilización de fosfatos, fijación de nitrógeno, producción de ácido indolacético (AIA) y síntesis de α-cetobutirato fueron verificadas. Además fue evaluada la tolerancia bacteriana al estrés osmótico mediante el análisis de producción de prolina, actividades enzimáticas antioxidantes [ascorbato peroxidasa (APX) y catalasa (CAT)] y la producción de poli-β-hidroxibutirato (PHB).

Seguidamente, se evaluó la posibilidad de estimular o promover el crecimiento vegetal, la nutrición, los valores fisiológicos y bioquímicos, y la tolerancia a la sequía de plantas de *L. sativa* a través de la inoculación de dichas bacterias autóctonas.

Los resultados obtenidos nos permiten obtener una mejor comprensión de la selección de ciertas bacterias de la rizosfera que pasan desapercibidos, y que su aplicación posee una gran relevancia en mejorar el crecimiento vegetal y por consiguiente, el rendimiento de cultivos y de zonas degradadas. La limitación de agua y el estrés osmótico afectan negativamente al crecimiento de las plantas, pero la inoculación bacteriana fue capaz de atenuar estos efectos perjudiciales por varios mecanismos moleculares, fisiológicos y bioquímicos. Este estudio revela que las especies bacterianas autóctonas aisladas fueron tolerantes al estrés osmótico (40% PEG), y que dichos aislados bacterianos pertenecen a los géneros *Bacillus* y *Enterobacter*, fueron de gran resistencia al estrés osmótico porque son cepas bacterianas que están predispuestos a adaptarse a estas condiciones.

Además, es importante desde un punto de vista práctico, saber que *Bacillus thuringiensis* fue la cepa bacteriana autóctona capaz de sobrevivir y multiplicarse para llegar a una población suficiente y expresar sus actividades bajo condiciones de estrés. La limitación de agua y estrés osmótico afectan negativamente el crecimiento de plantas, pero la inoculación de *B. thuringiensis* fue capaz de atenuar estos efectos perjudiciales, mejorando el crecimiento, la absorción de nutrientes y la calidad fisiológica de las plantas y por lo tanto, pueden ayudar a las

plantas de *L. sativa* en los procesos de osmoregulación y en la mejora de los mecanismos homeostáticos al desafío del estrés (Dimkpa et al, 2009; Miller et al., 2010). Sin embargo, se necesitan más estudios de investigación para establecer los principales procesos por los que estas cepas bacterianas autóctonas aislados de las zonas semiáridas y en particular *B. thuringiensis* mejora el rendimiento de las plantas en condiciones de sequía.

CAPÍTULO 2.

El restablecimiento de una cobertura vegetal sobre la base de especies vegetales autóctonas adaptadas a las condiciones ambientales locales, constituye la estrategia más eficaz para la recuperación de áreas degradadas en ambientes mediterráneos semiáridos (Vallejo et al., 1999). El éxito de los programas de revegetación de las zonas semiáridas se basa en el uso de tecnologías que benefician el establecimiento de las plantas y mejoran su tolerancia a la sequía. Las plantas dependen de sus sistemas de protección natural, incluyendo además, la ayuda de las actividades microbianas que intervienen en la adaptación al estrés, y la gestión de las comunidades microbianas asociada a la planta que tiende a ser una estrategia para atenuar el efecto negativo de los factores perjudiciales, tales como la sequía (Azcón et al., 2013; Dimkpa et al., 2009).

Por lo tanto, para llevar a cabo programas de reforestación con éxito, es necesario aplicar las tecnologías de inoculación que refuerzan el potencial microbiano limitado en estas áreas degradadas (Marulanda et al., 2003; 2009; Medina & Azcón, 2012). Observando la competitividad de las poblaciones de rizobacterias autóctonas, como una estrategia eficaz que contribuye a la creación de microorganismos beneficiosos preseleccionados en estos suelos semiáridos pobres e infértiles, mediante el establecimiento de bacterias de manera temprana, en la rizosfera por inoculación en el estado de plántula. La inoculación bacteriana, la selección de microorganismos específicos adaptados y eficaces, ha sido reconocida como una posibilidad interesante para aumentar el crecimiento vegetal (Zahir et al., 2004). Sin embargo, las respuestas de crecimiento de las plantas a la inoculación bacteriana implica desde la cepa bacteriana a la especie de planta, e incluso el ecotipo y la especificidad de la zona (Marulanda et al., 2009). Ciertos autores informaron de que los efectos de las variables se determinaron en función de las especies de plantas, el cultivar y las condiciones ambientales (Nowak et al., 1998).

Las comunidades microbianas juegan un papel importante en el suelo, debido a las numerosas funciones que desempeñan en el ciclo de nutrientes, simbiosis de plantas, la descomposición, y otros procesos de los ecosistemas (Nannipieri et al., 2003). Varios estudios

han demostrado que las especies vegetales tienen una importante influencia selectiva sobre las comunidades microbianas que conforman sus rizosferas (Garland, 1996; Smalla et al., 2001). Los microorganismos del suelo sintetizan y secretan enzimas extracelulares, que constituyen una parte importante de la matriz del suelo (Sinsabaugh et al., 1993). Actividades enzimáticas del suelo, se han sugerido como posibles indicadores del cambio en la calidad del suelo (Bastida et al., 2008; Hu et al., 2011). Hay ciertas informaciones de que las actividades enzimáticas disminuyeron en los ecosistemas mediterráneos debido a condiciones severas de sequía (Caravaca et al., 2002), lo que podría tener un efecto negativo en la disponibilidad de nutrientes.

La calidad del suelo está fuertemente influenciada por los procesos microbianos, y la función puede estar relacionado con la diversidad, es probable que la estructura de la comunidad microbiana tiene el potencial de servir como una indicación temprana de la degradación o mejora del suelo (Jackson et al., 2003; Aboim et al., 2008; Peixoto et al., 2010).

Las técnicas basadas en la biología molecular nos han dado una manera de caracterizar la estructura de la comunidad microbiana, y por lo tanto controlar su dinámica. Hay interés ecológico en la diversidad de los hongos micorrízicos arbusculares (MA) y las bacterias PGPR presentes en las raíces de las diferentes especies de plantas, en particular en los programas de revegetación para los ecosistemas utilizando arbustos autóctonos (Armada et al., 2014; Mengual et al., 2014).

Este capítulo se subdivide en tres subcapítulos;

2.1) Caracterización y gestión de las cepas bacterianas autóctonas de suelos semiáridos de España y sus interacciones con los residuos fermentados para mejorar la tolerancia a la sequía en especies arbustivas nativas.

El objetivo del presente estudio fue aislar y caracterizar tres cepas bacterianas autóctonas tolerantes a la sequía (denominadas *Enterobacter* sp.; *Bacillus thuringiensis; Bacillus* sp., fueron aisladas de la rizosfera de especies arbustivas mediterráneas que crecen en un ambiente semiárido), además de analizar sus efectos en comparación con una cepa de referencia (*Bacillus megaterium* utilizado como cepa alóctona tolerante a la sequía). Para analizar su eficiencia como inoculantes se seleccionaron cuatro especies de arbustos (*Thymus vulgaris*, *Santolina chamaecyparissus*, *Lavandula dentata* y *Salvia officinalis*) predominantes en dicha zona de estudio y adaptadas a la aridez, y su modulación por la aplicación de un residuo agrícola fermentado (compost).

Además, las bacterias autóctonas pueden interactuar positivamente con los hongos micorrízicos arbusculares (MA) nativos, existente en el suelo natural, por lo tanto el desarrollo de los hongos MA también se evaluó, ya que tales interacciones microbianas pueden afectar a la

tolerancia al déficit hídrico de las plantas. Algunas bacterias han sido nombrados como bacterias que ayudan a la micorriza, por su capacidad de promover el crecimiento de micelios y la formación de micorrizas (Frey-Klett et al., 2007).

Las rizobacterias que promueven el crecimiento vegetal (PGPR) juegan un importante papel en ayudar a resolver los problemas del medio ambiente, y por lo tanto puede ayudar al establecimiento y crecimiento de las plantas por varios mecanismos directos e indirectos (Kasim et al., 2013), esto lleva a aumentar la tolerancia de las plantas en situaciones de estrés como los causados por la escasez de agua (Naveed et al., 2014). De hecho, las PGPR han demostrado que afectan al balance hídrico de las plantas bien regadas y estresadas (Kohler et al., 2008). Y por ello, las variables fisiológicas como la conductancia estomática, la tasa de transpiración y el potencial hídrico foliar generalmente se ven afectados por la inoculación bacteriana en condiciones limitadas de agua (Benabdellah et al., 2011). Los factores de estrés ambiental que afectan al ecosistema semiárido, disminuyen la diversidad y la densidad de las poblaciones microbianas pero los propágulos microbianos no desaparecen por completo, y ello es una indicación de adaptación al estrés (Azcón et al, 2013; Barea et al., 2011). Los ecotipos microbianos tolerantes y adaptados a la sequía son los mejores candidatos para ser utilizados como inoculantes en los programas de reforestación bajo condiciones semiáridas y limitadas de agua (Alguacil et al., 2003; Caravaca et al., 2002).

Para evaluar las habilidades PGPR y la capacidad de resistencia a la sequía de estos aislamientos bacterianos autóctonos, fue determinado bajo condiciones de estrés osmótico, mediante las variables relacionadas con la bioestimulación de la planta (producción de hormonas (SA, ABA, JA y AIA) y la solubilización de fosfato) y también con la tolerancia celular a la sequía, como la producción de prolina, poli-ß-hidroxibutirato (PHB), actividades enzimáticas antioxidantes [APX; CAT] y 1-aminocyclopropane-1-carboxylate (ACC) deaminasa. El crecimiento potencial de las células bacterianas bajo condiciones de no estrés y de estrés por sequía también se evaluó.

Todas las bacterias inoculadas mejoraron la nutrición y las variables fisiológicas y bioquímicas relacionadas con la tolerancia a la sequía en estas plantas de prueba. La aplicación del residuo agrícola fermentado (compost) obtenido de la remolacha azucarera también se ensayó y resultó eficaz. Esta modificación también interactuó positivamente con las bacterias mediante el aumento de la absorción de nutrientes de las plantas y tolerancia a la sequía, pero la eficacia depende de las especies de plantas y bacterias implicadas. La aplicación dual de *B. megaterium* y el residuo agrícola fermentado aumentaron la captación de P y K en *S. chamaecyparissus*, *L. dentata* y *S. officinalis*. Sin enmienda, la bacteria nativa *B. thuringiensis*

fue la cepa bacteriana más eficiente para aumentar el contenido de P en *T. vulgaris* y *S. officinalis*, y el contenido de K en *L. dentata*, el cual decrece la conductancia estomática.

Los resultados muestran que en condiciones axénicas, la tensión aplicada no suprime las capacidades PGPR de las bacterias ensayadas lo que indica su potencial para ser probado como inoculantes en condiciones perjudiciales. La actividad de las bacterias específicas y/o residuo agrícola fermentado parece estar asociada a la protección de las plantas, para evitar así la sequía y la consiguiente alteración de las propiedades fisiológicas de las plantas. Las bacterias y el residuo agrícola fermentado protegen (dependiendo de la planta implicada) contra el estrés por sequía, y podrían ser utilizados como una herramienta biotecnológica para aliviar la deficiencia de agua de la planta. La multiplicidad y complejidad de las actividades bacterianas y las características intrínsecas de reacción de la planta a la sequía, podrían explicar los resultados impredecibles de la inoculación bacteriana. Hay muchos factores que están controlando el efecto PGPR, lo que hizo difícil generalizar y explicar la causa/efecto de las variables respuestas obtenidas. Con todo ello, los resultados apoyan que las bacterias dianas y el residuo agrícola fermentado pueden ayudar a las plantas y a la reforestación en tierras semiáridas.

2.2) Actividad diferencial de bacterias autóctonas en el control de estrés por sequía en especies de plantas nativas *Lavandula* y *Salvia* bajo condiciones de sequía en suelo áridos naturales.

Prosiguiendo el estudio anterior, se evaluó la eficacia de las rizobacterias autóctonas identificadas (*Enterobacter* sp.; *Bacillus thuringiensis*; *Bacillus* sp.) y la bacteria de referencia (*Bacillus megaterium*), que promueven el crecimiento vegetal (PGPR) que se estudió en las especies vegetales *L. dentata* y *S. officinalis* creciendo en un suelo árido Mediterráneo natural bajo condiciones de sequía. Ambas especies de plantas, constituyen ser importantes para los programas de revegetación en una zona mediterránea semiárida, y para mejorar el establecimiento de las plantas mediante la aplicación directa de los inóculos bacterianos siendo una práctica recomendada.

Cada bacteria tiene diferente potencial para mejorar la limitación de agua y aliviar el estrés por sequía en estas dos especies de plantas. *B. thuringiensis* promueve el crecimiento y evita la sequía en *L. dentata*, al aumentar el contenido de K, deprimiendo la conductancia estomática y controlando la acumulación de prolina en la parte aérea vegetal. Este efecto bacteriano en el aumento de la tolerancia a la sequía se relacionó con la disminución de la actividad antioxidante glutatión reductasa (GR) y APX, que dieron como resultado, índices de sensibilidad de menor daño celular oxidativo involucrado en la respuesta adaptativa a la sequía en plantas de *L. dentata* inoculadas con *B. thuringiensis*.

En cambio, en *S. officinalis*, que tiene una menor relación intrínseca tallo/raíz, mayor conductancia estomática y menor actividad antioxidante APX y GR que *L. dentata*, los efectos bacterianos en la nutrición, fisiología y sistemas enzimáticos antioxidantes fueron menores. La característica particular de las especies de plantas con tan baja relación tallo/raíz y alta conductancia estomática, son factores importantes que controlan la efectividad bacteriana, mejorando la nutrición, fisiología, y actividades metabólicas de la planta.

En conclusión, *L. dentata* demostró un mayor beneficio que *S. officinalis* para controlar el estrés por sequía cuando se inocularon con *B. thuringiensis*. La tolerancia bacteriana a la sequía se evaluó como supervivencia y producción de prolina y AIA, mostrando el potencial de esta bacteria para ayudar a las plantas a crecer en condiciones de sequía. *B. thuringiensis* puede ser utilizado para el establecimiento de las plantas de *L. dentata* en ambientes áridos.

2.3) Análisis de la comunidad microbiana por PLFA y pirosecuenciación en especies arbustivas autóctonas en estrés por sequía y el efecto de bacterias nativas de suelos Mediterráneos.

El objetivo de este apartado se centra en explorar y conocer la diversidad microbiana rizosférica que alberga determinadas especies vegetales autóctonas de dichos ambientes desérticos, y para ello se emplearon dos técnicas, la determinación de los perfiles de ácidos grasos de fosfolípidos [phospholipid fatty acid (PLFA)] y la pirosecuenciación.

Los biomarcadores de ácidos grasos se utilizan en estudios de ecología microbiana del suelo, ya que proporcionan información cualitativa y cuantitativa sobre las comunidades microbianas. El análisis de ácidos grasos de fosfolípidos (PLFA) de las membranas microbianas, derivan del fraccionamiento de lípidos, empleándose como método principal (Frostegård et al., 1993a,b; Frostegård & Bååth, 1996; Zelles, 1997). Ello nos proporciona un conjunto de marcadores moleculares para taxones microbianos e indicadores de estrés microbiano, que pueden ser utilizados para rastrear los cambios en la composición de la comunidad microbiana del suelo, y también da una medida de la biomasa microbiana total viable (Bossio & Scow, 1995; White et al., 1996). La separación de los lípidos también proporciona una fracción de ácido graso lipídico neutro [neutral lipid fatty acid (NLFA)], con información acerca de las reservas de energía eucariota útiles en estudios relacionados con el estado nutricional de los hongos (Baath, 2003).

Los recientes avances en la tecnología de secuenciación, como la secuenciación de próxima generación es un enfoque para evaluar la diversidad microbiana y la estructura de la comunidad microbiana en ambientes diferentes (Cristea-Fernstrom et al., 2007; Roesch et al., 2007).

Tras la obtención de datos y su respectivos análisis nos aportó la información sobre la identificación de las comunidades fúngicas destacando los hongos micorrízicos arbusculares (MA) (empleando primers específicos) y las comunidades bacterianas, y con ello estudiar el posible efecto de las distintas especies de plantas autóctonas seleccionadas (*T. vulgaris*; *S. chamaecyparissus*; *L. dentata*) en las comunidades de hongos MA y bacterias de los suelos naturales y los posibles cambios que conlleva en la diversidad microbiana.

A parte, tras una serie de estudios realizados previamente en el aislamiento e identificación de las especies bacterianas rizosféricas de dicha zona semiárida, y de su capacidad de ser promotoras de crecimiento vegetal (PGPR) y tolerantes al déficit hídrico (capítulo 1 y 2), se seleccionó una cepa bacteriana nativa beneficiosa (*B. thuringiensis*) para estudiar la posible influencia tras ser inoculada sobre el desarrollo y supervivencia de las comunidades fúngicas (MA) y bacterianas en cada una de las rizosferas de las dichas plantas autóctonas.

De acuerdo a los resultados obtenidos, se confirma que la comunidad microbiana del suelo fue significativamente diferente en las rizosferas de las tres especies de plantas. Esas diferencias fueron mostradas en los biomarcadores bacterianos (C17:1w8c; C18:1w9t) y biomarcadores fúngicos (C18:1w9c; C18:2w6c). Sin embargo, la inoculación bacteriana no influye significativamente en los perfiles de ácidos grasos.

Además los resultados nos confirman que las rizosferas de *S. chamaecyparissus* y *L. dentata* poseen una mayor diversidad fúngica con respecto a *T. vulgaris*, y nuestra evaluación nos demuestra que *S. chamaecyparissus* tiende a una mayor presencia de los phylum *Ascomycota* y *Basidiomycota*, y *L. dentata* destaca por el phylum *Glomeromycota*. La inoculación de *B. thuringiensis* promueve el incremento de orden *Glomus* en las tres especies vegetales, pero sobre todo *en L. dentata*.

S. chamaecyparissus presentó baja diversidad bacteriana, pero destaca por una elevada actividad deshidrogenasa y fosfatasa alcalina que se relaciona con el elevado contenido de P asimilable por la planta. La rizosfera de *S. chamaecyparissus* tras ser inoculada con la bacteria nativa *B. thuringiensis* es la que posee una mayor diversidad de la comunidad bacteriana. Es el tratamiento que expresa un incremento en la actividad ureasa por lo que se puede relacionar con la mayor diversidad bacteriana que posee, y por la comunidad fúngica que alberga.

La rizosfera de *L. dentata* que presenta una mayor actividad β-glucosidasa se relaciona con la presencia de la comunidad bacteriana pero sobre todo es debido a la comunidad fúngica. Es la especie vegetal que presenta en su rizosfera una mayor diversidad bacteriana y fúngica,

destacando sobre todo el phylum *Glomeromycota*. A diferencia de la especie *T. vulgaris* que destaca por una mayor diversidad bacteriana y *S. chamaecyparissus* por la diversidad fúngica. La inoculación de la bacteria nativa *B. thuringiensis* altera la diversidad microbiana en general, pero esencialmente en la rizosfera de *S. chamaecyparissus*.

En conclusión, nuestro estudio ha proporcionado cierta caracterización de los cambios en la composición de la rizosfera bacteriana y fúngica, y como esta responde a los distintos tipos de cobertura vegetal autóctona en condiciones ambientales de carácter semiárido. Podemos decir que las especies arbustivas autóctonas contribuyen significativamente al desarrollo y enriquecimiento de las comunidades de hongos y de bacterianas de estas zonas semiáridas, y en consecuencia, una mayor funcionalidad y diversidad del suelo. Además de como repercute el inocular una especie bacteriana nativa de la misma zona semiárida con capacidad PGPR, para fomentar la diversidad microbiana y por lo cual ser un posible método para fomentar el desarrollo vegetal, la disponibilidad de nutrientes y por consiguiente la tolerancia a soportar esas condiciones tan extremas de deficiencia de agua.

CAPÍTULO 3.

En este capítulo nos planteamos la hipótesis de que los tratamientos microbianos podrían conferir tolerancia a la sequía en la planta seleccionada, y mejorar el proceso de restablecimiento de la vegetación que conduce a mejorar el rendimiento de la planta.

Para ello el capítulo 3 se subdivide en dos subcapítulos;

3.1) *Bacillus thuringiensis* bacteria nativa promotora del crecimiento vegetal y la mezcla o individual de especies micorrízicas mejoraron la tolerancia a la sequía y el metabolismo oxidativo en plantas de *Lavandula dentata*

Evaluar las respuestas de *L. dentata* inoculada con distintas especies de hongos micorrízicos arbusculares (MA) autóctonos (cinco cepas de hongos: *Septoglomus constrictum* EEZ 198; *Diversispora aunantia* EEZ 199; *Archaeospora trappei* EEZ 200; *Glomus versiforme* EEZ 201; *Paraglomus ocultum* EEZ 202) o con su consorcio y/o mezcla, y su combinación con *B. thuringiensis* en condiciones de sequía.

Los hongos micorrízicos arbusculares (MA) tienen la capacidad de colonizar las raíces de la mayoría de las plantas vasculares, y dichas plantas colonizadas se enfrentan de manera más eficaz al déficit hídrico. Por ello las micorrizas pueden ayudar a las plantas a prosperar en ecosistemas semiáridos (Azcón et al., 2013). El efecto de las micorrizas se basa en mecanismos directos e indirectos, por ejemplo, el micelio micorrízico tiene acceso a los poros del suelo, por consiguiente, será más eficiente que las raíces para la extracción de nutrientes y agua (Azcón &

Barea, 2010). Es bien sabido que las plantas micorrizadas aumentaron la absorción de nutrientes, especialmente los nutrientes inmóviles. Existen varias evidencias de que los hongos MA se adaptan a las condiciones edáficas, pero las diferencias en el comportamiento de los hongos, la eficiencia en el crecimiento de la planta y la tolerancia al estrés, pueden ser al menos en parte, debido al hongo en cuestión.

No obstante, el objetivo es verificar el potencial de la co-inoculación en las plantas de *L. dentata*, para así incrementar la tolerancia a la sequía y aliviar el impacto de la escasez de agua. Basándonos en la selección de microorganismos del suelo, que nos proporcionan ayuda en el establecimiento de la cubierta vegetal autóctona bajo condiciones ambientales áridas, dichos microorganismos fueron tolerantes a la sequía e incrementaron el crecimiento y la nutrición de las plantas, y sus interacciones altamente reducen el daño oxidativo a lípidos de la planta e incrementaron el desarrollo de las micorrizas, explicando el mayor potencial de las plantas inoculadas dualmente a tolerar el estrés por sequía. El consorcio y/o mezcla de hongos MA y *B. thuringiensis* maximizan la biomasa vegetal y compensan el estrés por sequía, mediante los valores de actividades antioxidantes [superóxido dismutasa (SOD), CAT y APX)] y el daño oxidativo a lípidos [malondialdehído (MDA)] que presentan.

B. thuringiensis (bacteria endofítica) no reduce su potencial para mejorar el crecimiento de las plantas en condiciones de estrés, testado como el AIA, la producción de ACC-deaminasa y la solubilización de fosfato, y el aumento de la formación de arbúsculos y la funcionalidad simbiótica.

Las cepas de hongos autóctonos mantienen su interacción especial con *B. thuringiensis* que refleja la diversidad y las capacidades intrínsecas de estos microorganismos. Las especies de hongos AM autóctonos y en particular su consorcio y/o mezcla con *B. thuringiensis*, demostró su potencial para la protección de las plantas contra la sequía y ayudar a las plantas a prosperar en ecosistemas semiáridos.

Así que este estudio demostró, el efecto protector de las plantas colonizadas por cepas micorrízicas autóctonas adaptadas, y fue reforzada por la asociación con la bacteria autóctona *B. thuringiensis*.

3.2) Perfiles fisiológicos de comunidades rizosféricas bacterianas (estructura funcional) de *Lavandula dentata*, después de la inoculación con hongos micorrízicos autóctonos tolerantes a la sequía, y *Bacillus thuringiensis*.

Investigar los perfiles fisiológicos de las comunidades bacterianas de la rizosfera de *L. dentata* crecido en condiciones de sequía, e inoculada con hongos autóctonos formadores de

micorrizas arbusculares (MA) (cada una de las cinco cepas individuales o la mezcla de ellos) y un cepa bacteriana nativa (*B. thuringiensis*).

La hipótesis planteada fue de que los diferentes inoculantes podrían modificar los parámetros de crecimiento de las plantas y cambiar la composición de los exudados de las raíces de *L. dentata* (y el micelio de los hongos) bajo las condiciones de sequía ensayadas y, por tanto, alterar los patrones de uso de las diferentes fuentes de C y N de las comunidades bacterianas de la rizosfera y micorrizosfera.

Así que los efectos de estos inoculantes microbianos se evaluaron mediante algunos parámetros biométricos de la planta, contenido de C en planta, micorrización total y porcentaje de colonización, así como los agregados estables en la proximidad de la raíz de la planta. Estos microorganismos nativos inoculados fueron tolerantes a la limitación de agua, y los hongos MA incrementaron los parámetros de crecimiento de la planta y el contenido de C, pero la interacción con *B. thuringiensis* no siempre mejoró el efecto de la sola inoculación de hongos MA. Sin embargo, el consorcio y/o mezcla de hongos MA más la inoculación de *B. thuringiensis*, causó una mayor biomasa aérea y colonización micorrízica y el contenido de C vegetal ser altamente eficaz en la protección de las plantas contra la sequía.

En lo que se refiere a los perfiles fisiológicos de las comunidades bacterianas de la rizosfera, hubo claras diferencias encontradas entre los tratamientos con los diferentes hongos MA, los cuales no se modifican de forma sustancial por la co-inoculación con *B. thuringiensis*. Este efecto indica que el principal factor que impulsa los patrones de utilización de sustratos, en este experimento, es la especie de hongo MA. Por lo tanto, el consumo de hidratos de carbono parece ser más importante en *Septoglomus constrictum* y *Paraglomus occultum* con y sin la co-inoculación de *B. thuringiensis*. El uso de ácidos orgánicos predominan en la rizosfera de plantas colonizadas con el consorcio y/o mezcla de MA y con *Diversispora aunantia* (tanto solo, como con *B. thuringiensis*) pero el consumo de aminoácidos predominan en la rizosfera de plantas tratadas con *Archaeospora trappei*.

Más allá de las diferencias en la diversidad funcional de las rizosferas, nuestros resultados sugieren que existe una alteración en la exudación de la planta, que se refleja en el consumo de diferentes compuestos por las comunidades bacterianas rizosféricas de *L. dentata*, debido a la simbiosis micorrízica por el hongo específico. La comprensión de estos efectos como parte de los procesos de los ecosistemas, es esencial para obtener el máximo beneficio para el crecimiento vegetal y por consiguiente, la mejora en la zonas semiáridas, donde los efectos de la sequía afectan profundamente la capacidad de la planta.

CAPÍTULO 4.

Potencial del inóculo micorrízico para estimular el crecimiento, nutrición y actividades enzimáticas en plantas de *Retama sphaerocarpa* comparado con la fertilización química bajo condiciones de sequía.

En este apartado se evaluó el crecimiento de *Retama sphaerocarpa* en condiciones de sequía, que fue similarmente incrementado por la colonización de las micorrizas arbusculares (MA) [consorcio fúngico de MA nativo (M) o *Rhizophagus intraradices* alóctona (RI)] o la aplicación de fertilizante de H_3PO_4 [25 ppm de P (1P) o 50 ppm de P (2P)], pero RI fue la más efectiva mejorando el contenido de P.

La hipótesis que se planteó es de que bajo condiciones de sequía, la planta autóctona se beneficiará sobre todo de la inoculación con todo un consorcio de hongos MA nativos, debido a la diversidad y la funcionalidad de la comunidad micorrízica autóctona, que de la inoculación con un solo tipo de aislamiento micorrízico adaptado como *R. intraradices* (EEZ 195). Presumiblemente, la inoculación con una comunidad fúngica compleja tendría una mayor capacidad de amortiguación contra el estrés hídrico que un sólo inóculo fúngico (Caravaca et al., 2005).

Una tendencia general del efecto beneficioso de la micorrización se asocia con la adquisición de fósforo en las plantas colonizadas. La micorrización aumenta la absorción de P de la planta y esto es un mecanismo importante en relación con la tolerancia de las plantas a la sequía (Augé, 2004; Subramanian et al., 2006).

La micorriza arbuscular fue determinante, afectando a la actividad GR en plantas de similar biomasa. La actividad antioxidante APX se incrementó por la fertilización de P y disminuyó en plantas colonizadas por AM de tamaño similar lo cual reveló la protección de dichos hongos AM contra la sequía. Los hongos nativos y la fertilización 2P producen similar biomasa aérea y nutrición, sin embargo, las AM reducen las actividades antioxidantes CAT y APX indicando no obstante un estrés hídrico más bajo.

En un estudio posterior se valoró cómo el inóculo autóctono [consorcio de hongos MA (M) más *B. thuringiensis* (B)] es capaz de fortificar la fertilización de K_2SO_4 [5 mM K (1K) o 10 mM de K (2K)] en *R. sphaerocarpa* bajo condiciones de sequía. La dual inoculación incrementó el contenido de nutrientes solamente en plantas fertilizadas con 1K, mientras tanto, la fertilización 2K incluso disminuyó el desarrollo arbuscular. La reducción de la actividad superóxido dismutasa (SOD) y APX, y la eliminación de las actividades CAT y GR se

encuentran en las plantas co-inoculadas y fertilizadas con K, ello indica un alto potencial de los inóculos para hacer frente a la sequía, independientemente de la nutrición.

Las actividades enzimáticas del suelo mejoraron, especialmente la β-glucosidasa, por los inóculos en ambos experimentos. La mejora de las propiedades del suelo puede estimular indirectamente el crecimiento de las plantas. Los inóculos microbianos cuentan con mecanismos, que pueden ayudar a las plantas a que se desarrollen en condiciones de sequía.

Los resultados muestran que la inoculación con hongos MA nativos y bacterias puede ser una herramienta eficaz para la revegetación de tierras semiáridas. Las cepas autóctonas están presumiblemente pre-adaptados a las condiciones semiáridas y, por tanto, son colonizadores competitivos en su suelo original y medio ambiente. Curiosamente, en condiciones menos fértiles (1K) las cepas nativas resultaron ser colonizadores más eficaces, sobre todo en cuanto a la abundancia arbuscular y a la riqueza e intensidad de micorrizas. Estos desarrollos simbióticos promovieron un aumento diferencial en la captación de N y P. Leguminosas leñosas como *R. sphaerocarpa* son plantas colonizadoras útiles para suelos áridos deficientes en nutrientes. El restablecimiento de la vegetación con plantas nativas como *R. sphaerocarpa*, ha demostrado ser más eficaz que con plantas exóticas bajo tales condiciones limitadas de agua e infértiles (Caravaca et al., 2004).

CAPÍTULO 5.

Hongos micorrízicos arbusculares autóctonos y *Bacillus thuringiensis* de zonas Mediterráneas degradas puede ser usadas para mejorar los rasgos fisiológicos y de resistencia de una planta de interés agronómico bajo condiciones de sequía.

Tras los estudios presentados en los anteriores capítulos, se ha demostrado que algunos microorganismos autóctonos de ambientes estresantes son beneficiosos cuando se utiliza con plantas autóctonas, pero estos microorganismos rara vez se han probado con plantas alóctonas de interés agronómico.

Este estudio investiga la eficacia de microorganismos autóctonos adaptados a la sequía [*B. thuringiensis* (Bt) y un consorcio de hongos micorrízicos arbusculares (MA)] de una zona mediterránea degradada, para mejorar el crecimiento y la fisiología en *Zea mays* L. en condiciones de déficit hídrico.

Las plantas de maíz (*Zea mays* L.) fueron inoculadas o no con *B. thuringiensis*, un consorcio de hongos MA o una combinación de ambos microorganismos. Las plantas fueron cultivadas bajo condiciones de buen riego o sometidas a estrés por sequía. Se midieron varios

parámetros fisiológicos, incluyendo entre otros, crecimiento de las plantas, la eficiencia fotosintética, contenido de nutrientes, el daño oxidativo a lípidos, la acumulación de prolina y compuestos antioxidantes, la conductividad hidráulica de la raíz y la expresión de genes acuaporina de la planta.

Los resultados obtenidos bajo condiciones de sequía, fueron que la inoculación de Bt aumentó significativamente la acumulación de nutrientes. La inoculación combinada de ambos microorganismos disminuyó el daño oxidativo a lípidos y la acumulación de prolina, inducida por la sequía. Varios acuaporinas de maíz son capaces de transportar agua, CO_2 y otros compuestos, que son regulados por los inoculantes microbianos. El impacto de estos microorganismos en la tolerancia de la planta a la sequía era complementario, ya que Bt aumentó principalmente la nutrición de las plantas, y los hongos MA eran más activos mejorando los mecanismos homeostáticos y de tolerancia al estrés, incluyendo la regulación de acuaporinas de la planta con varias supuestas funciones fisiológicas.

Por lo tanto, el uso de microorganismos beneficiosos autóctonos de un área mediterránea degradada, es útil para proteger no sólo las plantas nativas contra la sequía, sino también una planta agronómicamente importante, tal como el maíz.

CONCLUSIONES GENERALES.

- Las especies bacterianas aisladas de las rizosferas de arbustos autóctonos de zonas semiáridas, pertenecientes a los géneros *Bacillus* y *Enterobacter*, en condiciones *in vitro*, sometidas a altos niveles de estrés osmótico, mostraron su capacidad de tolerar el estrés y las habilidades que podrían describirse como potencial PGPR.
- La actividad de las bacterias específicas y/o residuo agrícola fermentado parece estar asociada a la protección de las plantas, para evitar así la sequía y la consiguiente alteración de componentes antioxidantes y las propiedades fisiológicas de las plantas. Podría ser un posible método para fomentar el desarrollo vegetal y la disponibilidad de nutrientes, y por consiguiente la tolerancia a soportar esas condiciones tan extremas de deficiencia de agua.
- *Lavandula dentata* demostró una mayor capacidad de soportar el estrés por sequía cuando se inoculó con *Bacillus thuringiensis*. Dicha tolerancia bacteriana a la sequía se evaluó como supervivencia y producción de prolina y ácido indolacético (AIA), mostrando el potencial de esta bacteria para ayudar a las plantas a crecer en condiciones

de estrés hídrico. La inoculación de *B. thuringiensis* en plantas de *L. dentata* puede ser utilizado en los programas de revegetación de ecosistemas semiáridos.

- Cambios en la composición rizosférica (bacteriana y fúngica), y como esta responde a los distintos tipos de cobertura vegetal autóctona en condiciones ambientales de carácter semiárido. La inoculación de *B. thuringiensis* fomenta la diversidad microbiana.
- El consorcio y/o mezcla de hongos MA autóctonos con *B. thuringiensis*, demostró su potencial para la protección de las plantas contra la sequía y ayudar a las plantas a prosperar en ecosistemas semiáridos.
- Los patrones de uso de las diferentes fuentes de C y N de las comunidades bacterianas rizosféricas, se vieron alterados por el tipo de especie de hongo MA. No fue modificada por la co-inoculación con *B. thuringiensis*.
- El uso de microorganismos beneficiosos autóctonos de un área mediterránea degradada, protege no sólo las plantas nativas contra la sequía, sino también una planta agronómicamente importante, como el maíz. Destacando que *B. thuringiensis* interviene en la nutrición vegetal, y los hongos MA mejoran los procesos homeostáticos y de tolerancia, y participando en la regulación de las acuaporinas de la planta.

Referencias

Aboim, M.C.R., Coutinho, H.L.C., Peixoto, R.S., Barbosa, J.C., Rosado, A.S., 2008. Soil bacterial community structure and soil quality in a slash-and-burn cultivation system in Southeastern Brazil. Applied Soil Ecology 38, 100-108.

Alguacil, M.M., Caravaca, F., Azcón, R., Pera, J., Díaz, G., Roldán, A., 2003. Improvements in soil quality and performance of mycorrhizal *Cistus albidus* L. seedlings resulting from addition of microbially treated sugar beet residue to a degraded semiarid Mediterranean soil. Soil Use and Management 19, 277-283.

Armada, E., Roldán, A., Azcón, R., 2014. Differential activity of autochthonous bacteria in controlling drought stress in native *Lavandula* and *Salvia* plants species under drought conditions in natural arid soil. Microbial Ecology 67, 410-420.

Augé, R.M., 2004. Arbuscular mycorrhizae and soil/plant water relations. Canadian Journal of Soil Science 84, 373-381.

Azcón, R., Barea, J.M., 2010. Mycorrhizosphere interactions for legume improvement. In: Khan MS, Zaidi A, Musarrat J, editors. Microbes for Legume Improvement. Vienna, New York: Springer-Verlag, pp. 237-271.

Azcón, R., Medina, A., Aroca, R., Ruíz-Lozano, J.M., 2013. Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast interactions, in: de Bruijn, F.J. (Ed.), Molecular Microbial Ecology of the Rhizosphere. John Wiley & Sons, Hoboken, New Jersey, USA, pp. 991-1002.

Barea, J.M., Palenzuela, J., Cornejo, P., Sánchez-Castro, I., Navarro-Fernández, C., Lopéz-García, A., Estrada, B., Azcón, R., Ferrol, N., Azcón-Aguilar, C., 2011. Ecological and functional roles of mycorrhizas in semi-arid ecosystems of Southeast Spain. Journal of Arid Environments 75, 1292-1301.

Bastida, F., Kandeler, E., Moreno, J.L., Ros, M., García, C., Hernández, T., 2008. Application of fresh and composted organic wastes modifies structure, size and activity of soil microbial community under semiarid climate. Applied Soil Ecology 40, 318-329.

Beck, E.H., Fettig, S., Knake, C., Hartig, K., Bhattarai, T., 2007. Specific and unspecific responses of plants to cold and drought stress. Journal of Biosciences 32, 501-510.

Benabdellah, K., Abbas, Y., Abourouh, M., Aroca, R., Azcon, R., 2011. Influence of two bacterial isolates from degraded and non-degraded soils and arbuscular mycorrhizae fungi isolated from semi-arid zone on the growth of *Trifolium repens* under drought conditions: Mechanisms related to bacterial effectiveness. European Journal of Soil Biology 47, 303-309.

Bossio, D.A., Scow, K.M., 1995. Impact of carbon and flooding on the metabolic diversity of microbial communities in soils. Applied and Environmental Microbiology 61, 4043-4050.

Caravaca, F., Alguacil, M.M., Barea, J.M., Roldán, A., 2005. Survival of inocula and native AM fungi species associated with shrubs in a degraded Mediterranean ecosystem. Soil Biology and Biochemistry 37, 227-233.

Caravaca, F., Barea, J.M., Figueroa, D., Roldán, A., 2002. Assessing the effectiveness of mycorrhizal inoculation and soil compost addition for enhancing reaforestation with *Olea europaea* subsp. *sylvestris* through changes in soil biological and physical parameters. Applied Soil Ecology 20, 107-118.

Caravaca, F., Figueroa, D., Barea, J.M., Azcon-Aguilar, C., Roldan, A., 2004. Effect of mycorrhizal inoculation on nutrient acquisition, gas exchange, and nitrate reductase activity of two Mediterranean-autochthonous shrub species under drought stress. Journal of Plant Nutrition 27, 57-74.

Caravaca, F., Masciandaro, G., Ceccanti, B., 2002. Land use in relation to soil chemical and biochemical properties in a semiarid Mediterranean environment. Soil and Tillage Research 68, 23-30.

Cristea-Fernström, M., Olofsson, M., Chryssanthou, E., Jonasson, J., Petrini, B., 2007. Pyrosequencing of a short hypervariable 16S rDNA fragment for the identification of nontuberculous mycobacteria - A comparison with conventional 16S rDNA sequencing and phenotyping. APMIS 115, 1252-1259.

Denby, K., Gehring, C., 2005. Engineering drought and salinity tolerance in plants: lessons from genome-wide expression profiling in *Arabidopsis.* Trends in Biotechnology 23, 547-552.

Dimkpa, C., Weinand, T., Asch, F., 2009. Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell & Environment 32, 1682-1694.

Frey-Klett, P., Garbaye, J., Tarkka, M., 2007. The mycorrhiza helper bacteria revisited. New Phytologist 176, 22-36.

Frostegård, A., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59-65.

Frostegård, Å., Bååth, E., Tunlio, A., 1993a. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology and Biochemistry 25, 723-730.

Frostegard, A., Tunlid, A., Baath, E., 1993b. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Applied and Environmental Microbiology 59, 3605-3617.

Garland, J.L., 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biology and Biochemistry 28, 213-221.

Hu, J., Lin, X., Wang, J., Dai, J., Chen, R., Zhang, J., Wong, M., 2011. Microbial functional diversity, metabolic quotient, and invertase activity of a sandy loam soil as affected by long-term application of organic amendment and mineral fertilizer. Journal of Soils and Sediments 11, 271-280.

Jackson, L.E., Calderon, F.J., Steenwerth, K.L., Scow, K.M., Rolston, D.E., 2003. Responses of soil microbial processes and community structure to tillage events and implications for soil quality. Geoderma 114, 305-317.

Kasim, W.A., Osman, M.E., Omar, M.N., Abd El-Daim, I.A., Bejai, S., Meijer, J., 2013. Control of drought stress in wheat using plant-growth-promoting bacteria. Journal of Plant Growth Regulation 32, 122-130.

Kohler, J., Hernández, J.A., Caravaca, F., Roldán, A., 2008. Plant-growth-promoting rhizobacteria and arbuscular mycorrhizal fungi modify alleviation biochemical mechanisms in water-stressed plants. Functional Plant Biology 35, 141-151.

Marulanda, A., Azcón, R., Ruíz-Lozano, J.M., 2003. Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress. Physiologia Plantarum 119, 526-533.

Marulanda, A., Barea, J.M., Azcón, R., 2009. Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments. Mechanisms related to bacterial effectiveness. Journal of Plant Growth Regulation 28, 115-124.

Medina, A., Azcón, R., 2012. Reclamation strategies of semiarid mediterranean soil: improvement of the efficiency of arbuscular mycorrhizal fungi by inoculation of plant growth promoting microorganisms and organic amendments. In: Hafidi M, Duponnois R (Eds) The Mycorrhizal Symbiosis in Mediterranean Environment: Importance in Ecosystem Stability and in Soil Rehabilitation Strategies, Nova Science Publishers New York, pp 87-106.

Mengual, C., Schoebitz, M., Azcón, R., Roldán, A., 2014. Microbial inoculants and organic amendment improves plant establishment and soil rehabilitation under semiarid conditions. Journal of Environmental Management 134, 1-7.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions. European Journal of Soil Science 54, 655-670.

Naveed, M., Hussain, M.B., Zahir, Z.A., Mitter, B., Sessitsch, A., 2014. Drought stress amelioration in wheat through inoculation with Burkholderia phytofirmans strain PsJN. Plant Growth Regulation 73, 121-131.

Nowak, J., 1998. Benefits of in vitro "biotization" of plant tissue cultures with microbial inoculants. In Vitro Cellular & Developmental Biology-Plant 34, 122-130.

Peixoto, R.S., Chaer, G.M., Franco, N., Junior, F.B.R., Mendes, I.C., Rosado, A.S., 2010. A decade of land use contributes to changes in the chemistry, biochemistry and bacterial community structures of soils in the Cerrado. Antonie Van Leeuwenhoek 98, 403-413.

Roesch, L.F.W., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K.M., Kent, A.D., Daroub, S.H., Camargo, F.A.O., Farmerie, W.G., Triplett, E.W., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. ISME Journal 1, 283-290.

Sinsabaugh, R.L., Antibus, R.K., Linkins, A.E., McClaugherty, C.A., Rayburn, L., Repert, D., Weiland, T., 1993. Wood Decomposition: Nitrogen and Phosphorus Dynamics in Relation to Extracellular Enzyme Activity. Ecology 74, 1586-1593.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G., 2001. Bulk and Rhizosphere Soil Bacterial Communities Studied by Denaturing Gradient Gel Electrophoresis: Plant-Dependent Enrichment and Seasonal Shifts Revealed. Applied and Environmental Microbiology 67, 4742-4751.

Subramanian, K.S., Santhanakrishnan, P., Balasubramanian, P., 2006. Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress. Scientia Horticulturae 107, 245-253.

Vallejo, V.R., Bautista, S., Cortina, J., 1999. Restoration for soil protection after disturbances, in: Trabaud, L. (Ed.), Life and Environment in the Mediterranean. Advances in Ecological Sciences. WIT Press, Wessex, pp. 301-343.

Vinocur, B., Altman, A., 2005. Recent advanced in engineering plant tolerance to abiotic stress: achievements and limitations. Current Opinion Biotechnology 16, 123-132.

Warren G.F., 1998. Spectacular increases in crop yields in the twentieth century. Weed Technology 12, 752-760.

White, D.C., Pinkart, H.C., Ringelberg, D.B., 1996. Biomass measurements: biochemical approaches. In: In: Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. (Eds.), Manual of Environmental Microbiology. American Society for Microbiology Press, Washington, DC, pp. 91 - 101. (Ed.).

Zahir, Z.A., Arshad, M., Frankenberger, W.T., 2004. Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Advances in Agronomy 81, 97-168.

Zelles, L., 1997. Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35, 275-294.

Tabla de contenido

INTRODUCTION

This work of investigation focuses on the importance of microorganisms on plants that inhabit semiarid soils located in the southeast of Spain. The study site location corresponds to Mediterranean climate, characterized by a very dry summer period, and winters with low and erratic rainfalls which have contributed in the intense effect of soil degradation processes. Drought is one of the most important abiotic stress factors limiting plant growth and performance in large areas of world, because it causes a series of detrimental changes in plant nutrition and plant physiology. All these alterations are originated as consequence of the environmental changes (the loss of natural plant communities, the degeneration of physical and chemical soil properties as well as by the loss or reduction of the microbial communities of soil).

The rhizosphere is a soil volume that is under the influence of plant root (Nadeem et al., 2014). The term 'rhizosphere' for the first time was described by Hiltner (1904), as a zone of maximum microbial activity. The microbial population present in this environment is relatively different from that of its surroundings due to the presence of root exudates that serve as a source of nutrition for microbial growth (Burdman et al., 2000). Rhizosphere soil influenced by plant roots may select specifically adapted microbial communities (Appuhn and Joergensen, 2006; Bais *et al.*, 2006). The microorganisms colonizing the plant roots generally include bacteria, fungi, actinomycetes, protozoa and algae. Enhancement of plant growth and development by application of these microbial populations is well evident (Zahir et al., 1997; Gray and Smith, 2005; Hayat et al., 2010; Bhattacharyya and Jha, 2012).

Of different microbial populations present in the rhizosphere, bacteria are the most abundant in most of the soil (Kaymak, 2010). Several studies have demonstrated that the bacterial diversity (Fig. 1) in rhizospheres can be influenced by a number of different factors, i.e., the plant species, varietal differences within a species, plant age, plant genotype, agricultural management, or soil properties (Beneduzi *et al.*, 2008; Castellanos *et al.*, 2009). Plants can interact with several genera of bacteria (*Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Variovorax*) that provoke the effect of stimulate plant growth and are termed plant growth promoting rhizobacteria (PGPR). These beneficial bacteria can be isolated, selected and applied as inocula. PGPR are free-living microorganisms that exert beneficial effects on plants by colonizing their rhizospheres or phyllospheres (Bashan and de-Bashan, 2005). PGPR are characterized by the following inherent distinctiveness's: (i) they must be proficient to colonize the root surface, (ii) they must survive, multiply and compete with other microbiota, at least for the time needed to express their plant growth promotion/protection activities, and (iii) they must promote plant growth (Kloepper, 1993). They may stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or

inhibiting them, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (Braud *et al.*, 2009; Hayat *et al.*, 2010; Rajkumar *et al.*, 2010; Ahemad, 2012). Several mechanisms are involved in the plant growth promotion by PGPR and the most important are: biofertilization, phytostimulation, rhizoremediation and stress control and biocontrol.

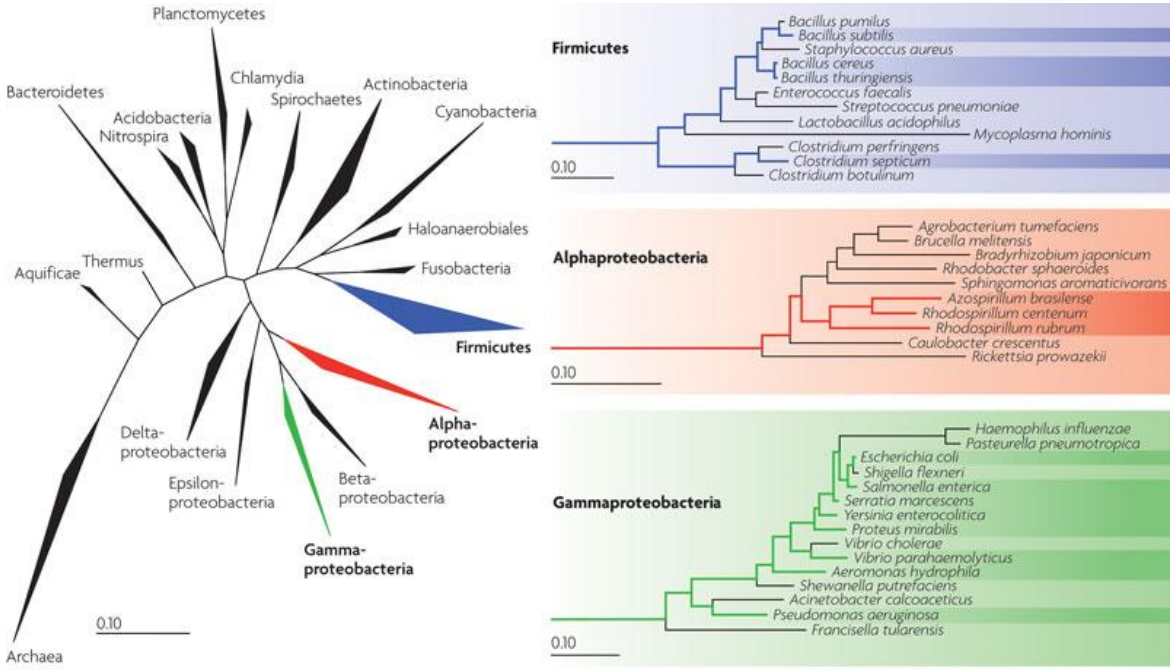


Fig. 1. Bacterial phylogeny based on the 16S rRNA gene (Kearns, 2010).

In addition to bacterial population, saprophytic fungi also represent a very significant portion of rhizosphere life (Fig. 2), in which they function as decomposers, pathogens and mycorrhizal mutualists. The obligate symbiotic association generated by fungi with plant roots is termed mycorrhizae. It represents an important soil component that increases the root surface area, and therefore enables the plant to absorb water and nutrients more efficiently from large soil volume. Two types of mycorrhizae: ecto- and endo-myccorrhizae have been reported in a great number of plants species. The mycorrhizal association not only increases the nutrient and water availability, but also protects the plant from a variety of abiotic stresses (Evelin *et al.*, 2009; Miransari, 2010). Due to the beneficial effects of rhizosphere microorganisms numerous studies are being conducted to evaluate plant effects by the application of different combinations or consortium of microorganisms, such arbuscular mycorrhizal (AM) fungi-PGPR, symbiotic-nitrogen-fixing rhizobia-PGPR or different PGPR (Swarnalakshmi et al., 2013).

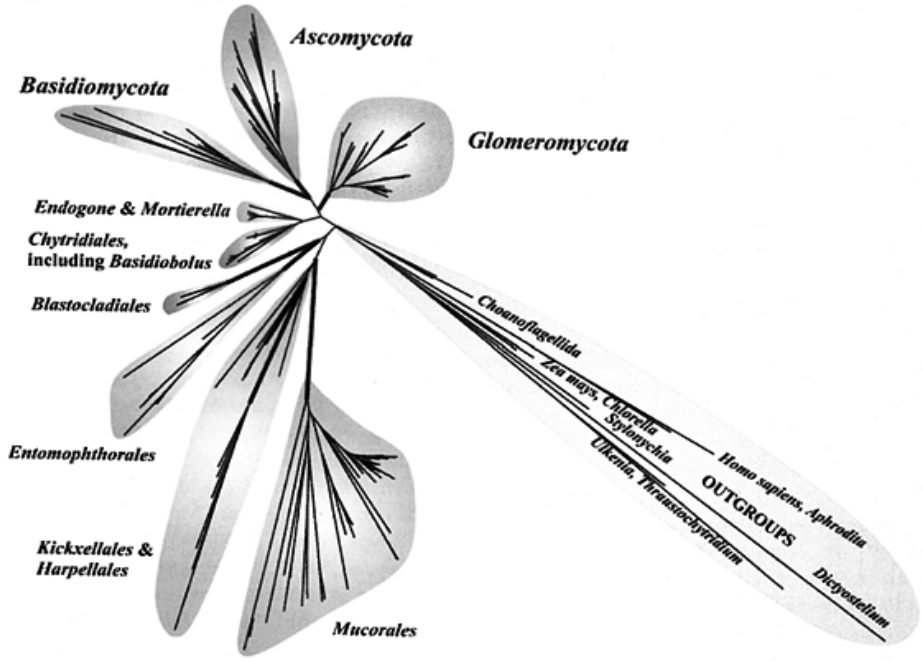


Fig. 2. Phylogeny showing the major clades of the kingdom fungi. (The group marked "outgroups" are non-fungi, and show the point where the phylogeny is rooted (Schüβler *et al.*, 2001).

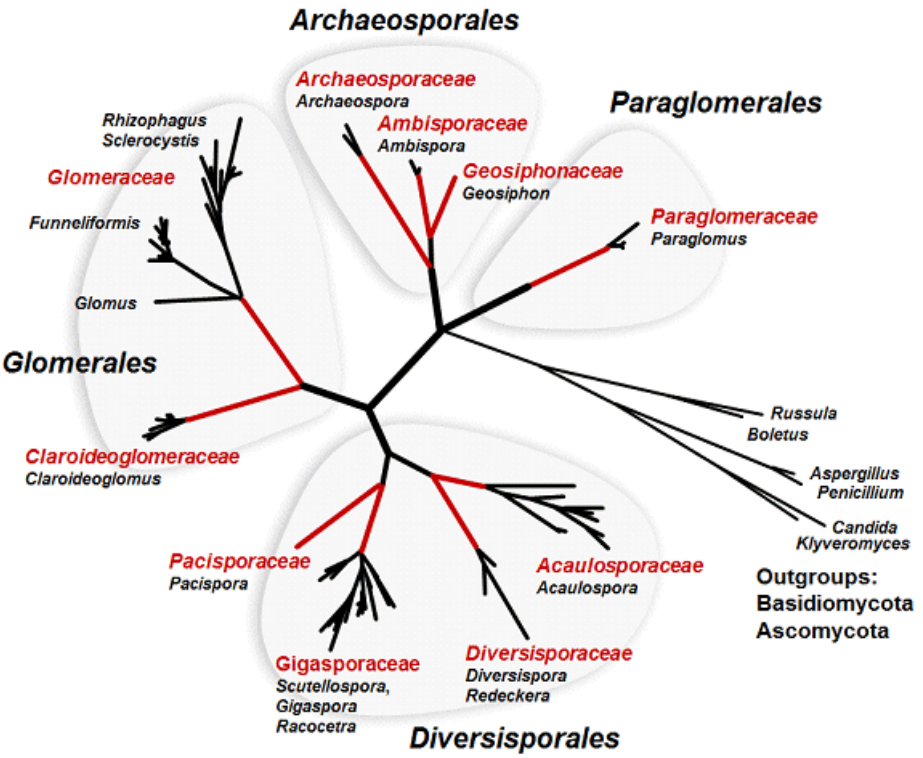


Fig. 3. Phylogenetic tree of 'AM fungi' (*Glomeromycota*), including *Geosiphon* (modified and updated from Schüßler *et al.* (2001); Schüßler and Walker (2010); see http://www.amf-phylogeny.com).

Mechanisms for the plant growth promotion by PGPR

PGPR promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Glick, 2012).

Biofertilization

Nitrogen fixation

Nitrogen (N) is one of the principal plant nutrients. There is about 78% N_2 in the atmosphere but it is unavailable to the growing plants. The atmospheric N_2 is converted into plant-utilizable forms by biological N_2 fixation (BNF) which is able to reduce nitrogen to ammonia by nitrogen fixing microorganisms using a complex enzyme system known as nitrogenase (Kim and Rees, 1994). Atmospheric N_2-fixing bacteria such as *Rhizobium* and *Bradyrhizobium* can establish symbiosis forming nodules on roots of leguminous plants such as soybean, pea, peanut and alfalfa, in with they convert N_2 into ammonia, which can be used by the plant as a nitrogen source (Murray, 2011) and non-leguminous trees that establish symbiosis with genera *Frankia* and non-symbiotic of free living, associative or endophytes such as cyanobaceria (*Anabaena*, *Nostoc*), *Azotobacter*, *Azospirillum*, *Gluconoacetobacter diazotrophicus* and *Azocarus*, etc (Bhattacharyya and Jha, 2012). Non-symbiotic PGPR that fix N_2 associated with non-leguminous plants are also called as diazotrophs since are capable of forming a non-obligate interaction with the host plants (Glick et al., 1999).

The process of N_2 fixation is carried out by a complex enzyme system, the nitrogenase complex (Kim and Rees, 1994). Structure of nitrogenase was elucidated by Dean and Jacobson (1992) as a two-component metalloenzyme: dinitrogenase reductase (iron protein) and dinitrogenase (metal cofactor). Dinitrogenase reductase provides electrons with high reducing power while dinitrogenase uses these electrons to reduce N_2 to NH_3. Based on the metal cofactors three different N fixing systems have been identified: Mo-nitrogenase, V-nitrogenase and Fe-nitrogenase. Most biological nitrogen fixation is carried out by the activity of the Mo-nitrogenase, which is found in all diazotrophs (Bishop and Joerger, 1990). So that, BNF represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al.*, 1997).

Phosphate solubilization

Phosphorus (P) is an essential nutrient for plants, but is often not available due to its fixation in soil. The low availability of phosphorous to plants is because the majority of soil P is found in insoluble forms, and the plants absorb it only in two soluble forms, the monobasic ($H_2PO_4^-$) and the diabasic (HPO_4^{2-}) ions (Bhattacharyya and Jha, 2012). The levels of P in soil are generally between 400 and 1200 mg kg^{-1} of soil, however, the concentration of soluble P in soil is usually ~ 1 mg kg^{-1} or less (Goldstein, 1994). The insoluble P is present as an inorganic mineral such as apatite (in soil with high pH) or as one of several organic forms including inositol phosphate (soil phytate), phosphomonesters and phosphotriesters (Glick, 2012).

The P deficiency in soils is compensated by the frequently application of phosphatic fertilizers in agricultural fields. However the plants absorb fewer amounts of applied phosphatic fertilizers and the rest is rapidly converted or transformed into insoluble complexes in soils (McKenzie and Roberts, 1990). The application of fertilizers is costly and environmentally detrimental, so that search of solutions should focus really on the safety and protection of the environment and a reasonable economic cost.

The application of phosphate solubilizing microorganisms (PSM), may provide the available forms of P to the plants and hence a viable substitute to chemical phosphatic fertilizers (Khan and Zaidi, 2006). Phosphate solubilizing bacteria (PSB) solubilize insoluble phosphate and make it available to the plants (Vessey, 2003). PSB secrete organic acids and enzymes that act on insoluble phosphates and convert it into soluble form thus providing phosphorus to plants (Fig. 4). Certain PGPR are able to solubilize those inorganic P forms through acidification (Richardson *et al.*, 2009), chelation and the organic P by enzymatic transformation (Hameeda *et al.*, 2008). Bacteria such as *Azospirillum*, *Bacillus*, *Burkholderia*, *Erwinia*, *Pseudomonas*, *Rhizobium* or *Serratia* are reported as PSB (Sudhakar *et al.*, 2000; Mehnaz and Lazarovits, 2006).

The solubilization of inorganic phosphorus occurs as a consequence of the action of low molecular weight organic acids which are synthesized by various soil bacteria (Zaidi *et al.*, 2009). However, the mineralization of organic phosphorus occurs through the synthesis of a variety of different phosphatases, catalyzing the hydrolysis of phosphoric esters (Glick, 2012). The solubilization of phosphate and mineralization can coexist in the same bacterial strain (Tao *et al.*, 2008).

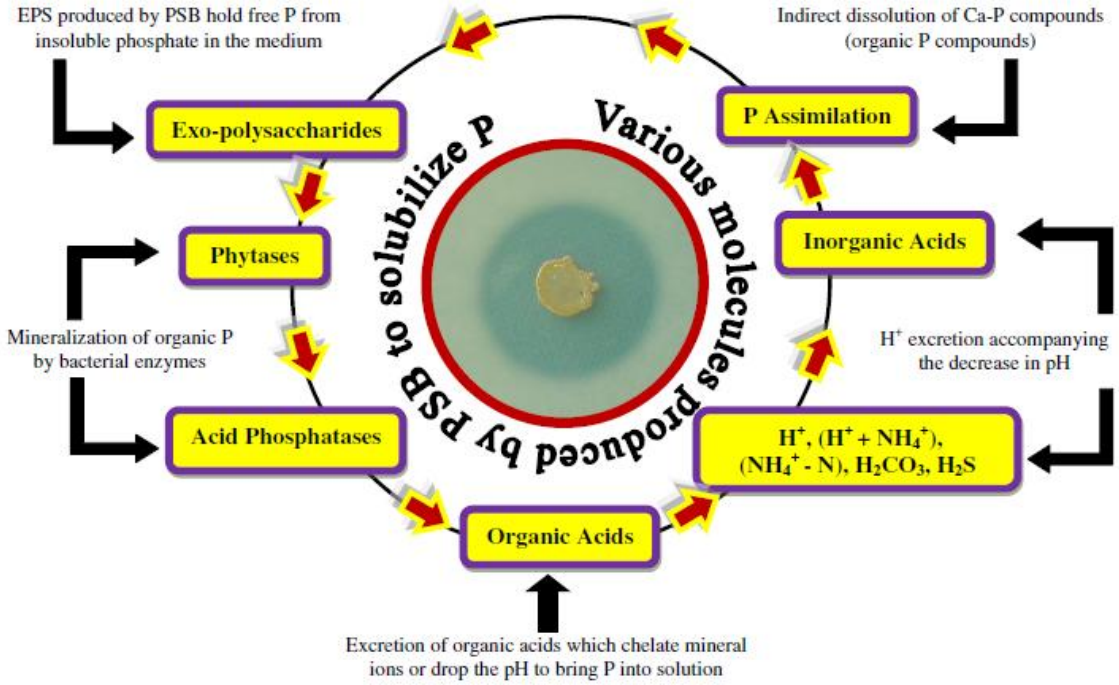


Fig. 4. The phosphate solubilization in soils by substances organic/inorganic produced by PSB (Ahemad and Kibret, 2014).

Other nutrients such as K, Ca, Mn, Fe, Cu and Zn can be increased in plants by inoculation of PGPR. This nutrient uptake usually occurs during acidification of the soil rhizosphere via organic acid production or via stimulation of proton pump ATPase (Mantelin and Touraine, 2004). In any case, the soil pH decrease improves solubilization of these (Pérez-Montaño *et al.*, 2014).

Siderophore production

Iron is an essential nutrient for almost all forms of life. In the aerobic environment, iron exists principally as ferric state (Fe^{3+}) and reacts to form insoluble hydroxides and oxyhydroxides, thus making it generally unavailable to plants and microorganisms.

Some bacteria and AM fungi produce low-molecular mass iron chelators with high affinity for iron termed siderophores (Machuca *et al.*, 2007; Miethke and Marahiel, 2007). The siderophores act as solubilizing agents for iron from organic compounds or minerals under conditions of iron limitation. Generally form 1:1 complexes with Fe^{3+}, which are then taken up by the cell membrane of bacteria, where the Fe^{3+} is reduce to Fe^{2+} and released from the siderophore into the cell (Boukhalfa and Crumbliss, 2002). Rhizobacteria differs regarding the siderophore cross-utilizing ability; some are proficient in using siderophores of the same genus (homologous siderophores) while others could utilize those produced by other rhizobacteria of different genera (heterologous siderophores) (Khan *et al.*, 2009). The roots could then take up

iron from siderophores-Fe complexes possibly via the mechanisms such as chelate degradation and release of iron, the direct uptake of siderophore-Fe complexes, and/or a ligand exchange reaction (Schmidt, 1999; Rajkumar *et al.*, 2010). Several studies of increased Fe uptake in plants with concurrent stimulation of plant growth as a result of plant growth promoting bacteria (PGPB) inoculation have been reported (Burd *et al.*, 2000; Carrillo-Castañeda *et al.*, 2003).

Phytostimulation

Phytohormone production

Diverse PGPR can alter root architecture and promote plant development due to their ability to synthesize and secrete plant hormones like indole-3-acetic acid (IAA), gibberellins (GAs), cytokinins and certain volatiles, hence they are termed phytostimulators (Bloemberg and Lugtenberg, 2001). This capacity is considered bacterial strain specific (Boiero *et al.*, 2007) (Fig. 5B).

IAA secreted by rhizobacteria interferes with the many plant developmental processes because the endogenous pool of plant IAA may be altered by the acquisition of IAA that has been secreted by soil bacteria (Spaepen *et al.*, 2007; Glick, 2012). Bacterial IAA increases root surface area and length, and thereby provides the plant greater access to soil nutrients, and also loosens plant cell walls as result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Glick, 2012). Plant-microbe interactions were determined by different IAA biosynthesis pathways: the beneficial plant-associates bacteria synthesize IAA via the indole-3-pyruvate (IPyA) pathways, whereas pathogenic bacteria mainly use the indole-3-acetamide (IAM) pathway (Patten and Glick, 1996; Hardoim *et al.*, 2008). Rhizobacterial IAA is identified as an effector molecule in plant-microbe interactions, both in pathogenesis and phytostimulation (Spaepen and Vanderleyden, 2011).

The GAs bacterial seem to be secondary metabolites that may play a role as signaling factors towards the host plant. There are many studies where GA production by *Azospirillum* or *Bacillus* sp. induces growth promotion in plants (Piccoli *et al.*, 1997; Gutiérrez-Mañero *et al.*, 2001; Bottini *et al.*, 2004).

It is important the involvement of cytokinins bacterial in root initiation, cell division, cell enlargement and increase in root surface area of crop plants through enhanced formation of lateral and adventitious roots (De Garcia Salamone *et al.*, 2006). Some PGPR release volatile signals (Ping and Boland, 2004), the rhizobacterial-produced volatile organic compounds (VOCs) like 2,3-butanediol, acetoin, terpenes, jasmonates, etc, are phytostimulators of great interest. The VOCs produced by the PGPR can act as signaling molecule to mediate plant-

microbe interaction as volatiles compounds mediating the roots colonization. They are generated at sufficient concentration to trigger the plant responses (Ryu *et al.*, 2003).

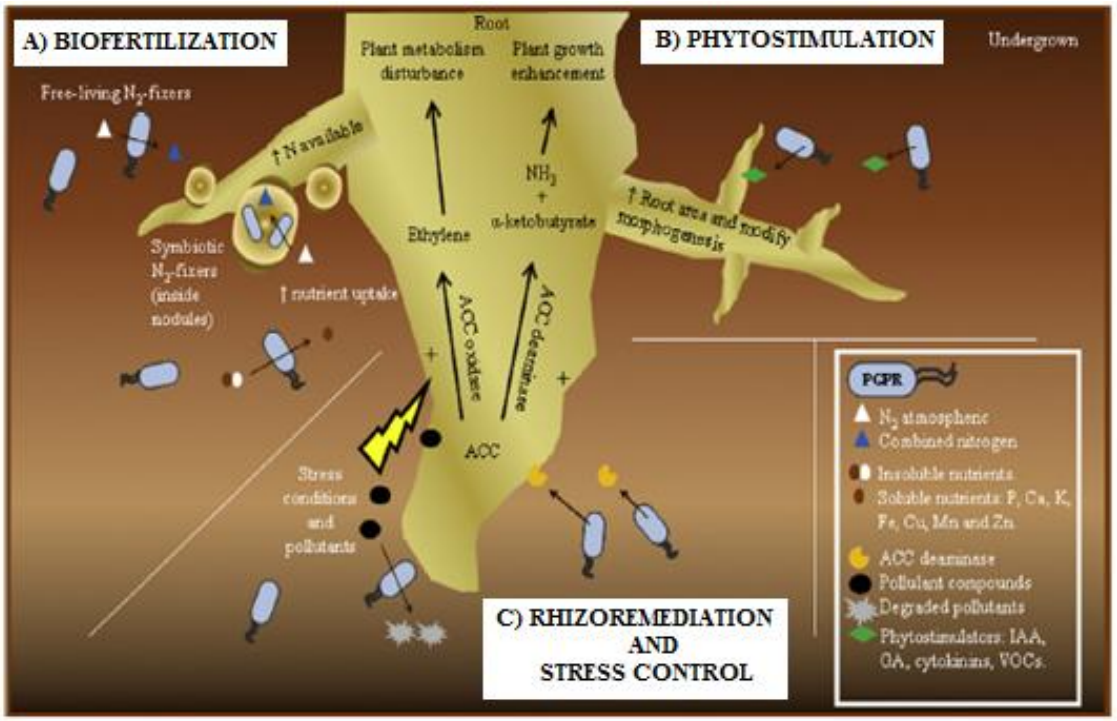


Fig. 5. Some mechanisms of plant growth promotion by PGPR. (A) Biofertilization; (B) Phytostimulation; (C) Rhizoremediation and stress control (Pérez-Montaño *et al.*, 2014).

Rhizoremediation and stress control

The ethylene (C_2H_4) is a phytohormone that has a central role in modulating the growth and cellular metabolism of plants (Ping and Boland, 2004). When plants are exposed to stress conditions they responded increasing ethylene levels that lead to an increase in cell and plant damage (Argueso *et al.*, 2007), induces defoliation and other cellular processes that may affect crop development (Desbrosses *et al.*, 2009). The 1-aminocyclopropane-1-carboxylic acid (ACC) is involved in biosynthetic pathway of ethylene, as an intermediate in the conversion of methionine to ethylene following biosynthetic sequence: methionine-S-adenosylmethionine (SAM)-ACC-C_2H_4 (Adams and Yang, 1979). Since SAM is converted by ACC synthase to ACC, the ACC synthase protein seems to play a main controlling role in ethylene biosynthesis pathway. ACC is oxidized by ACC oxidase to form ethylene, cyanide, and CO_2.

Bacteria are capable of alleviating the stress-mediated impact on plants by enzymatic hydrolysis of ACC (Glick *et al.*, 2007). ACC is exuded from plant roots or seeds and then taken up by the ACC-utilizing bacteria before its oxidation by the plant ACC oxidase (Contesto *et al.*, 2008) and cleaved by ACC deaminase to α-ketobutyrate and ammonia (Fig. 6). The bacteria

utilize the ammonia evolved from ACC as a sole nitrogen source and there by decrease ACC within the plant (Penrose and Glick, 2001) with the concomitant reduction of plant ethylene (Glick *et al.*, 1998; Belimov *et al.*, 2002). The decreased ethylene levels in plants hosting ACC-utilizing bacteria derive benefit by stress alleviation and enhanced plant productivity (Dell'Amico *et al.*, 2008; Hardoim *et al.*, 2008).

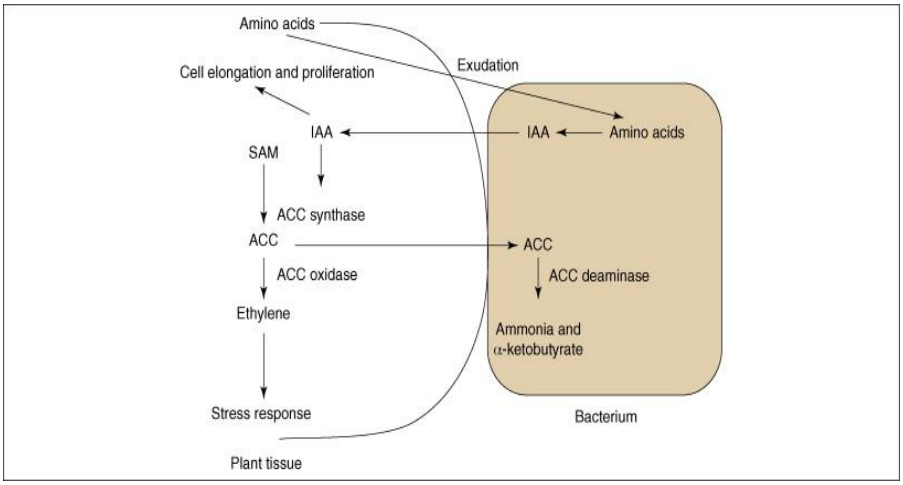


Fig. 6. Schematic representation of how bacteria containing ACC deaminase activity, lower the ethylene concentration in plant (Arshad *et al.*, 2007).

Bacteria strains exhibiting ACC deaminase activity (Fig. 5C) have been identified in a wide range of genera: *Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium*, etc. (Nadeem *et al.*, 2007; Shaharoona *et al.*, 2007a; Shaharoona *et al.*, 2007b; Zahir *et al.*, 2008; Zahir *et al.*, 2009). As a result, the major noticeable effects of seed or root inoculation with ACC deaminase-producing rhizobacteria are the plant root elongation, promotion of shoot growth and enhancement in N, P and K uptake as well as mycorrhizal colonization or rhizobial nodulation in various crops (Nadeem *et al.*, 2007; Glick, 2012).

Root endophytic bacterial colonization

Endophytic bacteria have been defined as bacteria colonizing the internal tissues of plants without causing symptomatic infections or negative effects on their host (Schulz and Boyle, 2006). Galippe in 1887, postuled that soil microorganisms can penetrate tissues of healthy plants and that the involved colonization mechanisms needed to be investigated (Galippe, 1887). Endophytic bacteria have been isolated from many different species (Lodewyckx *et al.*,

2002; Idris *et al.*, 2004; Barzanti *et al.*, 2007; Mastretta *et al.*, 2009). In some cases, they may confer to the plant higher tolerance to heavy metal stress and may stimulate host plant growth through several mechanisms including biological control, induction of systemic resistance in plants to pathogens, nitrogen fixation, production of growth regulators, and enhancement of mineral nutrients and water uptake (Ryan *et al.*, 2008). Beneficial effects due to bacterial endophytes inoculation are plant physiological changes including accumulation of osmolytes and osmotic adjustment, stomatal regulation, reduced membrane potentials, as well as changes in phospholipid content in the cell membranes (Compant *et al.*, 2005b). Following rhizosphere and rhizoplane colonization, some soil-borne microorganisms can enter roots, and establish subpopulations ranging from 10^5-10^7 cfu g^{-1} fresh weight (Hallmann, 2001).

The penetration process does not necessarily involve active mechanisms and thus all rhizosphere bacteria can be expected to be endophytic at one stage of their life (Hardoim et al., 2008). Passive penetration can take place at cracks, such as those occurring at root emergence sites or created by deleterious microorganisms as well as by root tips (Reinhold-Hurek and Hurek, 1998). Once a bacterium reaches the root cortical zone, a barrier such as the endodermis can block further colonization as only few bacteria are able to pass through the endodermis (Gregory, 2006) (Fig. 7). It is likely that endophytes able to pass through the endodermis can secrete cell-wall degrading enzymes (CWDEs) allowing them to continue colonization inside the endorhiza (James et al., 2002). Alternatively, some bacteria may passively enter as a portion of this endodermal cell layer is often disrupted, such as during the growth of secondary roots, which derive from the pericycle, located just below the endodermis barrier (Gregory, 2006). Under natural conditions some deleterious bacteria can moreover disrupt the endodermis, allowing endophytic bacteria at the same time to pass into the central cylinder. After passing through the endodermis barrier, endophytic bacteria have to penetrate the pericycle to further reach the root xylem vessels of their hosts (Compant et al., 2010) (Fig. 8.). Beneficial bacteria can pass from one xylem element to another using the perforated plates the size of the plate holes allows the passage of bacteria without requiring the activity of CWDEs (Bartz, 2005). A few studies reported that endophytic bacteria colonize flowers, fruits and seeds (Hallmann, 2001).

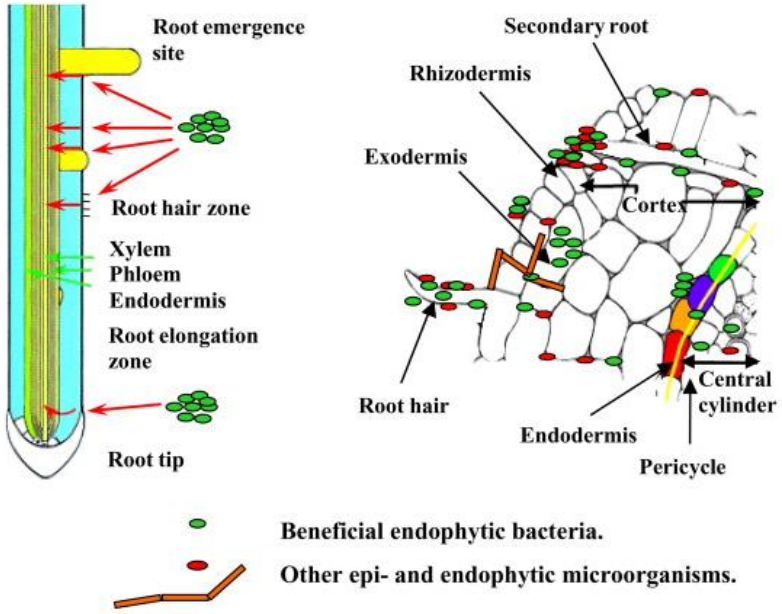


Fig. 7. Sites of plant colonization by endophytic bacteria. Drawing modified from Reinhold-Hurek (1998) and Compant (2007).

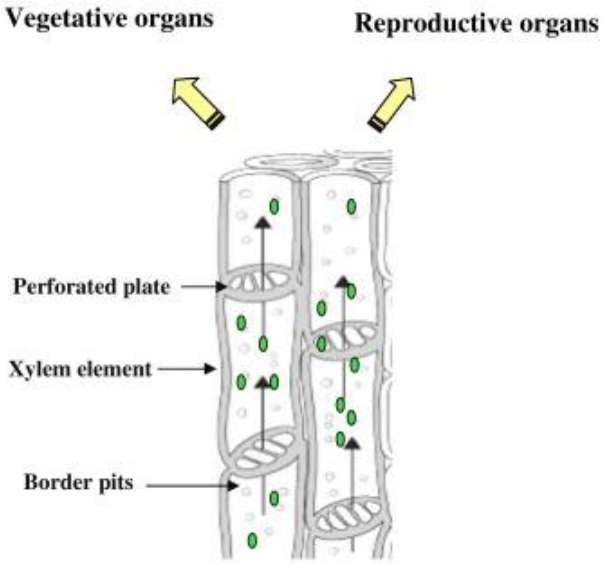


Fig. 8. Bacterial spread inside xylem vessels in aerial plant parts (Compant et al., 2010).

Biocontrol

Carbon fixed by plant photosynthesis is known to be partly translocated into the root zones and released as root exudates (Bais *et al.*, 2006). Root exudates and mucilage-derived nutrients are released in the rhizosphere, attract deleterious rhizobacteria as well as beneficial and neutral bacteria, fungi and other soil organisms, which provide a source of nutrients for root-associated bacteria (Walker *et al.*, 2003). The PGPR have to be highly competitive to successfully colonize the root zone. Secondary metabolites involved in biocontrol (Fig. 9), which are known to confer the producing bacteria a selective and competitive advantage against other microorganisms, further contribute to their rhizocompetence and root site colonization (Raaijmakers *et al.*, 2002; Compant *et al.*, 2005a; Haas and Défago, 2005).

Plants systemic resistance responses are induced systemic resistance (ISR) and systemic adquired resistance (SAR), are activated by certain microorganism molecules termed elicitors. The elicitors are cell wall polysaccharides, flagella, salicylic acid, cyclic lipopeptides, siderophores, antibiotics, the signal molecule N-acyl-homoserine-lactones (AHLs) or VOCs (Schuhegger *et al.,* 2006; Van Loon, 2007; Ramos Solano *et al.,* 2008; Berg, 2009). ISR is triggered by non-pathogenic microorganisms and starts in the root, extending to the shoot (Ramos Solano *et al.,* 2008). The defense response is dependent on ethylene and jasmonic acid signaling in the plant (Van Loon, 2007). SAR is activated by necrotic pathogenic bacteria and the molecule of defense response is salicylic acid (SA). Both ISR and SAR can overlap in some cases (López-Baena *et al.,* 2009). Several strains from *Azospirillum*, *Bacillus* and *Pseudomonas* genera are the group of PGPR that have been described eliciting IRS response, thus are biocontrol method (Fig. 9A).

PGPR have been demonstrated as enhancing the plant-growth producing very efficient extracellular siderophores which allow control of several plant diseases by depriving the pathogen of nutrition, thus resulting in increased crop yield (O'Sullivan and O'Gara, 1992) (Fig. 9B). Also, plants grown in metal-contaminated soils are often iron deficient and the bacteria may help plants to obtain sufficient iron (Burd *et al.,* 2000). Siderophores and lytic enzymes secreted by PGPR may reduce the growth of phytopathogens present in the rhizosphere.

Microbial isolates from plant-associate habitats, between 1 and 35% showed antagonistic capacity to inhibit the growth of pathogens in vitro (Berg, 2009). Antagonistic activity include inhibition of the pathogen by antibiotics, toxins and surface-active compounds (biosurfactans); competition for nutrients, minerals and colonization sites; and a mechanisms that develops production of extracellular cell wall degrading enzymes such as chitinase and β-1,3-glucanase (Whipps, 2001; Compant et al., 2005a; Haas and Défago, 2005) (Fig. 9C).

Many bacteria regulate their gene expression in response to changes in their population density in a process called Quorum Sensing (QS), which involves communication between cells mediated by small diffusible signal molecules termed autoinducers (Fuqua *et al.*, 1994). The most common autoinducers molecules are AHLs, regulate the expression of genes implied in the production of the virulence factor or biofilm formation in several plant pathogens (Quiñones *et al.*, 2005). Several bacteria produce acylase (*Ralstonia*) or lactonase (*Bacillus*) enzymes that degrade the AHL molecules and regulated virulence factors (Dong *et al.*, 2002). For example, the virulence *Erwinia carotovora*, whose virulence factors are regulated by QS, is attenuaded in the presence of the lactonase enzyme produced by *Bacillus* (Dong *et al.*, 2002). Many plant interfered in the QS systems of plant associated bacteria, are able produced molecules enhances o inhibit the phenotypes, depending on the bacterium being detected as a pathogen or as a beneficial microorganisms (Pérez-Montaño *et al.*, 2013) (Fig. 9D).

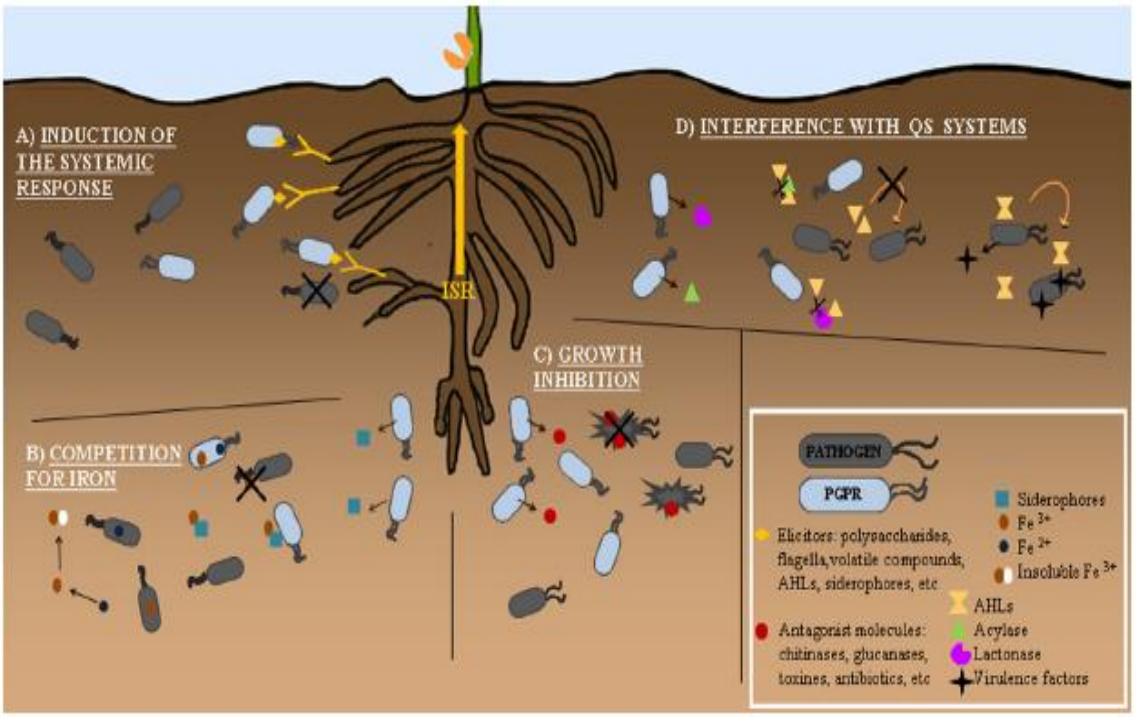


Fig. 9. Mechanisms of PGPR antagonism against plant pathogens (Biocontrol). (A) Induction of the systemic response. (B) Competition for iron. (C) Growth inhibition. (D) Interference with QS systems. (Pérez-Montaño *et al.*, 2014).

Mycorrhizae

Mycorrhiza is an obligate symbiotic association between plant roots and fungi. The two common types of fungi involved in such association are and ectomycorrhizae (ECM) and endomycorrhizae.

ECM the most advanced symbiotic association between higher plants and fungi, involving about 3% of seed plants including the majority of forest trees. In this association the plant root system is completely surrounded by a sheath of fungal tissue which can be more than 100 µm thick, though it is usually up to 50 µm thick. The hyphae penetrate between the outermost cell layers forming what is called the Hartig net. A network of hyphal elements (hyphae, strands and rhizomorphs) extends out to explore the soil domain and interface with the fungal tissue of the root (Moore *et al.*, 2011) (Fig. 10 and 11).

Endomycorrhizae (Fig. 11), in which the fungal structure is almost entirely within the host root comprising three major and two minor groupings: arbuscular endomycorrhizas (AM), orchidaceous endomycorrhizas and ericoid endomycorrhizas (arbutoid and monotropoid) (Fig. 10).

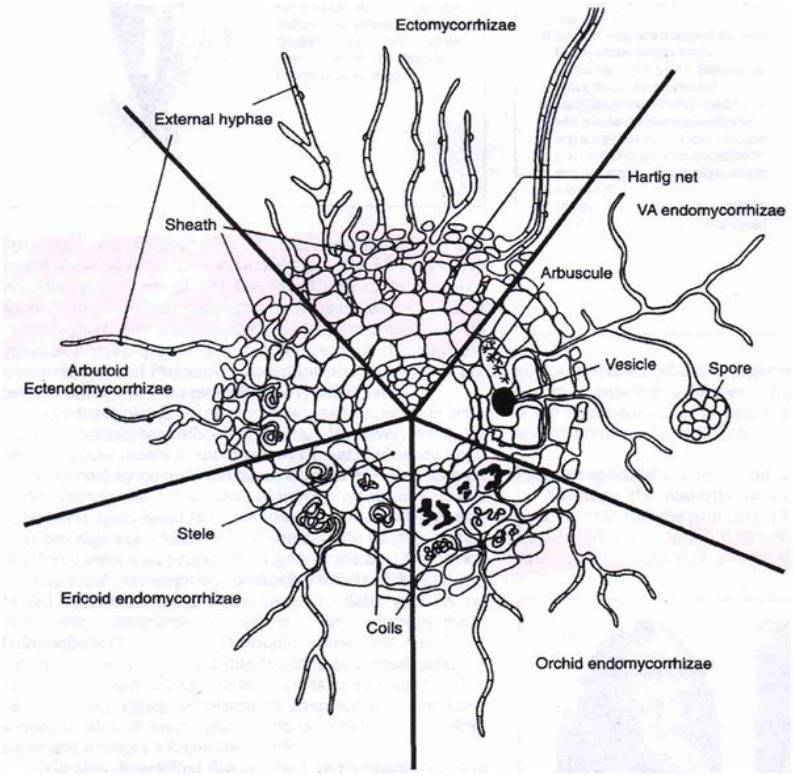


Fig. 10. The principle structural features of the five main types of mycorrhiza (Selosse and Le Tacon, 1998).

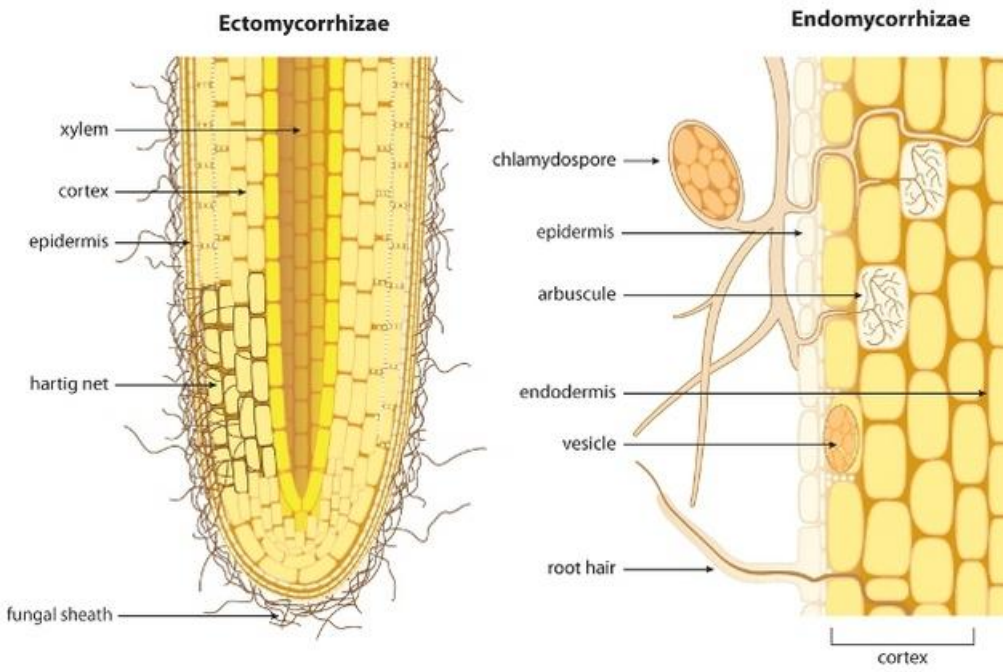


Fig. 11. Schematic showing the difference between ectomycorrhizae and endomycorrhyzae colonization of plant roots (Bonfante and Genre, 2010).

AM fungi (phylum Glomeromycota) (Fig.3) are probably the most abundant fungi that are commonly present in agricultural soils. These have been referred to as vesicular-arbuscular mycorrhiza (VAM), this name has been modified in favour of AM, since not all of the fungi form vesicles. But it can still be found in certain texts irrespectively as 'VAM' or 'VA' mycorrhizas (Fig. 10). About 80% of all terrestrial plants, including most agricultural, horticultural, and hardwood crop species are able to establish this mutualistic association (Giovannetti *et al.*, 2006). These fungi form symbiotic association with terrestrial plants as well as aquatic plants (Nielsen *et al.*, 2004; Wang *et al.*, 2015).

Phases of the establishment of arbuscular mycorrhizal symbiosis

To understand the molecular dialogue between these two symbionts, arbuscular mycorrhizal fungi (AMF) and plants, the process of establishment of the symbiotic relationship has been divided into three phases:

Pre-symbiotic dialogue (recognition and anticipation)

The period before physical contact and formation of appressoria involves recognition and attraction of certain signals. Spores of AM fungi persist in the soil and under accurate physical-chemical soil conditions they germinate spontaneously, independent of plant-derived signals (Mosse, 1959). However, root exudates and volatiles may promote or suppress spore germination, indicating the existence of spore "receptors" compounds responsive to alterations

in the chemical composition of the environment (Giovannetti and Sbrana, 1998; Bécard *et al.*, 2004; Harrison, 2005). After germination, the hyphal germ tube grows through the soil. In the absence of a potential host (asymbiotic phase), hyphal growth is limited by the utilization of low amounts of stored carbon (Becard and Piche, 1989; Bago *et al.*, 1999; Bago *et al.*, 2000) and eventually ceases; however, the spore retains sufficient carbon to allow repeated germination and further possibilities to encounter an appropriate host. Particularly large spores of *Gigaspora gigantean* can germinate up to 10 times (Koske, 1981).

In the vicinity of a host root, fungal morphology shifts towards enhanced hyphal growth and extensive hyphal branching (Giovannetti *et al.*, 1993b; Buee *et al.*, 2000). Such a response can be triggered by host root exudates or volatiles compounds. It suggest that the fungus senses a host-derived signal "branching factor", leading to intensified hyphal ramification that is likely to increase the probability of contact with a host root. A recent major breakthrough was the identification of the host branching factor as 5-deoxy-strigol, belonging to the strigolactones (Akiyama *et al.*, 2005). Strigolactones have been isolated from a wide range of mono- and dicotyledonous plants and were previously found to stimulate seed germination of parasitic weeds such as *Striga* and *Orobanche* (Bouwmeester *et al.*, 2003). AM fungi produce and release mycorrhizal factors "Myc" which cause changes in the calcium concentration in the epidermal cells of the root (Kosuta *et al.*, 2008), which in turn induce activation of genes related to the symbiosis (Kosuta *et al.*, 2003) (Fig. 12).

Early symbiotic phase (contact and penetration)

The symbiosis is marked morphologically by the formation of appressoria, termed hyphopodium the first cell-to-cell contact between fungus and plant, and the site of fungal ingress into the host root (Fig. 12). The development of appressoria can be considered the successful result of pre-symbiotic recognition when fungal and plants are committed to an interaction (Giovannetti *et al.*, 1993a). Because the plant produced a transcellular apoplastic compartment termed pre-penetration apparatus (PPA), the fungal hyphae enters the preformed channel and follows the route outlined by the plant cell nucleus (Fig. 13A). The transcellular migration of the nucleus was directed toward the point of entry of the fungus, from which they create a transcellular tunnel surrounded by a membrane, called perifungal membrane (Fig. 13B). Hence, infection only occurs after preparatory activities in the plant cell. Transcellular passage of the outer root cell layers appears to be a bottleneck in the development of the AM symbiosis, while entrance to the cortex apoplast permits rapid spread of the fungus along the axes of the root (Parniske, 2004).

Mature symbiotic phase

The fungus invaginates inner cortex cells where it undergoes extensive dichotomous branching into a tree-like fungal structure, termed arbuscule that may entirely fill the cortical cell. Consequently the architecture of the host cell undergoes remarkable changes; the nucleus moves from periphery to a central position, the vacuole becomes fragmented and an extensive periarbuscular membrane is synthesized that is in continuum with the plant plasma membrane (Harrison, 1999). PPA allows fungal hyphae to penetrate from hyphopodium to the cortex, where hyphae will branch to form arbuscules or vesicles (fungal storage organs) (Genre *et al.*, 2008).

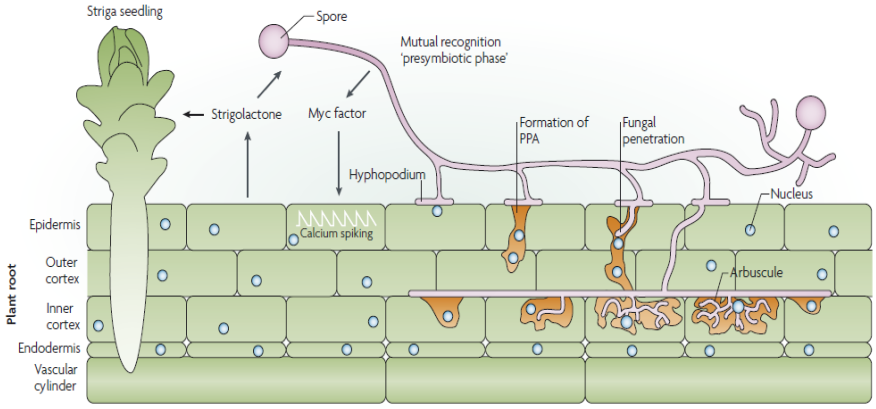


Fig. 12. It represents the various stages of establishment of symbiosis (Parniske, 2008).

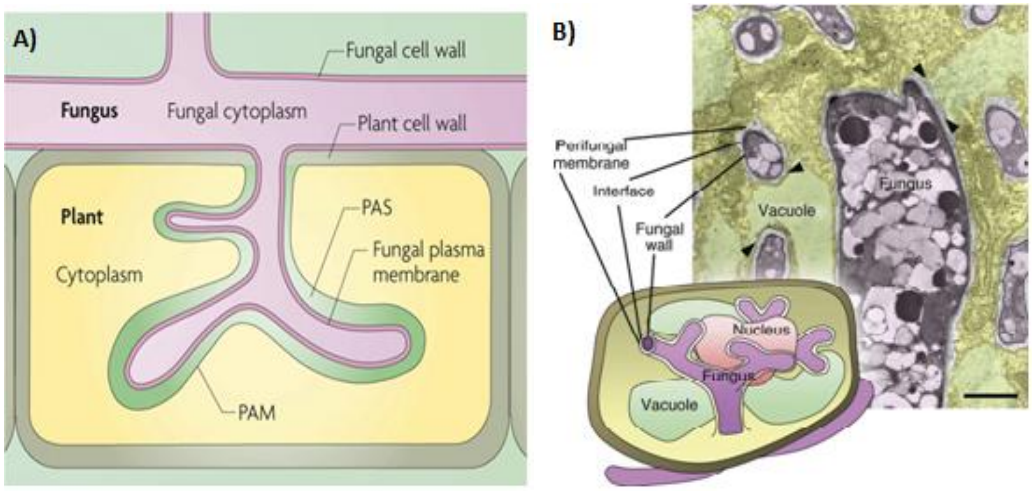


Fig. 13. A) Schematic drawing of an arbuscule. Each fungal branch within a plant cell is surrounded by a plant-derived periarbuscular membrane (PAM) that is continuous with the plant plasma membrane and excludes the fungus from the plant cytoplasm. The apoplastic interface between the fungal plasma membrane and the plant-derived PAM is called the periarbuscular space (PAS) (Parniske, 2008). **B**) The transmission electron image in shows the details of the fungal accommodation process inside an arbusculated cell of carrot. The interface compartment (uncoloured) is clearly visible all around the fungus (pink), whereas plant organelles are observed all around the perifungal membrane. In particular, the tonoplast occasionally seems to be in direct connection (arrows) with the perifungal membrane (Bar: 2 μm) (Bonfante and Genre, 2010).

Nutritional acquisition and stability of soil

These fungi penetrate into root cortical cells and form an arbuscule that serves as a mediator for the exchange of metabolites between fungus and host cytoplasm (Oueslati, 2003). The AM fungal hyphae also proliferate into the soil (Bethlenfalvay and Linderman, 1992) which helps plants to acquire mineral nutrients and water from the soil and also contribute to improving soil structure (Rillig and Mummey, 2006; Javaid, 2009). Mycorrhizal roots can explore more soil volume due to their extrametrical hyphae (Joner and Jakobsen, 1995; Guo *et al.,* 2010). Arbuscular mycorrhizal fungi transfer inorganic nutrients and water to the plant and receive carbohydrates in exchange. By driving this bidirectional nutrient transport between soil and plants, AM fungi are highly relevant for global phosphorus (P), nitrogen (N) and CO2 cycles (Fig. 14). It has been estimated that about 80% of the phosphorus taken up by a mycorrhizal plant is supplied by the fungus (Marschner and Dell, 1994). AM fungi play a very important role in ecosystems through nutrient cycling (Barea and Jeffries, 1995; Shokri and Maadi, 2009; Wu *et al.*, 2011).

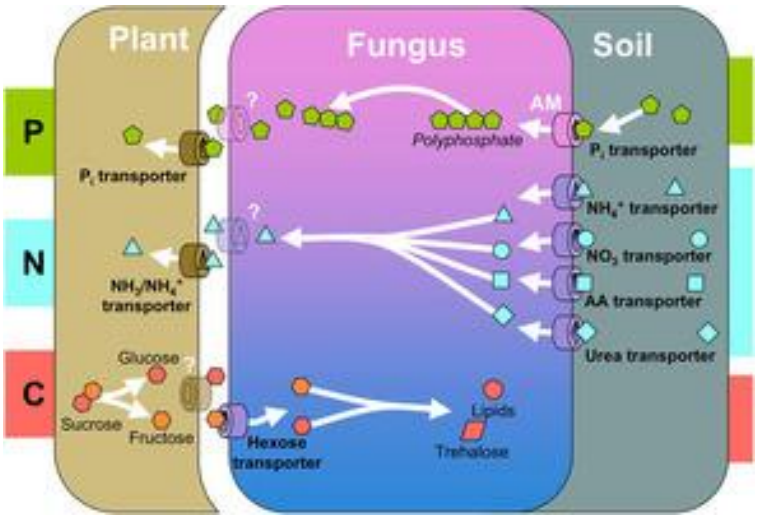


Fig. 14. Scheme summarizing the main nutrient exchange processes in extraradical mycelium (EM) and AM symbiosis (Bonfante and Genre, 2010).

AM colonization also play an important role in improving soil physical properties. The external mycorrhizal mycelium along with other soil organisms forms stable aggregates thereby improving soil aggregation (Bethlenfalvay and Schüepp, 1994; Borie *et al.*, 2008; Rillig *et al.*, 2010; Singh, 2012). Be due to production of an insoluble glycoprotein glomalin (Gadkar and Rillig, 2006) that plays an important role in soil stability (Rillig *et al.*, 2003).

Stress tolerance mediated by mycorrhizae

Mycorrhizae increases the root surface area, and therefore enables the plant absorb water and nutrients more efficiently from large soil volume, necessary for plant growth and also help the plant to tolerate stress environmental (Fig. 15). Moreover, they affect directly and indirectly the diversity and productivity of land-plant communities by their central role at the soil-plant interface (Van Der Heijden *et al.,* 1998). They can also improve host plant pathogen resistance (Vigo *et al.,* 2000; De La Peña *et al.,* 2006) and drought stress tolerance (Michelson and Rosendahl, 1990; Aroca *et al.,* 2007). Also of providing nutritional and structural benefits to plants, they also impart other benefits to them including production and /or accumulation of secondary metabolites (amino acids, phytohormones, vitamins), osmotic adjustment under osmotic stress, improved nitrogen fixation, enhanced photosynthesis rate, and increased resistance against biotic and abiotic stresses (Ruiz-Lozano, 2003; Wu and Xia, 2006; Schliemann *et al.,* 2008). AM fungi can improve plant tolerance to heavy metals, drought, and salinity, and also protect plants from pathogens (Azcón-Aguilar *et al.*, 2002; Marulanda *et al.*, 2006; Gamalero *et al.*, 2009; Marulanda *et al.*, 2009).

A number of studies have shown that AM-plant association improved P nutrition under salinity and water deficit environment. This is a primary mechanism for promoting stress tolerance in plants (Ruiz-Lozano *et al.*, 1996; Subramanian *et al.*, 1997), but AM symbiosis also affects the physiological processes of plants by increasing proline contents (Ruiz-Lozano *et al.*, 1995). Proline is known to act as osmoregulator under stress conditions (Irigoyen *et al.*, 1992; Ashraf and Foolad, 2007). Physiological processes involved in osmoregulation like enhanced carbon dioxide exchange rate, water use efficiency, and stomatal conductance are also influenced by the activities of AM fungi (Ruiz-Lozano and Aroca, 2010; Birhane *et al.*, 2012). It is also well documented that AM fungi affect the expression of a number of antioxidant enzymes, which protect the plants from reactive oxygen species produces under stress conditions (Gamalero *et al.*, 2009). The plants associating AM fungi showed more drought tolerance in terms of higher shoot biomass production and leaf water potential than that by non-AM plants. High proline contents in the root and low in the shoot were observed in drought-stressed AM plants, whereas low activity of lipid peroxidase was observed in the shoots of drought-stressed AM plants (Porcel and Ruiz-Lozano, 2004). These authors demonstrated that AM symbiosis enhanced osmotic adjustment in roots that helped to maintain favorable water potential gradient for water movement from soil to roots. It results in high water potential under drought stress and, therefore, protects plants from the drastic effects of drought.

Plants have to face with the problem of acquiring sufficient amount of water from the soil under drought conditions (Ouziad *et al.*, 2006), and aquaporins participate in this process (Maurel *et al.,* 2008). Aquaporins are water channel proteins that facilitate and regulate the

passive movement of water molecules down a water potential gradient (Kruse *et al.*, 2006). These proteins are present in all kingdoms and belong to the major intrinsic protein (MIP) family of transmembrane proteins. In maize two major classes of plant aquaporins are located in the plasma membrane (PIPs) and in the tonoplast (TIPs). PIPs and TIPs isoforms have been recognized as central pathways for transcellular and intracellular water transport (Maurel *et al.*, 2008).

Thus, aquaporins seem to play a specifically important role in controlling transcellular water transport in plant tissues (Javot and Maurel, 2002). In any case, the relationship between aquaporins and plant responses to water deficit is still elusive and with contradictory results (Aharon *et al.*, 2003; Lian *et al.*, 2004). In addition, although many aquaporins are highly selective for water, uptake experiments with *Xenopus laevis* oocytes clearly showed that certain aquaporins are permeable to small solutes such as glycerol, urea, amino acids, CO_2 and/or NH_3/NH_4 or even small peptides and ions (Kaldenhoff *et al.*, 2007; Uehlein *et al.*, 2007), which opens many questions about the physiological roles of aquaporins, especially in AM plants (Maurel and Plassard, 2011). Interestingly, several maize aquaporins have been shown to be regulated by the AM symbiosis under different drought scenarios, and their regulation has been related with the exchange of water and other molecules of physiological importance between the host plant and the AM fungus (Bárzana *et al.*, 2014).

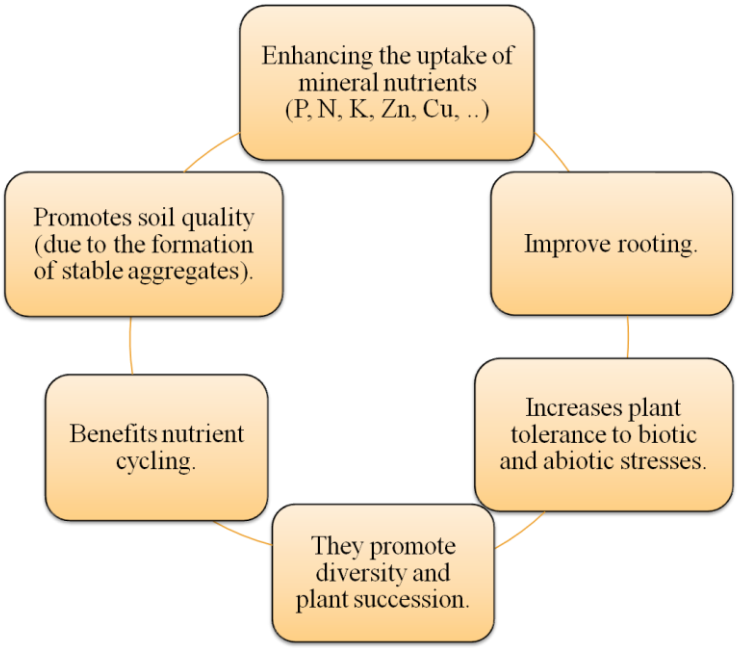


Fig. 15. Benefits provided by AM fungi to plants and the ecosystem.

Role of PGPR and mycorrhizal fungi in stress tolerance

The combined inoculation of PGPR and mycorrhizae is reported to be helpful to enhance plant growth under normal conditions and could be very useful to reduce the negative impact of a stress on plant growth and development (Fig. 16). In addition, stress conditions causes adverse effects on microbial functions can be reduced by combined inoculations. Interactions between plant and fungus/bacteria in which both partners get benefits as mutualistic association (Beattie, 2006; Finlay, 2008). AM fungi interact with others microorganisms like bacteria and synergistic interaction between them not only promotes plant growth but also enhances the population of each other (Artursson *et al.*, 2006; Yusran *et al.*, 2009). Bacteria can produce compounds to increase cell permeability so as to enhance the rate of root exudation that stimulates the hyphal growth and facilitates root penetration by the fungus (Jeffries *et al.*, 2003). Furthermore, PGPR improve the development of the mycosimbionts and facilitate the colonization of plant roots by AMF (Hildebrandt *et al.*, 2002; Jäderlund *et al.*, 2008; Armada *et al.*, 2014b). Barea *et al.*, (1998) demonstrated that *Pseudomonas* sp. had the ability to produce antifungal metabolites but did not cause any negative effect on *Glomus mosseae*, and an effective biocontrol agent against *Fusarium* sp., is also helpful for promoting symbiosis between *Medicago truncatula* and *G. mosseae* (Pivato *et al.*, 2009). On one hand, mycorrhizae help the plant to resist against biotic and abiotic stresses by increasing surface area of roots nutrient acquisition or through more specific mechanisms (Artursson *et al.*, 2006; Asif and Bhabatosh, 2013) and enhance the activities of nitrogen fixing and phosphorus solubilizing bacteria (Linderman, 1992). For example, root colonization of lettuce by AM fungus was reduced under drought stress, but dual application of fungus and bacteria improved it (Vivas *et al.*, 2003). *Bacillus* genera caused a significant stimulatory effect on *Glomus intraradices* development by enhancing the mycelium growth, was further evaluated by inoculating it with drought tolerant and drought sensitive species of AM fungus under water stress environment (Marulanda *et al.*, 2006).

Under drought stress, reduction in plant water uptake occurs. This reduced water uptake decreased nitrogen and carbon metabolism and ultimately changed the plant physiology (Ruiz-Lozano and Azcón, 2000). Dual inoculation of PGPR and mycorrhizae proved more useful for enhancing water and nutrient content. Improved water content of drought stressed *Trifolium repens* inoculated with PGPR and mycorrhizal fungi decreased stomatal conductance and increased the relative water content, both are important for plants growing in water limited environment (Benabdellah *et al.*, 2011; Armada *et al.*, 2014a; Ortiz *et al.*, 2015).

Plants colonized by growth promoting microorganisms showed higher root hydraulic conductance and/or increased tolerance against drought and salinity (Aroca *et al.*, 2007; Dimkpa *et al.*, 2009). It was proposed that decreased expression of plasma membrane aquaporin genes

during drought stress can be a regulatory mechanism to limit water loss from cells (Barrieu *et al.*, 1999). Ouziad *et al.,* (2006) found reduced transcript levels of both a tonoplast and a plasmalemma aquaporin gene in the roots of *Lycopersicon esculentum* colonized by a mixture of *Glomus intraradices* and *Glomus geosporum*, with this reduction being even greater in plants living under sustained salt stress. But AMF colonization increased transcript levels of three aquaporin genes analyzed (*LePIP1*, *LePIP2* and *LeTIP*) in tomate leaves upon salt stress, while genes encoding two Na^+/H^+ transporters were unaffected. Aroca *et al.,* (2007) found that root hydraulic conductivity (L_{pr}) of *Phaseolus vulgaris* mycorrhized plants under control conditions was about half that of non-AM plants, and that this parameter decreased as a result of drought, cold or salinity in non-AM plants, while it remained almost unchanged in AM plants. The effects of the microbial inoculants on the PIP genes in maize may be related to a possible role of these aquaporins in root water uptake from soil (Armada *et al.*, 2015). Indeed, under drought stress conditions the root hydraulic conductivity of AM- or AM + *B. thuringiensis*-inoculated plants was significantly higher than that of inoculated control plants, and this correlated with the up-regulation of several PIP genes in root of these plants (Armada *et al.*, 2015). These results give to understand, differential expression of various PIP genes, suggesting that each PIP gene has specific functions and regulation mechanisms under certain environmental stresses.

The rhizospheric microorganisms can affect plant ABA flows by the release or consumption of other plant growth regulators or their precursors (Jiang and Hartung, 2008; Yang *et al.*, 2009), as well as by the release of volatile compounds involved in ABA sensing (Zhan et al 2008) (Fig. 17). Under drought conditions, AM symbiosis regulates ABA content (Goicoechea *et al.*, 1997; Ludwig-Müller, 2000; Estrada-Luna and Davies Jr, 2003) and expression of some host plant aquaporin genes (Ruiz-Lozano *et al.*, 2006; Aroca *et al.*, 2008). At the same time, abscisic acid has been found to be necessary for arbuscular development (Herrera-Medina *et al.*, 2007).

These beneficial microorganisms induced physical and chemical changes that lead to increased root hydraulic conductivity and enhanced drought and salinity tolerance. In addition, those related the regulation of root aquaporins, an effect that is probably mediated by complex hormonal mechanisms in which plant ABA levels may play a central role (Fig. 17).

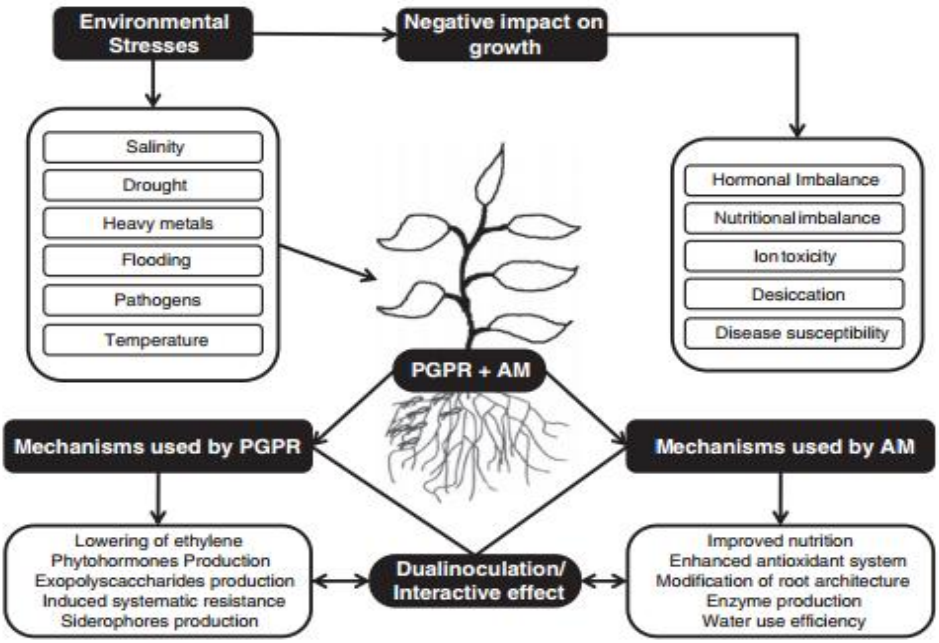


Fig. 16. Mechanisms used by plant growth promoting rhizobacteria (PGPR) and mycorrhizae for enhancing plant growth under stress (Nadeem *et al.*, 2014).

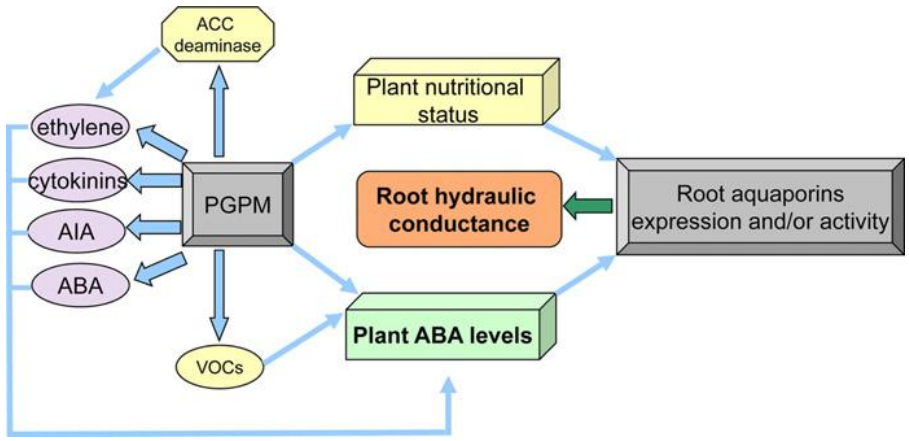


Fig. 17. Plant growth promoting microorganisms (PGPM) may affect root hydraulic conductance through a complex network of biochemical interactions. Microbial biosynthesis of plant hormones and microbial-mediated regulation of endogenous plant hormone levels, particularly of abscicic acid, which in turn is involved in aquaporin gene expression, seems to be of capital relevance in these processes. Additionally, changes in the nutritional status of colonized plants and volatile organic compounds (VOCs) released by certain microorganisms may directly or indirectly impact on root aquaporins expression and/or activity, and therefore, on root hydraulic conductance (Groppa *et al.*, 2012).

Application of Mycorrhizae-PGPR and their constraints in natural environmental conditions

The application of PGPR and/or mycorrhizae is beneficial for plants. In co-inoculation, each strain not only competes successfully with indigenous rhizosphere population, but also proves helpful for promoting the growth of each other (Fig. 18). However, the principle obstacle to the commercial use of microbial inoculants is his inconsistent performance under field conditions. The inoculums efficiency depends upon a number of factors like soil mineral content, type of crop and competition with indigenous strains (Jefwa *et al.*, 2010). It has been observed that microbial performance in the rhizosphere was significantly affected due to competition with an indigenous population for nutrient and niches (Strigul and Kravchenko, 2006) also be due to certain edaphic conditions and a number of abiotic factors (Schreiner, 2007). For example, tillage practice is recommended as a soil management practice, but it causes a negative impact on AM fungus by disrupting mycelia network (Jasper *et al.*, 1991), and high phosphorus content in soil reduces the activity of AM fungus (Habte and Manjunath, 1987). Such interactions might be due to incompatibility and /or pathogenicity of one partner to the other as observed by Dewey *et al.,* (1999), that associated bacteria enhanced the fungal pathogenicity, although the bacterium itself was nonpathogenic. The PGPR-mycorrhizal interactions are very important from point of view of plant growth and development. The assays are based on study the combination of species of plants, bacteria and fungi most suitable, are a truly adequate solution for success in their application, both in the laboratory and field.

Regarding the information reported, the perspective of future in research on such subject should be focused in to; explore and know what strains of PGPR and fungus are beneficial for promoting plant growth and enhanced the microbial diversity of soil. To select the combination of these strains that interacts synergistically so as to achieve a maximum benefit. To clarify the mechanisms of interactions between PGPR and mycorrhizae in natural field conditions under stressful environments and to examine the effectiveness of co-inoculation in a multi-stressed natural environment. The final objective is the commercialization of microbial inocula with promising results.

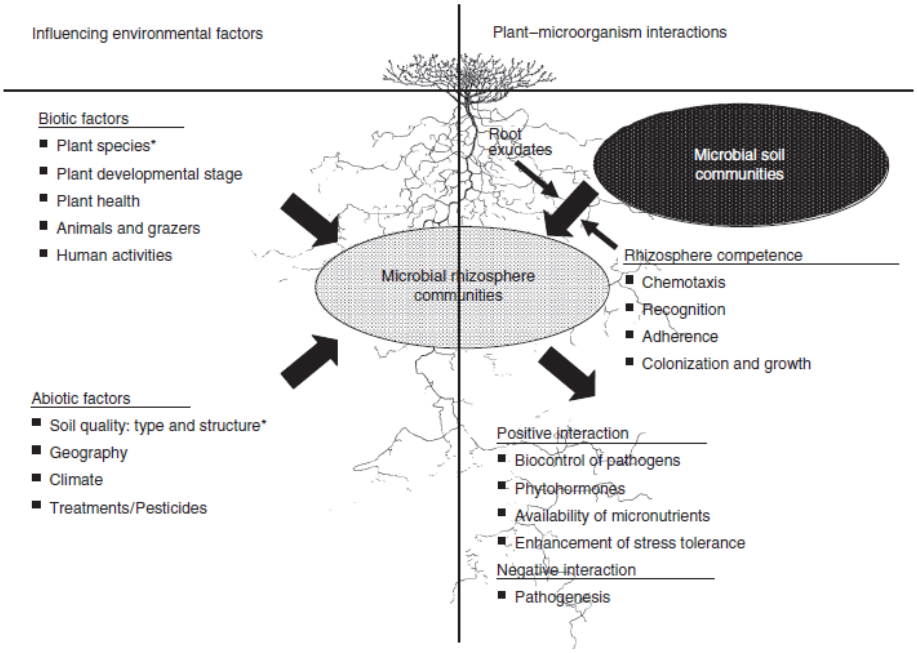


Fig. 18. Influencing factors of rhizosphere microbial communities and model how microbial communities were selected from soil: by root exudates and their rhizosphere competence (Berg and Smalla, 2009).

Organic amendments

Arid soils are generally characterized by poor structure, lack of organic matter and low water-holding capacity. The most important factor making the rhizosphere an attractive habitat for saprophyte microorganisms, like many bacteria, is the organic carbon provided by plant roots. Thus, the limited plant growth and C exudation under arid conditions may cause the poor surviving of saprophyte microbial inoculum and it is necessary to assure their establishment to be effective on plant growth in arid soils. In fact, the deterioration of biological properties of arid soils is in part due to their progressive decrease in organic matter content (Bashan and de-Bashan, 2010).

In this respect the application of organic amendments to desertified soil, prior to the microbial inoculation has been recommended (Medina et al., 2004a; 2004b; Trejo et al., 2012; Armada et al., 2014a). In previous studies the most important effects of organic amendments included not only the improvement of soil quality (nutrients, humus, water-holding capacity) but also an increase of microbial activities (Kloepper et al., 1999; Caravaca et al., 2005; 2006; Trejo et al., 2012; López et al., 2013).

Exist great variety of agricultural residues from the crops themselves and of industrial sources. In particular, sugar beet residues agro-industrial are generated in the industry to obtain sugar of beet. Large amounts of agrowastes are produced during the extraction of sugar from the sugar beet, but this product only can be used as organic amendment after biological

transformation processes. Sugar beet residue (Table 1), because of its lignocellulosic composition, may be mineralized by specific lignocellulosic microorganisms such as *Aspergillus niger*, resulting in a product rich in minerals for plant growth and also in sugars that can be used as energy sources for heterotrophic microorganisms, such as plant growth promoting bacteria (PGPB) as suggested by Bashan and Holguin (1998). Nevertheless, the fertilizer ability of this agrowaste can be increased when rock-phosphate (RP) is added to the fermentation medium (Medina et al., 2005). The rock-phosphate solubilization was carried out by the citric acid production by *A. niger* growing on the agrowaste residue.

Table 1. Characteristics of sugar beet waste (SB) (Vassilev *et al.*, 2006).

Cellulose (%)	**Hemicellulose (%)**	**Lignin (%)**	**C_{total} (g kg^{-1} DW)**	**N_{total} (g kg^{-1} DW)**	**P_{total} (g kg^{-1} DW)**
29	23	5	520	7	0.7

The application of this *A. niger* + RP treated product as amendment improved soil fertility and in previous studies this amendment was used in reclamation strategies of degraded systems particularly associated with AM fungi or yeast (Medina and Azcón, 2010) or bacteria (Armada *et al.*, 2014a). In addition to the nutritional abilities for plants and microorganisms, may affect water uptake by plants (Caravaca et al., 2006).

The application of this amendment could be considered as an interesting product to be used for revegetation in water-limited environments improving plant/soil quality and PGPB inocula survival.

References

Adams, D.O., Yang, S.F., 1979. Ethylene biosynthesis. Identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methione to ethylene. . Proceedings of the National Academy of Sciences USA 76, 170-174.

Aharon, R., Shahak, Y., Wininger, S., Bendov, R., Kapulnik, Y., Galili, G., 2003. Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. Plant Cell 15, 439-447.

Ahemad, M., 2012. Implications of bacterial resistance against heavy metals in bioremediation: A review. IIOAB Journal 3, 39-46.

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

Akiyama, K., Matsuzaki, K., Hayashi, H., 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435, 824-827.

Appuhn, A., Joergensen, R.G., 2006. Microbial colonisation of roots as a function of plant species. Soil Biology and Biochemistry 38, 1040-1051.

Argueso, C.T., Hansen, M., Kieber, J.J., 2007. Regulation of ethylene biosynthesis. Journal of Plant Growth Regulation 26, 92-105.

Armada, E., Azcón, R., López-Castillo, O.M., Calvo-Polanco, M., Ruiz-Lozano, J.M., 2015. Autochthonous arbuscular mycorrhizal fungi and Bacillus thuringiensis from a degraded Mediterranean area can be used to improve physiological traits and performance of a plant of agronomic interest under drought conditions. Plant Physiology and Biochemistry 90, 64-74.

Armada, E., Portela, G., Roldán, A., Azcón, R., 2014a. Combined use of beneficial soil microorganism and agrowaste residue to cope with plant water limitation under semiarid conditions. Geoderma 232-234, 640-648.

Armada, E., Roldán, A., Azcon, R., 2014b. Differential Activity of Autochthonous Bacteria in Controlling Drought Stress in Native Lavandula and Salvia Plants Species Under Drought Conditions in Natural Arid Soil. Microbial Ecology 67, 410-420.

Aroca, R., Porcel, R., Ruiz-Lozano, J.M., 2007. How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in Phaseolus vulgaris under drought, cold or salinity stresses? New Phytologist 173, 808-816.

Aroca, R., Vernieri, P., Ruiz-Lozano, J.M., 2008. Mycorrhizal and non-mycorrhizal Lactuca sativa plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. Journal of Experimental Botany 59, 2029-2041.

Arshad, M., Saleem, M., Hussain, S., 2007. Perspectives of bacterial ACC deaminase in phytoremediation. Trends in Biotechnology 25, 356-362.

Artursson, V., Finlay, R.D., Jansson, J.K., 2006. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environmental Microbiology 8, 1-10.

Ashraf, M., Foolad, M.R., 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany 59, 206-216.

Asif, M., ., Bhabatosh, M., 2013. Effects of inoculation with stress-adapted arbuscular mycorrhizal fungus *Glomus deserticola* on growth of *Solanum melogena* L. and *Sorghum sudanese* Staph. seedlings under salinity and heavy metal stress conditions. Archives Agronomy Soil Science 59, 173-183.

Azcón-Aguilar, C., Jaizme-Vega, M.C., Calvet, C., 2002. The contribution of arbuscular mycorrhizal fungi to the control of soil borne pathogens. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandre K, editors. Mycorrhizal techonology in agriculture. Switzerland: Birkhausser Verlag., 187-198.

Bago, B., Pfeffer, P.E., Douds Jr, D.D., Brouillette, J., Bécard, G., Shachar-Hill, Y., 1999. Carbon metabolism in spores of the arbuscular mycorrhizal fungus Glomus intraradices as revealed by nuclear magnetic resonance spectroscopy. Plant Physiology 121, 263-271.

Bago, B., Pfeffer, P.E., Shachar-Hill, Y., 2000. Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiology 124, 949-957.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology, pp. 233-266.

Barea, J.M., Andrade, G., Bianciotto, V., Dowling, D., Lohrke, S., Bonfante, P., O'Gara, F., Azcon-Aguilar, C., 1998. Impact on arbuscular mycorrhiza formation of Pseudomonas strains used as inoculants for biocontrol of soil-borne fungal plant pathogens. Applied and Environmental Microbiology 64, 2304-2307.

Barea, J.M., Jeffries, P., 1995. Arbuscular mycorrhizas in sustainable soil-plant systems. In: Varma A, Hock B, editors. Mycorrhiza: structure, function, olecular biology and biotechnology. Berlin Heidelberg, New York; Springer., 521-561.

Barrieu, F., Marty-Mazars, D., Thomas, D., Chaumont, F., Charbonnier, M., Marty, F., 1999. Desiccation and osmotic stress increase the abundance of mRNA of the tonoplast aquaporin BobTIP26-1 in cauliflower cells. Planta 209, 77-86.

Bartz, J.A., 2005. Internalization and infiltration. In: SSapers, G.M., Gorny. J.R., Yousef, A.E. (Eds.). Microbiology of fruits and vegetables. CRC Press/Taylor and Francis Group., 77-94.

Bárzana, G., Aroca, R., Bienert, G.P., Chaumont, F., Ruíz-Lozano, J.M., 2014. New insights into the regulation of aquaporins by the arbuscular mycorrhizal symbiosis in maize plants under drought stress and possible implications for plant performance. Mol. Plant-Microbe Interact. 27, 349-363.

Barzanti, R., Ozino, F., Bazzicalupo, M., Gabbrielli, R., Galardi, F., Gonnelli, C., Mengoni, A., 2007. Isolation and characterization of endophytic bacteria from the nickel hyperaccumulator plant *Alyssum bertolonii*. Microbial Ecology 53, 306-316.

Bashan, Y., de-Bashan, L.E., 2005. BACTERIA | Plant Growth-Promoting. In: Hillel, D. (Ed.), Encyclopedia of Soils in the Environment. Elsevier, Oxford, pp. 103-115.

Bashan, Y., de-Bashan, L.E., 2010. How the plant growth-promoting bacterium azospirillum promotes plant growth-a critical assessment. Advances in Agronomy, pp. 77-136.

Bashan, Y., Holguin, G., 1998. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: Biocontrol-PGPB (Plant Growth-Promoting Bacteria) and PGPB. Soil Biol. Biochem. 30, 1225-1228.

Beattie, G., 2006. Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. In: Gnanamanickam, S. (Ed.), Plant-Associated Bacteria. Springer Netherlands, pp. 1-56.

Bécard, G., Kosuta, S., Tamasloukht, M., Séjalon-Delmas, N., Roux, C., 2004. Partner communication in the arbuscular mycorrhizal interaction. Canadian Journal of Botany 82, 1186-1197.

Becard, G., Piche, Y., 1989. New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, Gigaspora margarita. New Phytologist 112, 77-83.

Belimov, A.A., Safronova, V.I., Mimura, T., 2002. Response of spring rape (*Brassica napus* var. *oleifera* L.) to inoculation with plant growth promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase depends on nutrient status of the plant. Canadian Journal of Microbiology 48, 189-199.

Benabdellah, K., Abbas, Y., Abourouh, M., Aroca, R., Azcón, R., 2011. Influence of two bacterial isolates from degraded and non-degraded soils and arbuscular mycorrhizae fungi isolated from semi-arid zone on the growth of Trifolium repens under drought conditions: Mechanisms related to bacterial effectiveness. European Journal of Soil Biology 47, 303-309.

Beneduzi, A., Peres, D., Vargas, L.K., Bodanese-Zanettini, M.H., Passaglia, L.M.P., 2008. Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil. Applied Soil Ecology 39, 311-320.

Berg, G., 2009. Plant-microbe interactions promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84, 11-18.

Berg, G., Smalla, K., 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiology Ecology 68, 1-13.

Bethlenfalvay, G.J., Linderman, R.G., 1992. Mycorrhize in sustainable agriculture. ASA special publication no 54. Madison. pp. 8-13.

Bethlenfalvay, G.J., Schüepp, H., 1994. Arbuscular mycorrhizas and agrosystem stability. In: Gianinazzi S, Schüepp H, editors. Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Basel: Birkhauser., 117-131.

Bhattacharyya, P.N., Jha, D.K., 2012. Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. World Journal of Microbiology and Biotechnology 28, 1327-1350.

Birhane, E., Sterck, F.J., Fetene, M., Bongers, F., Kuyper, T.W., 2012. Arbuscular mycorrhizal fungi enhance photosynthesis, water use efficiency, and growth of frankincense seedlings under pulsed water availability conditions. Oecologia 169, 895-904.

Bishop, P.E., Joerger, R.D., 1990. Genetics and molecular biology of alternative nitrogen fixation systems. Annual Review of Plant Physiology and Plant Molecular Biology 41, 109-125.

Bloemberg, G.V., Lugtenberg, B.J.J., 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Current Opinion in Plant Biology 4, 343-350.

Boiero, L., Perrig, D., Masciarelli, O., Penna, C., Cassán, F., Luna, V., 2007. Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Applied Microbiology and Biotechnology 74, 874-880.

Bonfante, P., Genre, A., 2010. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. Nat Commun 1, 48.

Borie, F., Rubio R., Morales, A., 2008. Arbuscular mycorrhizal fungi and soil aggregation. Journal Soil Science Plant Nutrient 8, 9-18.

Bottini, R., Cassán, F., Piccoli, P., 2004. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. Applied Microbiology and Biotechnology 65, 497-503.

Boukhalfa, H., Crumbliss, A.L., 2002. Chemical aspects of siderophore mediated iron transport. BioMetals 15, 325-339.

Bouwmeester, H.J., Matusova, R., Zhongkui, S., Beale, M.H., 2003. Secondary metabolite signalling in host-parasitic plant interactions. Current Opinion in Plant Biology 6, 358-364.

Braud, A., Jézéquel, K., Bazot, S., Lebeau, T., 2009. Enhanced phytoextraction of an agricultural Cr- and Pb-contaminated soil by bioaugmentation with siderophore-producing bacteria. Chemosphere 74, 280-286.

Buee, M., Rossignol, M., Jauneau, A., Ranjeva, R., Bécard, G., 2000. The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Molecular Plant-Microbe Interactions 13, 693-698.

Burd, G.I., Dixon, D.G., Glick, B.R., 2000. Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Canadian Journal of Microbiology 46, 237-245.

Burdman, S., Jurkevitch, E., Okon, Y., 2000. Recent advances in the use of plant promoting rhizobacteria (PGPR) in agriculture. In: Subba Rao NS,Dommergues YR, editors. Microbial interactions in agriculture and forestry. Enfield, NH: Science Publishers; p.229-50.

Caravaca, F., Alguacil, M.M., Azcón, R., Roldán, A., 2006. Formation of stable aggregates in rhizosphere soil of *Juniperus oxycedrus*: Effects of AM fungi and organic amendments. Appl. Soil Ecol. 33, 30-38.

Caravaca, F., Alguacil, M.M., Díaz, G., Marín, P., Roldán, A., 2005. Nutrient acquisition and nitrate reductase activity of mycorrhizal *Retama sphaerocarpa* L. seedlings afforested in an amended semiarid soil under two water regimes. Soil Use Manage 21, 10-16.

Carrillo-Castañeda, G., Muñoz, J.J., Peralta-Videa, J.R., Gomez, E., Gardea-Torresdey, J.L., 2003. Plant growth-promoting bacteria promote copper and iron translocation from root to shoot in alfalfa seedlings. Journal of Plant Nutrition 26, 1801-1814.

Castellanos, T., Dohrmann, A.B., Imfeld, G., Baumgarte, S., Tebbe, C.C., 2009. Search of environmental descriptors to explain the variability of the bacterial diversity from maize rhizospheres across a regional scale. European Journal of Soil Biology 45, 383-393.

Compant, S., 2007. Interaction between grapevine, *Vitis vinifera* L., and the endophytic bacterium *Burkholderia phytofirmans* strain PsJN: colonization, induced defense responses and systemic resistance towards *Botrytis cinerea*. URCA, Remis, 210 pp.

Compant, S., Clément, C., Sessitsch, A., 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry 42, 669-678.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E.A., 2005a. Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. Applied and Environmental Microbiology 71, 4951-4959.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., Barka, E.A., 2005b. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Applied and Environmental Microbiology 71, 1685-1693.

Contesto, C., Desbrosses, G., Lefoulon, C., Béna, G., Borel, F., Galland, M., Gamet, L., Varoquaux, F., Touraine, B., 2008. Effects of rhizobacterial ACC deaminase activity on Arabidopsis indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria. Plant Science 175, 178-189.

De Garcia Salamone, I.E., Hynes, R.K., Nelson, L.M., 2006. Role of cytokinins in plant growth promotion by rhizosphere bacteria. PGPR: Biocontrol and Biofertilization, pp. 173-195.

De La Peña, E., Echeverría, S.R., Van Der Putten, W.H., Freitas, H., Moens, M., 2006. Mechanism of control of root-feeding nematodes by mycorrhizal fungi in the dune grass *Ammophila arenaria*. New Phytologist 169, 829-840.

Dean, D.R., Jacobson, M.R., 1992. Biochemical genetics of nitrogenase. . In: Stacey, G., Burris, R.H., Evans, H.J. (Eds.), Biological Nitrogen Fixation. Chapman and Hall, New York, pp.763-834.

Dell'Amico, E., Cavalca, L., Andreoni, V., 2008. Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. Soil Biology and Biochemistry 40, 74-84.

Desbrosses, G., Contesto, C., Varoquaux, F., Galland, M., Touraine, B., 2009. PGPR-*Arabidopsis* interactions is a useful system to study signaling pathways involved in plant developmental control. Plant Signaling and Behavior 4, 321-322.

Dewey, F.M., Li Wong, Y., Seery, R., Hollins, T.W., Gurr, S.J., 1999. Bacteria associated with Stagonospora (Septoria) nodorum increase pathogenicity of the fungus. New Phytologist 144, 489-497.

Dimkpa, C., Weinand, T., Asch, F., 2009. Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant, Cell and Environment 32, 1682-1694.

Dong, Y.H., Gusti, A.R., Zhang, Q., Xu, J.L., Zhang, L.H., 2002. Identification of quorum-quenching N-acyl homoserine lactonases from Bacillus species. Applied and Environmental Microbiology 68, 1754-1759.

Estrada-Luna, A.A., Davies Jr, F.T., 2003. Arbuscular mycorrhizal fungi influence water relations, gas exchange, abscisic acid and growth of micropropagated chile ancho pepper (Capsicum annuum) plantlets during acclimatization and post-acclimatization. Journal of Plant Physiology 160, 1073-1083.

Evelin, H., Kapoor, R., Giri, B., 2009. Arbuscular mycorrhizal fungi in alleviation of salt stress: A review. Annals of Botany 104, 1263-1280.

Finlay, R.D., 2008. Ecological aspects of mycorrhizal symbiosis: with special emphasis on the functional diversity of interactions involving the extraradical mycelium. J. Exp. Bot. 59, 1115-1126.

Fuqua, W.C., Winans, S.C., Greenberg, E.P., 1994. Quorum sensing in bacteria: The LuxR-LuxI family of cell density- responsive transcriptional regulators. Journal of Bacteriology 176, 269-275.

Gadkar, V., Rillig, M.C., 2006. The arbuscular mycorrhizal fungal protein glomalin is a putative homolog of heat shock protein 60. FEMS Microbiology Letters 263, 93-101.

Galippe, V., 1887. Note sur la présence de micro-organismes dans les tissus végétaux. Comptes Rendus Hebdomadaires de la Société de Biologie, Paris., 410-416.

Gamalero, E., Berta, G., Glick, B.R., 2009. The use of microorganisms to facilitate the growth of plants in saline soils. In: Khan MS, Zaidi A, Musarrat J, editors. Microbial strategies for crop improvement. Dordrecht Heidelberg, London: Springer., 1-22.

Genre, A., Chabaud, M., Faccio, A., Barker, D.G., Bonfante, P., 2008. Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both Medicago truncatula and *Daucus carota*. Plant Cell 20, 1407-1420.

Giovannetti, M., Avio, L., Fortuna, P., Pellegrino, E., Sbrana, C., Strani, P., 2006. At the root of the wood wide web: Self recognition and non-self incompatibility in mycorrhizal networks. Plant Signaling and Behavior 1, 1-5.

Giovannetti, M., Ayio, L., Sbrana, C., Silvia Citernesi, A., 1993a. Factors affecting appressorium development in the vesicular–arbuscular mycorrhizal fungus Glomus mosseae (Nicol. & Gerd.) Gerd. & Trappe. New Phytologist 123, 115-122.

Giovannetti, M., Sbrana, C., 1998. Meeting a non-host: The behaviour of AM fungi. Mycorrhiza 8, 123-130.

Giovannetti, M., Sbrana, C., Avio, L., Citernesi, A.S., Logi, C., 1993b. Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytologist 125, 587-593.

Glick, B.R., 2012. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Scientifica 2012, 15.

Glick, B.R., Cheng, Z., Czarny, J., Duan, J., 2007. Promotion of plant growth by ACC deaminase-producing soil bacteria. European Journal of Plant Pathology 119, 329-339.

Glick, B.R., Patten, C.L., Holguin, G., Penrose, G.M., 1999. Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria

Imperial College Press, London.

Glick, B.R., Penrose, D.M., Li, J., 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. Journal of Theoretical Biology 190, 63-68.

Goicoechea, N., Antolín, M.C., Sánchez-Díaz, M., 1997. Gas exchange is related to the hormone balance in mycorrhizal or nitrogen-fixing alfalfa subjected to drought. Physiologia Plantarum 100, 989-997.

Goldstein, A.H., 1994. Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by gram-negative bacteria. . In: Torriani-Gorini A, Yagil E, Silver S (eds) Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, pp 197-203.

Gray, E.J., Smith, D.L., 2005. Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes. Soil Biology and Biochemistry 37, 395-412.

Gregory, P.J., 2006. Plant roots:Growth, Activity and Interaction with Soils. Black-wel Publishing, Oxford, 318 pp.

Groppa, M.D., Benavides, M.P., Zawoznik, M.S., 2012. Root hydraulic conductance, aquaporins and plant growth promoting microorganisms: A revision. Applied Soil Ecology 61, 247-254.

Guo, Y., Ni, Y., Huang, J., 2010. Effects of rhizobium, arbuscular mycorrhiza and lime on nodulation, growth and nutrient uptake of lucerne in acid purplish soil in China. Tropical Grasslands 44, 109-114.

Gutiérrez-Mañero, F.J., Ramos-Solano, B., Probanza, A., Mehouachi, J., Tadeo, F.R., Talon, M., 2001. The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiologia Plantarum 111, 206-211.

Haas, D., Défago, G., 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology 3, 307-319.

Habte, M., Manjunath, A., 1987. Soil solution phosphorus status and mycorrhizal dependency in *Leucaena leucocephala*. Applied and Environmental Microbiology 53, 797-801.

Hallmann, J., 2001. Plant interactions with endophyric bacteria. In: Jeger, M.J., Spence, N.J. (Eds.), Biotic Interactions in Plant-Pathogen Associations. CABI Publishing, Wallingford, United Kingdom., 87-119.

Hameeda, B., Harini, G., Rupela, O.P., Wani, S.P., Reddy, G., 2008. Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. Microbiological Research 163, 234-242.

Hardoim, P.R., van Overbeek, L.S., Elsas, J.D.v., 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends in Microbiology 16, 463-471.

Harrison, M.J., 1999. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annual Review of Plant Biology, pp. 361-389.

Harrison, M.J., 2005. Signaling in the arbuscular mycorrhizal symbiosis. Annual Review of Microbiology 59, 19-42.

Hayat, R., Ali, S., Amara, U., Khalid, R., Ahmed, I., 2010. Soil beneficial bacteria and their role in plant growth promotion: A review. Annals of Microbiology 60, 579-598.

Herrera-Medina, M.J., Steinkellner, S., Vierheilig, H., Ocampo Bote, J.A., Garcia Garrido, J.M., 2007. Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. New Phytol. 175, 554-564.

Hildebrandt, U., Janetta, K., Bothe, H., 2002. Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Applied and Environmental Microbiology 68, 1919-1924.

Hiltner L, 1904. Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung der Gründüngung und Brache. . Arb DLG 98:59-78.

Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W.W., Sessitsch, A., 2004. Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. Applied and Environmental Microbiology 70, 2667-2677.

Irigoyen, J.J., Einerich, D.W., Sánchez-Díaz, M., 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (Medicago sativd) plants. Physiologia Plantarum 84, 55-60.

Jäderlund, L., Arthurson, V., Granhall, U., Jansson, J.K., 2008. Specific interactions between arbuscular mycorrhizal fungi and plant growth-promoting bacteria: As revealed by different combinations. FEMS Microbiology Letters 287, 174-180.

James, E.K., Gyaneshwar, P., Mathan, N., Barraquio, W.L., Reddy, P.M., Iannetta, P.P.M., Olivares, F.L., Ladha, J.K., 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Molecular Plant-Microbe Interactions 15, 894-906.

Jasper, D.A., Abbott, L.K., Robson, A.D., 1991. The effect of soil disturbance on vesicular-arbuscular mycorrhizal fungi in soils from different vegetation types. New Phytologist 118, 471-476.

Javaid, A., 2009. Arbuscular mycorrhizal mediated nutrition in plants. Journal of Plant Nutrition 32, 1595-1618.

Javot, H., Maurel, C., 2002. The role of aquaporins in root water uptake. Ann. Bot. 90, 301-313.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., Barea, J.M., 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biology and Fertility of Soils 37, 1-16.

Jefwa, J., Vanlauwe, B., Coyne, D., Asten, P.V., Gaidashova, S., Rurangwa, E., Mwashasha, M., Elsen, A., 2010. Benefits and potential use of Arbuscular Mycorrhizal Fungi (AMF) in banana and plantain (Musa spp.) systems in Africa. Acta Horticulturae, pp. 479-486.

Jiang, F., Hartung, W., 2008. Long-distance signalling of abscisic acid (ABA): The factors regulating the intensity of the ABA signal. Journal of Experimental Botany 59, 37-43.

Joner, E.J., Jakobsen, I., 1995. Growth and extracellular phosphatase activity of arbuscular mycorrhizal hyphae as influenced by soil organic matter. Soil Biology and Biochemistry 27, 1153-1159.

Kaldenhoff, R., Bertl, A., Otto, B., Moshelion, M., Uehlein, N., 2007. Characterization of plant aquaporins. Methods Enzymol. 428, 505-531.

Kaymak, D.C., 2010. Potential of PGPR in agricultural innovations. In: Maheshwari DK, editr. Plant growth and health promoting bacteria. Berlin Heidelberg, Germany: Springer-Verlag.

Kearns, D.B., 2010. A field guide to bacterial swarming motility. Nat Rev Micro 8, 634-644.

Khan, M.S., Zaidi, A., 2006. Influence of composite inoculations of phosphate solubilizing organisms and an arbuscular mycorrhizal fungus on yield, grain protein and phosphorus and nitrogen uptake by greengram. Archives of Agronomy and Soil Science 52, 579-590.

Khan, M.S., Zaidi, A., Wani, P.A., Oves, M., 2009. Role of plant growth promoting rhizobacteria in the remediation of metal contamined soils. Environmental Chemical 7, 1-19.

Kim, J., Rees, D.C., 1994. Nitrogenase and biological nitrogen fixation. Biochemistry 33, 389-397.

Kloepper, J.W., 1993. Plant growth-promoting rhizobacteria as biological control agents. Soil microbial ecology, 255-274.

Kloepper, J.W., Rodriguez-Kabana, R., Zehnder, G.W., Murphy, J.F., Sikora, E., Fernandez, C., 1999. Plant root-bacterial interactions in biological control of soilborne diseases and potential extension to systemic and foliar diseases. Australas Plant Pathol 28, 21-26.

Koske, R.E., 1981. Multiple germination by spores of Gigaspora gigantea. Transactions of the British Mycological Society 76, 328-330.

Kosuta, S., Chabaud, M., Lougnon, G., Gough, C., Denarie, J., Barker, D.G., Becard, G., 2003. A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific MtENOD11 expression in roots of Medicago truncatula. Plant Physiology 131, 952-962.

Kosuta, S., Hazledine, S., Sun, J., Miwa, H., Morris, R.J., Downie, J.A., Oldroyd, G.E.D., 2008. Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. Proceedings of the National Academy of Sciences of the United States of America 105, 9823-9828.

Kruse, E., Uehlein, N., Kaldenhoff, R., 2006. The aquaporins. Genome Biol. 7.

Ladha, J.K., De Bruijn, F.J., Malik, K.A., 1997. Introduction: Assessing opportunities for nitrogen fixation in rice - A frontier project. Plant and Soil 194, 1-10.

Lian, H.L., Yu, X., Ye, Q., Ding, X., Kitagawa, Y., Kwak, S.S., Su, W.A., Tang, Z.C., 2004. Erratum: The role of aquaporin RWC3 in drought avoidance in rice. Plant Cell Physiol 45, 481-489.

Linderman, R.G., 1992. Vesicular-arbuscular mycorrhizae and soil microbial interactions. In: Bethlenfalvay GJ, Linderman RG, editors. Mycorrhizae in sustainable agriculture. Madison, Wis: ASA., 1-26.

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S., Mezgeay, M., Van der Lelie, D., 2002. Endophytic bacteria and their potential applications. Critical Reviews in Plant Sciences 21, 583-606.

López-Baena, F.J., Monreal, J.A., Pérez-Montaño, F., Guasch-Vidal, B., Bellogín, R.A., Vinardell, J.M., Ollero, F.J., 2009. The absence of nops secretion in sinorhizobium fredii HH103 increases GmPR1 expression in williams soybean. Molecular Plant-Microbe Interactions 22, 1445-1454.

López, B.R., Bashan, Y., Trejo, A., de-Bashan, L.E., 2013. Amendment of degraded desert soil with wastewater debris containing immobilized *Chlorella sorokiniana* and *Azospirillum brasilense* significantly modifies soil bacterial community structure, diversity, and richness. Biol. Fert. Soils 49, 1053-1063.

Ludwig-Müller, J. (Ed), 2000. Hormonal balance in plants during colonization by mycorrhizal fungi. In: Kapulnik, Y., Douds, D.D. (Eds.), Arbuscular Mycorrhizas: Physiology and Function. Kluer Academic Publisher, The Netherlands, pp. 263-285.

Machuca, A., Pereira, G., Aguiar, A., Milagres, A.M.F., 2007. Metal-chelating compounds produced by ectomycorrhizal fungi collected from pine plantations. Letters in Applied Microbiology 44, 7-12.

Mantelin, S., Touraine, B., 2004. Plant growth-promoting bacteria and nitrate availability: Impacts on root development and nitrate uptake. Journal of Experimental Botany 55, 27-34.

Marschner, H., Dell, B., 1994. Nutrient uptake in mycorrhizal symbiosis. Plant and Soil 159, 89-102.

Marulanda, A., Barea, J.M., Azcón, R., 2006. An Indigenous Drought-Tolerant Strain of Glomus intraradices Associated with a Native Bacterium Improves Water Transport and Root Development in Retama sphaerocarpa. Microbial Ecology 52, 670-678.

Marulanda, A., Barea, J.M., Azcón, R., 2009. Stimulation of plant growth and drought tolerance by native microorganisms (AM Fungi and bacteria) from dry environments: Mechanisms related to bacterial effectiveness. Journal of Plant Growth Regulation 28, 115-124.

Mastretta, C., Taghavi, S., van der Lelie, D., Mengoni, A., Galardi, F., Gonnelli, C., Barac, T., Boulet, J., Weyens, N., Vangronsveld, J., 2009. Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. International Journal of Phytoremediation 11, 251-267.

Maurel, C., Plassard, C., 2011. Aquaporins: for more than water at the plant-fungus interface? New Phytol. 190, 815-817.

Maurel, C., Verdoucq, L., Luu, D.-T., Santoni, V., 2008. Plant aquaporins: Membrane channels with multiple integrated functions. Annu. Rev. Plant Biol. 59, 595-624.

McKenzie, R.H., Roberts, T.L., 1990. Soil and fertilizers phosphorus update. In: Proceedings of Alberta Soil Science Workshop Proceedings, Feb. 20-22, Edmonton, Alberta, pp. 84-104.

Medina, A., Azcón, R., 2010. Effectiveness of the application of arbuscular mycorrhiza fungi and organic amendments to improve soil quality and plant performance under stress conditions. J. Soil Sci. Plant Nut. 10, 354-372.

Medina, A., Vassilev, N., Alguacil, M.M., Roldán, A., Azcón, R., 2004a. Increased plant growth, nutrient uptake, and soil enzymatic activities in a desertified mediterranean soil amended with treated residues and inoculated with native mycorrhizal fungi and a plant growth-promoting yeast. Soil Science 169, 260-270.

Medina, A., Vassilev, N., Barea, J.M., Azcón, R., 2005. Application of *Aspergillus niger*-treated agrowaste residue and *Glomus mosseae* for improving growth and nutrition of *Trifolium repens* in a Cd-contaminated soil. J. Biotechnol. 116, 369-378.

Medina, A., Vassileva, M., Caravaca, F., Roldán, A., Azcón, R., 2004b. Improvement of soil characteristics and growth of *Dorycnium pentaphyllum* by amendment with agrowastes and inoculation with AM fungi and/or the yeast *Yarowia lipolytica*. Chemosphere 56, 449-456.

Mehnaz, S., Lazarovits, G., 2006. Inoculation effects of Pseudomonas putida, Gluconacetobacter azotocaptans, and Azospirillum lipoferum on corn plant growth under greenhouse conditions. Microbial Ecology 51, 326-335.

Michelson, A., Rosendahl, S., 1990. The effect of VA mycorrhizal fungi, phosphorus and drought stress on the growth of *Acacia nilotica* and *Leucaena leucocephala* seedlings. Plant and Soil 124, 7-13.

Miethke, M., Marahiel, M.A., 2007. Siderophore-based iron acquisition and pathogen control. Microbiology and Molecular Biology Reviews 71, 413-451.

Miransari, M., 2010. Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. Plant Biology 12, 563-569.

Moore, D., Robson, G.D., Trinci A.P.J, 2011. 21st Century Guidebook to Fungi. Cambridge University Press: ISBN: 9780521186957.

Mosse, B., 1959. The regular germination of resting spores and some observations on the growth requirements of an Endogone sp. causing vesicular-arbuscular mycorrhiza. Transactions of the British Mycological Society 42, 273-286.

Murray, J.D., 2011. Invasion by invitation: rhizobial infection in legumes. Molecular Plant Microbe Interact 24, 631-639.

Nadeem, S.M., Ahmad, M., Zahir, Z.A., Javaid, A., Ashraf, M., 2014. The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. Biotechnology Advances 32, 429-448.

Nadeem, S.M., Zahir, Z.A., Naveed, M., Arshad, M., 2007. Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Canadian Journal of Microbiology 53, 1141-1149.

Nielsen, K.B., Kjøller, R., Olsson, P.A., Schweiger, P.F., Andersen, F.Ø., Rosendahl, S., 2004. Colonisation and molecular diversity of arbuscular mycorrhizal fungi in the aquatic plants *Littorella uniflora* and *Lobelia dortmanna* in southern Sweden. Mycological Research 108, 616-625.

O'Sullivan, D.J., O'Gara, F., 1992. Traits of fluorescent Pseudomonas spp. involved in suppression of plant root pathogens. Microbiological Reviews 56, 662-676.

Ortiz, N., Armada, E., Duque, E., Roldán, A., Azcón, R., 2015. Contribution of arbuscular mycorrhizal fungi and/or bacteria to enhancing plant drought tolerance under natural soil conditions: Effectiveness of autochthonous or allochthonous strains. Journal of Plant Physiology 174, 87-96.

Oueslati, O., 2003. Allelopathy in two durum wheat (Triticum durum L.) varieties. Agriculture, Ecosystems and Environment 96, 161-163.

Ouziad, F., Wilde, P., Schmelzer, E., Hildebrandt, U., Bothe, H., 2006. Analysis of expression of aquaporins and Na+/H+ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. Environmental and Experimental Botany 57, 177-186.

Parniske, M., 2004. Molecular genetics of the arbuscular mycorrhizal symbiosis. Curr. Opin. Plant Biol. 7, 414-421.

Parniske, M., 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Micro 6, 763-775.

Patten, C.L., Glick, B.R., 1996. Bacterial biosynthesis of indole-3-acetic acid. Canadian Journal of Microbiology 42, 207-220.

Penrose, D.M., Glick, B.R., 2001. Levels of ACC and related compounds in exudate and extracts of canola seeds treated with ACC deaminase-containing plant growth-promoting bacteria. Canadian Journal of Microbiology 47, 368-372.

Pérez-Montaño, F., Alías-Villegas, C., Bellogín, R.A., Del Cerro, P., Espuny, M.R., Jiménez-Guerrero, I., López-Baena, F.J., Ollero, F.J., Cubo, T., 2014. Plant growth promotion in cereal and leguminous agricultural important plants: From microorganism capacities to crop production. Microbiological Research 169, 325-336.

Pérez-Montaño, F., Jiménez-Guerrero, I., Contreras Sánchez-Matamoros, R., López-Baena, F.J., Ollero, F.J., Rodríguez-Carvajal, M.A., Bellogín, R.A., Espuny, M.R., 2013. Rice and bean AHL-mimic quorum-sensing signals specifically interfere with the capacity to form biofilms by plant-associated bacteria. Research in Microbiology 164, 749-760.

Piccoli, P., Lucangeli, C.D., Schneider, G., Bottini, R., 1997. Hydrolysis of [17,17-2H2]gibberellin A20-glucoside and [17,17-2H2]gibberellin A20-glucosyl ester by *Azospirillum lipoferum* cultured in a nitrogen-free biotin-based chemically-defined medium. Plant Growth Regulation 23, 179-182.

Ping, L., Boland, W., 2004. Signals from the underground: bacterial volatiles promote growth in *Arabidopsis*. Trends in Plant Science 9, 263-266.

Pivato, B., Offre, P., Marchelli, S., Barbonaglia, B., Mougel, C., Lemanceau, P., Berta, G., 2009. Bacterial effects on arbuscular mycorrhizal fungi and mycorrhiza development as influenced by the bacteria, fungi, and host plant. Mycorrhiza 19, 81-90.

Porcel, R., Ruiz-Lozano, J.M., 2004. Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. Journal of Experimental Botany 55, 1743-1750.

Quiñones, B., Dulla, G., Lindow, S.E., 2005. Quorum sensing regulates exopolysaccharide production, motility, and virulence in Pseudomonas syringae. Molecular Plant-Microbe Interactions 18, 682-693.

Raaijmakers, J.M., Vlami, M., de Souza, J.T., 2002. Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 81, 537-547.

Rajkumar, M., Ae, N., Prasad, M.N.V., Freitas, H., 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends in Biotechnology 28, 142-149.

Ramos Solano, B., Barriuso Maicas, J., Pereyra De La Iglesia, M.T., Domenech, J., Gutiérrez Mañero, F.J., 2008. Systemic disease protection elicited by plant growth promoting rhizobacteria strains: Relationship between metabolic responses, systemic disease protection, and biotic elicitors. Phytopathology 98, 451-457.

Reinhold-Hurek, B., Hurek, T., 1998. Life in grasses: Diazotrophic endophytes. Trends in Microbiology 6, 139-144.

Richardson, A.E., Barea, J.M., McNeill, A.M., Prigent-Combaret, C., 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant and Soil 321, 305-339.

Rillig, M.C., Maestre, F.T., Lamit, L.J., 2003. Microsite differences in fungal hyphal length, glomalin, and soil aggregate stability in semiarid Mediterranean steppes. Soil Biology and Biochemistry 35, 1257-1260.

Rillig, M.C., Mardatin, N.F., Leifheit, E.F., Antunes, P.M., 2010. Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates. Soil Biology and Biochemistry 42, 1189-1191.

Rillig, M.C., Mummey, D.L., 2006. Mycorrhizas and soil structure. New Phytologist 171, 41-53.

Ruiz-Lozano, J.M., 2003. Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhiza 13, 309-317.

Ruiz-Lozano, J.M., Aroca, R., 2010. Host response to osmotic stresses: stomatal behaviour and water use efficiency of arbucular mcorrhizal plants. In: Koltai H, Kapulnik Y, editors. Arbuscular mycorrhizas: physiology and function. Netherlands: Springer., 239-256.

Ruiz-Lozano, J.M., Azcón, R., 2000. Symbiotic efficiency and infectivity of an autochthonous arbuscular mycorrhizal Glomus sp. from saline soils and Glomus deserticola under salinity. Mycorrhiza 10, 137-143.

Ruiz-Lozano, J.M., Azcon, R., Gomez, M., 1995. Effects of arbuscular-mycorrhizal Glomus species on drought tolerance: Physiological and nutritional plant responses. Applied and Environmental Microbiology 61, 456-460.

Ruiz-Lozano, J.M., Azcon, R., Gomez, M., 1996. Alleviation of salt stress by arbuscular-mycorrhizal Glomus species in Lactuca sativa plants. Physiologia Plantarum 98, 767-772.

Ruiz-Lozano, J.M., Porcel, R., Aroca, R., 2006. Does the enhanced tolerance of arbuscular mycorrhizal plants to water deficit involve modulation of drought-induced plant genes? [2]. New Phytologist 171, 693-698.

Ryan, R.P., Germaine, K., Franks, A., Ryan, D.J., Dowling, D.N., 2008. Bacterial endophytes: recent developments and applications. FEMS MIcrobiology Letters 278, 1-9.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Kloepper, J.W., Paré, P.W., 2003. Bacterial volatiles promote growth in *Arabidopsis*. Plant Physiology 134, 1017-1026.

Schliemann, W., Ammer, C., Strack, D., 2008. Metabolite profiling of mycorrhizal roots of *Medicago truncatula*. Phytochemistry 69, 112-146.

Schmidt, W., 1999. Mechanisms and regulation of reduction-based iron uptake in plants. New Phytology 141, 1-26.

Schreiner, R.P., 2007. Effects of native and nonnative arbuscular mycorrhizal fungi on growth and nutrient uptake of 'Pinot Noir' (*Vitis vinifera* L.) in two soils with contrasting levels of phosphorus.. . Applied Soil Ecology 36, 205-215.

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., Hutzler, P., Schmid, M., Van Breusegem, F., Eberl, L., Hartmann, A., Langebartels, C., 2006. Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. Plant, Cell and Environment 29, 909-918.

Schulz, B., Boyle, C., 2006. What are endophytes? In: Schulz BJE, Boyle CIC, Sieber TN, editors. Microbial Root Endophytes. Berlin: Springer-Verlag., 1-3.

Schüßler, A., Walker, C., 2010. The *Glomeromycota*: a species list with new families and genera. . Edinburgh & Kew, UK: The Royal Botanic Garden; Munich, Germany: Botanische Staatssammlung Munich; Oregon, USA: Oregon State University.

Schüßler, A., Schwarzott, D., Walker, C., 2001. A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycological Research 105, 1413-1421.

Selosse, M.A., Le Tacon, F., 1998. The land flora: a phototroph-fungus partnership? Trends in Ecology & Evolution 13, 15-20.

Shaharoona, B., Arshad, M., Khalid, A., 2007a. Differential response of etiolated pea seedlings to inoculation with rhizobacteria capable of utilizing 1-aminocyclopropane-1-carboxylate or L-methionine. Journal of Microbiology 45, 15-20.

Shaharoona, B., Jamro, G.M., Zahir, Z.A., Arshad, M., Memon, K.S., 2007b. Effectiveness of various *Pseudomonas* spp. and *Burkholderia caryophylli* containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.). Journal of Microbiology and Biotechnology 17, 1300-1307.

Shokri, S., Maadi, B., 2009. Effects of arbuscular mycorrhizal fungus on the mineral nutrition and yield of Trifolium alexandrinum plants under salinity stress. Journal of Agronomy 8, 79-83.

Singh, R., 2012. Role of glomalin related soil protein produced by arbuscular mycorrhizal fungi: a review. Agriculture Science Research Journal 2, 119-125.

Spaepen, S., Vanderleyden, J., 2011. Auxin and plant-microbe interactions. Cold Spring Harbor perspectives in biology 3.

Spaepen, S., Vanderleyden, J., Remans, R., 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiology Reviews 31, 425-448.

Strigul, N.S., Kravchenko, L.V., 2006. Mathematical modeling of PGPR inoculation into the rhizosphere. Environmental Modelling and Software 21, 1158-1171.

Subramanian, K.S., Charest, C., Dwyer, L.M., Hamilton, R.I., 1997. Effects of arbuscular mycorrhizae on leaf water potential, sugar content, and P content during drought and recovery of maize. Canadian Journal of Botany 75, 1582-1591.

Sudhakar, P., Chattopadhyay, G.N., Gangwar, S.K., Ghosh, J.K., 2000. Effect of foliar application of Azotobacter, Azospirillum and Beijerinckia on leaf yield and quality of mulberry (Morus alba). Journal of Agricultural Science 134, 227-234.

Swarnalakshmi, K., Prasanna, R., Kumar, A., Pattnaik, S., Chakravarty, K., Shivay, Y.S., Singh, R., Saxena, A.K., 2013. Evaluating the influence of novel cyanobacterial biofilmed biofertilizers on soil fertility and plant nutrition in wheat. European Journal of Soil Biology 55, 107-116.

Tao, G.C., Tian, S.J., Cai, M.Y., Xie, G.H., 2008. Phosphate-Solubilizing and -Mineralizing Abilities of Bacteria Isolated from Soils1 1 Project supported by the Scientific Research

Foundation for the Returned Overseas Chinese Scholars, the Ministry of Education of the P.R. China. Pedosphere 18, 515-523.

Trejo, A., de-Bashan, L.E., Hartmann, A., Hernandez, J.-P., Rothballer, M., Schmid, M., Bashan, Y., 2012. Recycling waste debris of immobilized microalgae and plant growth-promoting bacteria from wastewater treatment as a resource to improve fertility of eroded desert soil. Environ. Exp. Bot. 75, 65-73.

Uehlein, N., Fileschi, K., Eckert, M., Bienert, G.P., Bertl, A., Kaldenhoff, R., 2007. Arbuscular mycorrhizal symbiosis and plant aquaporin expression. Phytochemistry 68, 122-129.

Van Der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 69-72.

Van Loon, L.C., 2007. Plant responses to plant growth-promoting rhizobacteria. European Journal of Plant Pathology 119, 243-254.

Vassilev, N., Medina, A., Azcon, R., Vassileva, M., 2006. Microbial solubilization of rock phosphate on media containing agro-industrial wastes and effect of the resulting products on plant growth and P uptake. Plant and Soil 287, 77-84.

Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255, 571-586.

Vigo, C., Norman, J.R., Hooker, J.E., 2000. Biocontrol of the pathogen *Phytophthora parasitica* by arbuscular mycorrhizal fungi is a consequence of effects on infection loci. Plant Pathology 49, 509-514.

Vivas, A., Marulanda, A., Gómez, M., M Barea, J., Azcón, R., 2003. Physiological characteristics (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by Bacillus thuringiensis inoculation under two phosphorus levels. Soil Biology and Biochemistry 35, 987-996.

Walker, T.S., Bais, H.P., Grotewold, E., Vivanco, J.M., 2003. Root exudation and rhizosphere biology. Plant Physiology 132, 44-51.

Wang, L., Wu, J., Ma, F., Yang, J., Li, S., Li, Z., Zhang, X., 2015. Response of Arbuscular Mycorrhizal Fungi to Hydrologic Gradients in the Rhizosphere of *Phragmites australis* (Cav.) Trin ex. Steudel Growing in the Sun Island Wetland. BioMed Research International.

Whipps, J.M., 2001. Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany 52, 487-511.

Wu, Q.S., Li, G.H., Zou, Y.N., 2011. Roles of arbuscular mycorrhizal fungi on growth and nutrient acquisition of peach (*Prunus persica* l. Batsch) seedlings. . Journal Animal Plant Science 21, 746-750.

Wu, Q.S., Xia, R.X., 2006. Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions. Journal of Plant Physiology 163, 417-425.

Yang, J., Kloepper, J.W., Ryu, C.-M., 2009. Rhizosphere bacteria help plants tolerate abiotic stress. Trends in Plant Science 14, 1-4.

Yusran, Y., Volker, R., Torsten, M., 2009. Effects of plant growth-promoting rhizobacteria and *Rhizobium* on mycorrhizal development and growth of *Paraserianthes falcataria* (L.) nielse seedlings in two types of soils with contrasting levels of pH. Proceedings of the international plant nutrition colloquium XVI. UC Davis: Department of Plant Sciences.

Zahir, Z.A., Arshad, M., Azam, M., Hussain, A., 1997. Effect of an auxin precursor tryptophan and Azotobacter inoculation on yield and chemical composition of potato under fertilized conditions. Journal of Plant Nutrition 20, 745-752.

Zahir, Z.A., Ghani, U., Naveed, M., Nadeem, S.M., Asghar, H.N., 2009. Comparative effectiveness of *Pseudomonas* and *Serratia* sp. containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. Archives of Microbiology 191, 415-424.

Zahir, Z.A., Munir, A., Asghar, H.N., Shaharoona, B., Arshad, M., 2008. Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (*Pisum sativum*) under drought conditions. Journal of Microbiology and Biotechnology 18, 958-963.

Zaidi, A., Khan, M.S., Ahemad, M., Oves, M., 2009. Plant growth promotion by phosphate solubilizing bacteria. Acta Microbiologica et Immunologica Hungarica 56, 263-284.

OBJETIVOS

El objetivo principal de la tesis doctoral es el conocimiento del funcionamiento de los microorganismos autóctonos (bacterias y hongos formadores de micorrizas arbusculares), que proporcionan un gran beneficio sobre el desarrollo vegetal en dichas zonas desertificadas.

Para lograr dicho objetivo, se realizaron los siguientes objetivos específicos que se presentan en diferentes capítulos que conforman este trabajo de investigación.

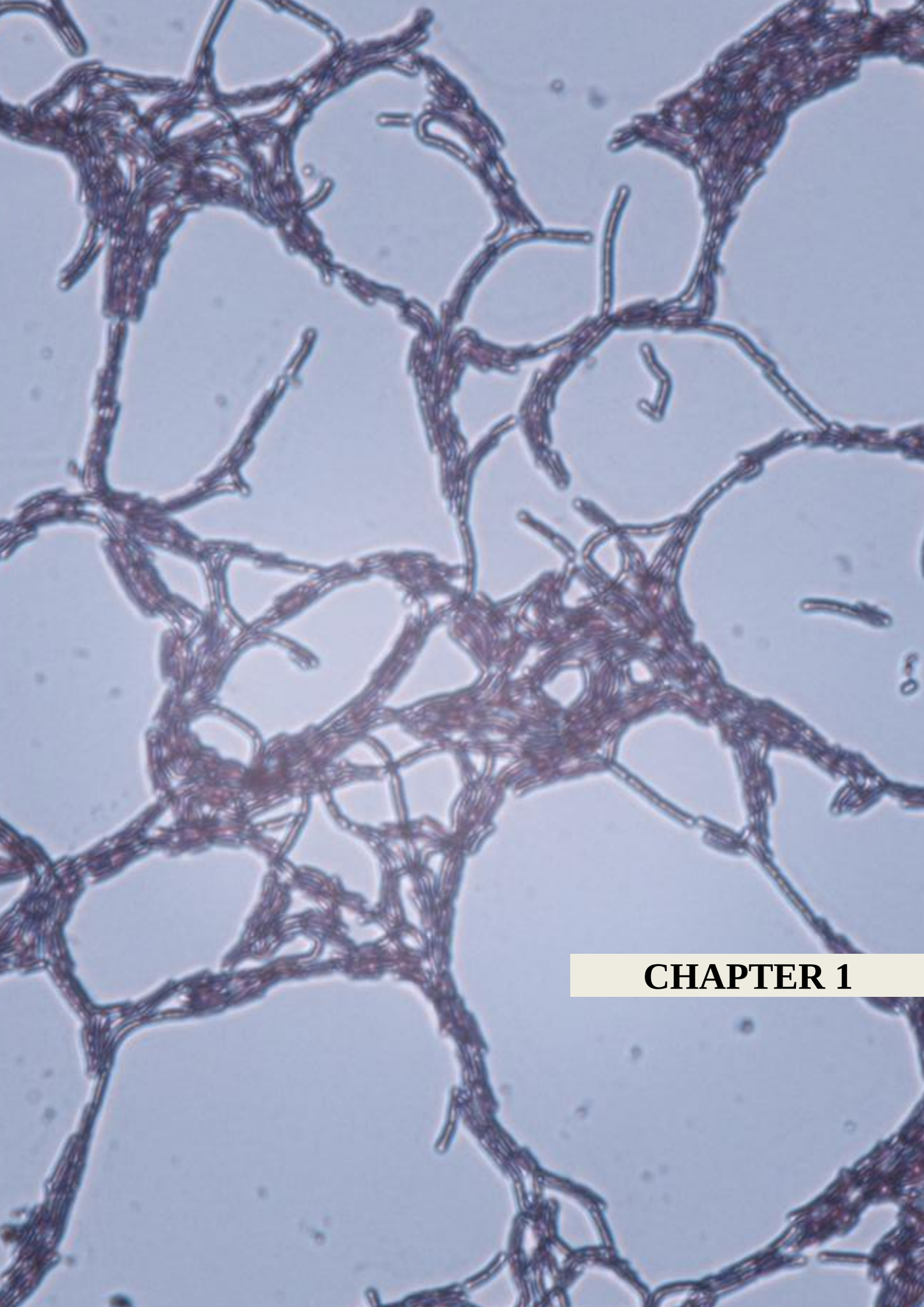
- Desarrollar tecnologías que faciliten la recuperación de la cubierta vegetal en zonas semiáridas mediante la selección de consorcios de microorganismos promotores del crecimiento que mejoren la nutrición y la eficiencia en el uso del agua en condiciones de estrés hídrico severo y prolongado.

- Determinar el carácter generalista o específico de la actividad PGPR de los inóculos seleccionados, así como las posibles sinergias derivadas de la interacción de diversos microorganismos beneficiosos.

- Determinación de los cambios en la biodiversidad microbiana en suelos rizosféricos, correspondientes a las diferentes especies vegetales y tras su inoculación microbiana.

- Efecto comparativo entre los microorganismos beneficiosos con los fertilizantes ante la tolerancia al estrés hídrico en planta.

- Validar los beneficios del uso de microorganismos autóctonos de un área Mediterránea degradada, en plantas de importancia agronómica como el maíz.



CHAPTER 1

Isolation and characterization of plant growth-promoting bacteria (PGPR) of semiarid areas of the Southeast peninsular of Spain

Armada E., López-Castillo O. M., Azcón R

1. Introduction

Drought stress is one of the most important abiotic factors limiting plant growth and performance in large areas of the Southeast of Spain. Limitation of water causes a series of detrimental changes in plant nutrition and plant physiology. Rhizosphere soil is influenced by plant roots which select for specifically adapted microbial communities (Appuhn and Joergensen, 2006; Bais et al., 2006). Several studies have demonstrated that the bacterial diversity in rhizospheres can be influenced by a number of different factors, i.e., the plant species, varietal differences within a species, plant age, plant genotype, agricultural management, or soil properties (Marschner et al., 2001; Costa et al., 2006; Beneduzi et al., 2008; Castellanos et al., 2009). Specific soil microbial communities play a key role in the survival and growth of plants by improving the uptake of nutrients, water and the quality of the soil.

Plants can interact with several soil microorganisms, including plant growth-promoting rhizobacteria (PGPR) that make the plant more tolerant to stress factors (Barea et al., 2002; Vessey, 2003; Barea et al., 2005). PGPR are free-living microorganisms that exert beneficial effects on plants by colonizing their rhizospheres or phyllospheres (Bashan and de-Bashan, 2005). These bacteria stimulate plant growth through mobilizing nutrients in soils, solubilize minerals such as phosphorus, making them more readily available for plant growth (Glick, 1995; 2007; Bashan and de-Bashan, 2010) by producing numerous plant growth regulators, such as auxins, cytokinins and gibberellins which can act to enhance or regulate various stages of plant growth by protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (Hayak et al., 2010; Rajkumar et al., 2010; Ahemad and Malik, 2011; Ahemad, 2012). To fix atmospheric nitrogen and to supply it to plants, although play a very important role in symbiotic bacteria legume associations, this is usually a minor the benefit in the non-symbiotic bacteria. The synthesis of siderophores which can sequester iron from the soil providing to plant cells siderophore-iron complex play an interesting effect in biocontrol. Bacteria may directly affect plant growth and development by using anyone or more than one of these mechanisms. These diverse mechanisms involved in the

PGPR activity are often specific and may affect the life cycle of plants. Plant-associated N_2-fixing and P-solubilizing bacteria are regarded as a possible alternative for inorganic nitrogen and phosphorus fertilizers (Azcón et al., 2013). Thus, PGPR strains have previously been attracted the attention of agriculturists as soil inocula to improve the plant growth and yield (Park et al., 2005; Çakmakçi et al., 2006; Hariprasad et al., 2009).

The study site location here selected was located in the southeast of Spain and it corresponds to Mediterranean climate. It is characterized by a very dry summer period and winters with low and erratic rainfall that it causes intense effect on soil degradation processes. This type of abiotic factors affects the plant water relation at cellular physiological and biochemical levels. Thus, in whole plant originates a series of adverse reactions and stresses that are unsuitable for plants growth. To counteract, the inoculation of plants with native beneficial microorganisms may increase drought tolerance of plants growing in arid or semiarid areas (Marulanda et al., 2007). Plants interact with fungi and bacteria contributing to their fitness (Azcón et al., 2013). Bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they manipulation may influence the plants performance to a greater extent, but it is important to consider their competitiveness in root colonization and survival abilities particularly under drought conditions (Barriuso et al., 2008).

The aim of this study was the isolation and characterization of autochthonous drought-adapted microorganisms from semiarid environments and their evaluation based on the growth promoting abilities in host plants under drought conditions. The finality of this study was to get the most appropriate and effective inocula to be used in revegetation programs of semiarid and degraded areas. To reach this objective, we selected and autochthonous bacteria tested the PGPR mechanisms and drought tolerance *in vitro* conditions through to creating increasing levels of osmotic stress conditions using by application of polyethylene glycol (PEG) in the culture medium. We also confirm the ability to enhance plant growth promotion, nutrition, physiological and biochemical values and drought tolerance in *Lactuca sativa* (a cultivable agronomic plant) to test the effectiveness of such autochthonous bacteria in colonized plants under drought conditions.

2. Materials and methods

Two independent experiments were carried out in the present study. First, autochthonous bacteria (five bacteria strains) isolated from the semiarid experimental soil of the southeast area of Spain (province Murcia), was identified using morphological and molecular methods, and subsequently an *in vitro* assays, we determine changes on maintenance of growth of the bacterial cells in axenic culture medium under non stress and drought stress conditions [by the

application of 40% polyethylene glycol (PEG)]. Their PGPR abilities such as solubilizing phosphate, nitrogen fixing, indole acetic acid (IAA) production and α-ketobutyrate (ACC deaminase) synthesis were also verified. Secondly, the bacterial drought tolerance was evaluated analyzing production of proline, antioxidant enzymatic activities and poly-β-hydroxybutyrate (PHB) production under non-stress and osmotic stress conditions.

The bacterial selection included subsequently, microcosm (pot) experiment using semiarid soil and drought conditions which analyzed the effectiveness of inoculation of five selected autochthonous bacteria species in improving plant growth, physiology, nutrition and antioxidant activities as indexes of plant drought tolerance.

2.1. Bacterial isolation and molecular identification

The autochthonous bacteria, assayed in the present study, were isolated from the semiarid experimental soil of the southeast area of Spain (Armada et al., 2014 a,b) The bacteria were isolated from the rhizosphere soils from several autochthonous shrub species. A homogenate of 1 g soil in 9 mL sterile water was diluted (10^{-2} to 10^{-4}), plated on three different growing media [Yeast Mannitol Agar, Potato Dextrose Agar, Luria-Bertani (LB) Agar] and then incubated at 28 ºC for 48 h, to isolate bacteria. The selected bacteria were the most abundant bacterial type in such arid soil.

The identification of the selected bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were collected, diluted, lysed, and genomic DNA extracted. The DNAs were used as a template in the PCR reactions. All reactions were conducted in 25 µL volume containing PCR buffer 10X, 50 mM $MgCl_2$, 10 µL each primers: 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), 5 U/µl of *Taq* polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with the following conditions: 5 min at 95 ºC, followed by 30 cycles of 45s at 95 ºC, 45s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. The products of PCR were analyzed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (NCBI) using BLAST program. (Identification of one bacterial strain through analysis of proteins “MALDI biotyper” (Bruker Daltonik).

2.2. Bacterial PGPR characteristics

Nitrogen fixation, each strain isolated was inoculated in plate containing nitrogen-free base (NFb) solid media to isolate free-living diazotrophic bacteria (Döbereiner and Day, 1976), and incubated 10 days at 28 °C. A strain of *Azospirillum brasilensis* was used as control.

Siderophores production, 1 µL of pure bacterial culture grown in Luria-Bertani (LB) broth were inoculated in plates containing agar Chrome Azurol Sulphonate (CAS) and incubated at 30 °C. Each plate was observed daily for 7 days to detect the appearance of change of CAS-iron complex (from blue to orange) after the iron chelation by siderophores (Schwyn and Neilands, 1987). Experiments were performed in triplicate.

2.3. Evaluation in axenic culture under non stress and stress (40% of PEG) conditions the bacterial growth, PGPR characteristics and stress tolerance abilities

Bacterial growth

Autochthonous bacterial isolates were grown at 28 ºC in nutrient broth (8 g L^{-1}) supplemented with PEG (40%) to generate osmotic stress (equivalent to -3.99 MPa). Number of viable cells was estimated after 4 and 6 days of growth following the conventional procedure: 1 mL of suspension was plated in nutrient broth medium. The bacterial growth was monitored by measuring optical density at 600 nm (Fig. 1).

Bacterial PGPR charactheristics [phosphate solubilization, indole acetic acid (IAA) and α -ketobutyrate (ACC deaminase) production]

The bacterial isolates were cultivated at 28 ºC at 120 rpm in 100 mL of liquid nutrient medium supplemented or not with 40% of PEG in order to induce drought stress.

To determine phosphate solubilization index (PSI), each bacterial culture was assayed on Pikovskaya agar plates (Pikovskaya, 1948) containing tricalcium phosphate ($Ca_3(PO_4)_2$) as insoluble phosphate source. Cells were grown overnight in LB medium, next they were washed twice with 0.9% NaCl and re-suspended in 0.9% NaCl to produce equal cell densities among all the isolates. Solutions were inoculated on the agar plates and incubated at 30 °C, and observed daily for 7 days for appearance of transparent “halos” (Katznelson and Bose, 1959). Experiments were performed in triplicate. Phosphorus solubilization index was measured using following formula (Edi-Premono et al., 1996).

$$PSI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

The production of indole-3- acetic acid (IAA) by these bacteria was determined using the Salper’s reagent (Gordon and Paleg, 1957). Three milliliters of fresh Salper’s reagent (1 mL 0.5 M $FeCl_3$ in 50 mL 37% $HClO_4$) was added to free-cell supernatant and kept in complete darkness for 30 minutes at ambient temperature, and the optical density at 535 nm was

measured in each treatment (Wöhler, 1997). A standard curve was also prepared for IAA determination.

The activity of ACC deaminase enzyme in isolates was measured as described by Penrose and Glick (2003). The enzyme activity was assayed according to a modification of the method of Honma and Shimomura (1978) which measures the amount of α -ketobutyrate produced when the enzyme ACC deaminase hydrolyses ACC. The quantity of µmol of α-ketobutyrate produced by this reaction was determined and comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 1.0 mmol and 1.0 µmol. Protein concentration of cellular suspension in the toluenized cells was determined by the method of Bradford (1976).

Bacterial stress tolerance abilities (proline production, antioxidant enzimatic activities and poly-β- hydroxybutyrate (PHB) production)

The accumulation of proline was estimated by spectrophotometric analysis at 530 nm (Bates et al., 1973). The bacterial extracts react with ninhydrin and glacial acetic acid during 1 h at 100 ºC. The reaction stops by introducing the tubes in ice bath. The reaction mixture is extracted with 2 mL of toluene, shaking vigorously for 20 seconds. A standard curve was prepared with known concentrations of proline.

The method for the extraction of antioxidant enzymes in the microbial cells was described by Azcón et al. (2010). Bacterial cells were homogenized in a cold mortar with 4 mL 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, 8 mM $MgCl_2$, 5 mM DTT, and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 6000 rpm for 15 min at 4 ºC, and the supernatant was used for enzyme activity determination. Catalase (CAT) activity was measured as described by Aebi (1984), conducted in 2 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µL of enzyme extract. It was determined the consumption of H_2O_2 and followed by decrease in absorbance at 240 nm for 1 min (extinction coefficient (ε_{240}) of 39.6 mM^{-1} cm^{-1}). Ascorbate peroxidase (APX) activity was measured in a 1 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide and 0.5 mM sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako et al., 1994). Total soluble protein amount was determined using Bradford method 1976, and bovine serum albumin as standard.

The poly-β- hydroxybutyrate (PHB) production of the bacterial strain on different osmotic concentrations (0% and 40% PEG) in N_2 deficient medium (pH 7) and incubated a 28 ºC for 72 h at 120 rpm was measured. PHB produced were extracted as described in the metod of Ramsay et al. (1994). The amount of PHB in the extracts was determined

spectrophotometrically at 235 nm (Law and Slepecky, 1961; Lee et al., 1995). A standard curve was prepared to determine PHB in mg mL^{-1}.

2.4. Biossay in *Lactuca sativa* plants under water limited greenhouse conditions

Experimental design

The bioassay study was based on design each one of the bacterial inoculation treatments [bacteria native isolated of study zone: uninoculated control (-); *Bacillus thuringiensis* (B. N2); *Bacillus* sp. (B. N9); *Sphingomona paucimobilis* (Sp. N5); *Pseudomona koreensis* (Ps. N1); *Enterobacter* sp. (E. N10)]. These bacteria were assayed in a pot experiment. The plant used in this study was *Lactuca sativa* grown under drought conditions for 3 months, in greenhouse. The soil used in this experiment is located from Granada (Spain) consisted of a mixture of loamy soil, sieved (5mm) and sterilized by steaming (100 ºC for 1 h for 3 days), and mixed with sterile quartz-sand in the ratio [1:1 (v/v)]. The main characteristics of the soil were pH 8.2; 1.5% organic matter, nutrient concentrations (g kg^{-1}): N 1.9; P 1; K 6.9. Substrate was disposed in pots of 0.3 kg of capacity.

One milliliter of pure bacterial culture (10^7 cfu mL^{-1}) grown in LB nutrient broth medium for 48 h at 28 ºC was applied to the appropriate pots at sowing time just below to plant seedlings, and 15 days later of the bacterial culture (1 mL, 10^7 cfu mL^{-1}) was applied around the plant on the soil. Four replicates by treatments were used, making a total of 24 pots.

Plant growth conditions

These plants were grown for 3 months in pots containing a mixture of sterile soil and sterile quartz-sand [1:1 (v/v)] under greenhouse conditions (temperature ranging from 15 to 21 ºC; 16/8 light/dark photoperiod, and a relative humidity of 50-70%). A photosynthetic photon flux density of 400-700 µmol m^{-2} s^{-1} was applied as supplementary light. Plants were grown along the experiment under drought conditions by keeping soil water capacity close to 50% each day after water application.

Stomatal conductance and photosynthetic efficiency

Stomatal conductance was determined 2 h after the light turned on by using a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the second youngest leaf from each plant.

Photosystem II efficiency was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll *a* fluorescence. FluorPen quantifies the

quantum yield of photosystem II as the ratio between the actual fluorescence yield in the light-adapted state (F_v) and the maximum fluorescence yield in the light-adapted state (F_m), according to Oxborough and Baker (1997). Measurements of photosynthetic efficiency were taken in the second youngest leaf of each plant.

Plant biomass and nutrients content

After three months of growth, plants were harvested (four replicates per each treatment) shoots and roots was weighed and dried for 48 h at 75 ºC to obtain dry weights.

Shoot content (mg plant^{-1}) of P, K, Ca and Mg as well as of Fe, Mn, Cu and Zn (µg plant^{-1}) were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) at Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

Water content

Water content (WC) of a plant is determined from fresh weight (FW) and dry weight (DW) and was calculated according by the following equation:

$$WC = [(FW-DW) / FW] \times 100$$

Antioxidant enzymatic activities in shoot (SOD, CAT, APX and GR)

The method followed for the extraction of antioxidant enzymes on shoot tissues was the described by Aroca et al. (2003). Thus, plant material was homogenized in a cold mortar with 4 mL 100 mM phosphate buffer (pH 7.2) containing 60 mM KH_2PO_4, 40 mM K_2HPO_4, 0.1 mM DTPA and 1 % (w/v) PVPP. The homogenate was centrifuged at 18,000 *g* for 10 min at 4 ºC, and the supernatant was used for enzyme activity determination. Total SOD activity (EC 1.15.1.1) (Burd et al., 2000) was measured on the basis of SOD's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25ºC. CAT activity (EC 1.11.1.6) was measured as described by Aebi (1984), conducted in 2 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µL of enzyme extract. It was determined the consumption of H_2O_2 and followed by decrease in absorbance at 240 nm for 1 min (extinction coefficient (ε_{240}) of 39.6 mM^{-1} cm^{-1}). APX activity (EC 1.11.1.11) was measured in a 1 mL reaction volume containing 80 mM potassium phosphate buffer (pH 7.0), 0.5 mM hydrogen peroxide and 0.5 mM sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako et al., 1994). GR activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to

the oxidation of NADPH (Carlberg and Mannervik, 1985). The reaction mixture (1 mL) contained 50 mM Tris buffer, 3 mM $MgCl_2$ (pH 7.5), 1 mM oxidized glutathione, 100 µL enzyme extract, and 0.3 mM NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in mmol NADPH oxidized mg^{-1} protein, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH (ε_{340}= 6.22 mM^{-1} cm^{-1}). Total soluble protein amount was determined using the Bradford method (Bradford, 1976) and bovine serum albumin as standard.

Endophytic bacterial colonization in roots

Roots of *L. sativa* containing rhizospheric soil were washed with sterile distilled water, desinfected with 70% ethanol, rinsed, disinfected superficially with 3% sodium hypochlorite, rinsed again to eliminate hypochlorite, and spread on nutritive agar to confirm root surface sterility. Finally, roots were added with 0.9% NaCl (1:10) and macerated with mortar and pestle (Forchetti et al., 2007). One gram of macerated tissue was placed in a tube containing 9 mL sterile 0.9% NaCl. One milliliter of appropriate (10^{-2} to 10^{-7}) dilution of tissue was plated on nutritive agar, maintained at 28 °C for 48 h.

The different bacteria strains from were tagged with mini-Tn7transposons by introducing the delivery plasmid and the helper plasmid pUX-BF13 (carrying the transposase genes), by conjugative transfer aided by the mobilizing plasmid pRK600 carrying the RP4/RK2 conjugation system (Kessler et al., 1992). Bacteria cells expressing fluorescent proteins were inspected by epi-fluorescence microscopy as fluorescent colonies on agar plates, or as single fluorescent cells on glass slides. It was possible to detect fluorescent signals of bacteria tagged with gfp (green fluorescent protein) in roots of *L. sativa*.

2.5. Statistical analyses

Data from both experiments were analyzed using SPSS 21 software package for Windows, were subjected to one-way general linear model ANOVA (analysis of variance) was used to determine the effect of each treatment. The Duncan's multiple-range test (Duncan, 1955) was used for post hoc analysis to determine differences between means. Differences were considered significant at $p \leq 0.05$.

3. Results

Five bacteria strains were isolated from a mixture of rhizosphere soils from several autochthonous shrub species naturally grow in a semiarid Mediterranean soil. For the molecular identification of each strain, each sequence obtained was compared with the database of 16S

rDNA from the NCBI/BLAST. The similarity unambiguously identified of the bacterial strain (Table 1) showed that two bacteria were *Bacillus* genera [*Bacillus thuringiensis* (B. N2); *Bacillus* sp. (B. N9)], one bacterium was *Sphingomonas* genera [*Sphingomona paucimobilis* (Sp. N5)], one bacterium was *Pseudomonas* genera [*Pseudomona koreensis* (Ps. N1)] and one bacterium was *Enterobacter* genera [*Enterobacter* sp. (E. N10)].

Table 1. Phylogenetic assignment and biochemical characteristic of the autochthonous bacterial strains isolation of arid Mediterranean soils.

	Isolated	Accession	Identity (%)	N-fixing	Siderophore production	Endophytic
B. N2	*Bacillus thuringiensis*	NR043403.1	(98%)	+	-	+
B. N9	*Bacillus* sp.	NR043403.1	(91%)	-	-	+
Sp. N5	*Sphingomona paucimobilis*	DSM 30198	2.04* (68%)	+	-	+
Ps. N1	*Pseudomona koreensis*	NR 025228.1	(99%)	+	+	+
E. N10	*Enterobacter* sp.	NR044977.1	(99%)	+	-	+

Based on completed sequencing of 16S rDNA gene and comparison with those NCBI by using Blast. *(MALDI Biotyper).

Many of bacterial strains isolated were able to fix atmospheric nitrogen, only one of these bacterial strains (*Pseudomona koreensis* (Ps. N1)) produced siderophores (Fig. 1, Table 1). The autochthonous bacteria were also characterized by their capacity of solubilize phosphate (Fig. 2), synthesis of α-ketobutyrate (ACC deaminase), PHB production and endophytic capacity (Fig. 3) under osmotic stress (40% PEG) conditions (Table 2 and 3).

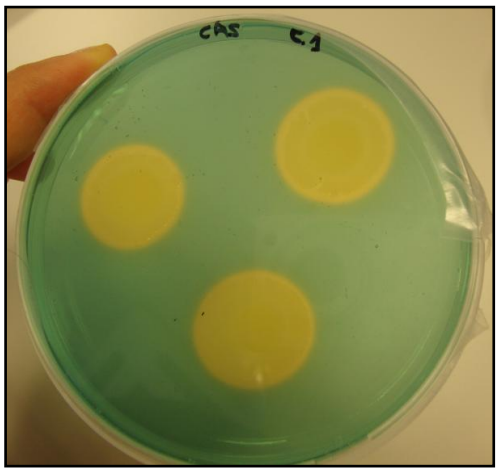


Fig. 1. Production of siderophores by the bacterial strains (*Pseudomona koreensis* (Ps. N1).

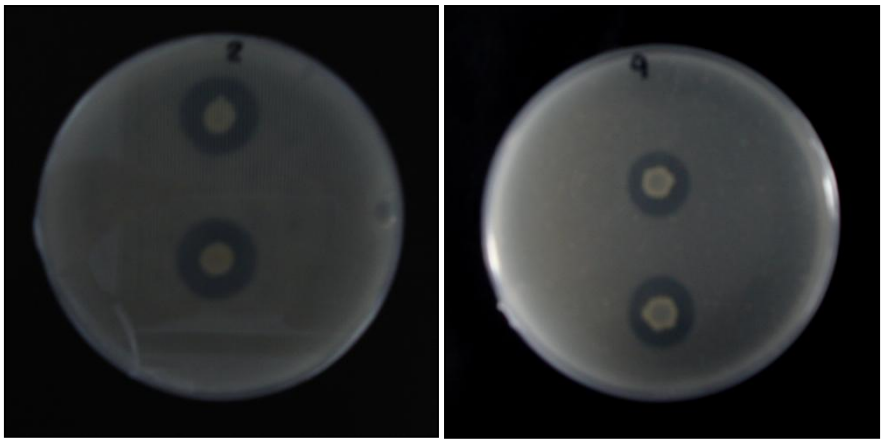


Fig. 2. Phosphate solubilizing capacity of autochthonous bacteria.

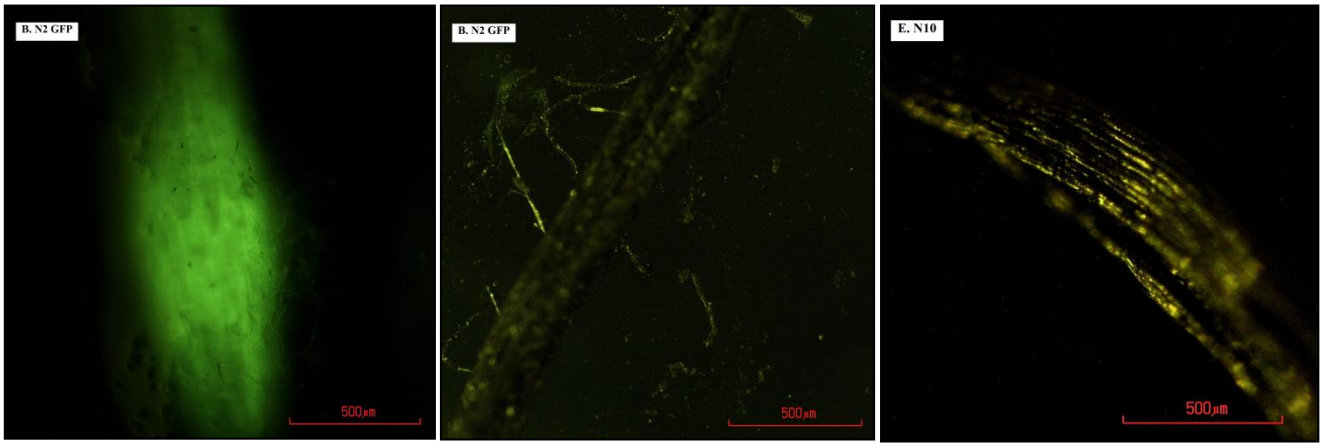


Fig. 3. Endophytic root colonization of *Lactuca sativa* with autochthonous bacterial strains observed by epi-fluorescence microscopy previous labeled gfp. Bar= 500 µm.

The increasing levels of PEG in the growing medium specifically affected bacterial growth. *Enterobacter* sp. was most stress tolerant bacteria showing the greatest growth under osmotic stress of 40% PEG, whereas *P. koreensis* (Ps. N1) and *Bacillus* sp. (B. N9) were the most sensitive to such conditions (Fig. 4).

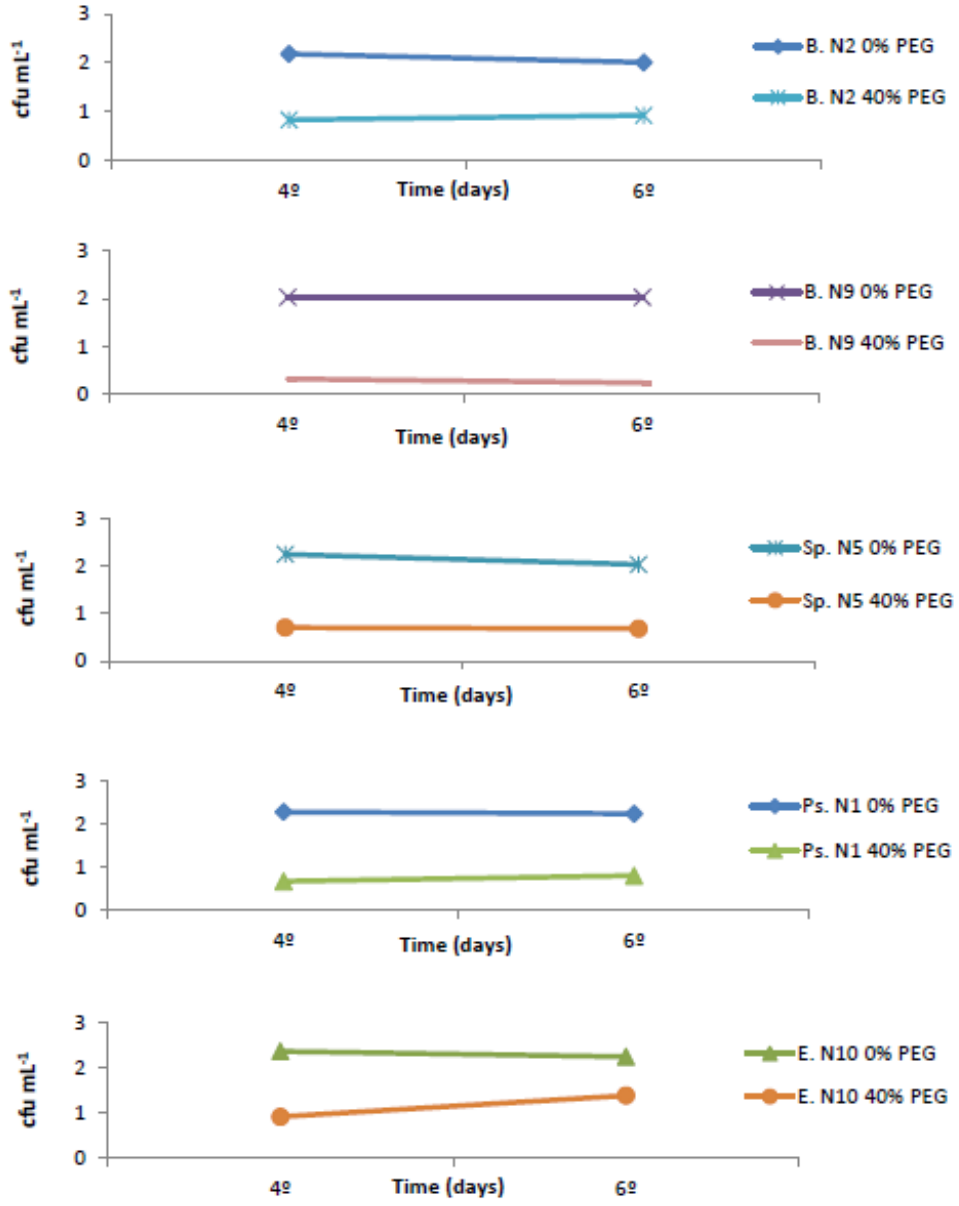


Fig.4. Viable cells (cfu mL^{-1}) of the autochthonous bacterial strains growing in axenic medium supplement or not with polyethylene glycol (PEG) at 40% at different time intervals (4º and 6º days).

Many of bacterial strains isolated were characterized as good solubilizing of phosphate and IAA production and they did not significantly reduce these abilities under stress. When subjected, *in vitro*, to osmotic stress conditions (40% PEG) the PGPR abilities of these autochthonous bacteria in general, were maintained or increased. However, E. N10 and B.N9 tends to produce the highest amount of IAA but under osmotic stress conditions the synthesis of IAA decreased meanwhile the phosphate solubilizing capacity did not change (B. N9) or decreased (E. N10) compared to non-stress conditions (Table 2).

The bacterium belonging to *Enterobacter* sp. shows, under normal conditions, elevated index of phosphate solubilization (PSI= 2.06) besides it is a good producers of IAA (0.25 mg mg^{-1}protein). This bacterium in conditions of osmotic stress (40% PEG) also decreased the production of PSI and IAA and the synthesis of ACC deaminase was also decreased by 40.5%. In contrast, B. N2, with 40% PEG increased the synthesis of ACC-deaminase by 105% compared to non-stress conditions. *S. paucimobilis* (Sp. N5) strain has a high PSI (1.55) but it lacks the ability to ACC-deaminase synthesis. Under osmotic stress conditions this bacteria (Sp. N5) decreased PSI by 35% compared to non-stress conditions but *P. koreensis* (Ps. N1) highly increased the production of IAA and ACC-deaminase activity but decreased its capacity of phosphate solubilizing (Table 2).

Table 2. Phosphate solubilization index (PSI), indole acetic acid (IAA) and α-ketobutyrate production by the autochthonous bacterial strains after four days of growth in axenic culture medium supplemented or not with 40% polyethylene glycol (PEG).

	PSI		**mg IAA mg^{-1}protein**		**mmoles α-ketobutyrate mg^{-1}protein**	
	0%PEG	**40%PEG**	**0%PEG**	**40%PEG**	**0%PEG**	**40%PEG**
B. N2	1.56 cd	1.37 bc	0.05 a	0.01 a	0.20 b	0.41 d
B. N9	1.00 a	1.00 a	0.15 b	0.02 a	0.41 d	1.09 e
Sp. N5	1.55 cd	1.00 a	0.02 a	0.03 a	-	-
Ps. N1	1.77 de	1.48 c	0.02 a	0.10 b	0.08 a	0.25 c
E. N10	2.06 de	1.00 a	0.25 c	0.05 a	0.37 d	0.22 b

Within parameters values with different letters are significantly different ($p \leq 0.05$) as determined by Duncan´s multiple-range test (n=3).

The drought tolerance abilities of bacterial strains were shown in Table 3. The levels of enzymatic activities (APX and CAT) were high in *B. thuringiensis* (B. N2) compared with the other bacterial strains. But under osmotic stress conditions considerably decreases both antioxidant enzymatic activities, by 95% and 92% respectively compared to non-stress conditions. However, we observe that *P. koreensis* (Ps. N1) under osmotic stress conditions highly increased proline production, enzymatic APX and CAT antioxidant activities and PHB production (Table 3). *Enterobacter* sp. increased PHB production by 700% compared to non-stress conditions. However, *S. paucimobilis* decreased by 95% PHB production and increased

by 566% proline levels compared to non-stress conditions. In both bacterial strains no significant differences in antioxidant enzymatic activities as well as in B. N9 strain (Table 3).

Table 3. Proline, antioxidant enzymatic [ascorbate peroxidase (APX) and catalase (CAT)] activities and poly-β-hydroxybutyrate (PHB) production by the autochthonous bacterial strains after four days of growth in axenic culture medium supplemented or not with 40% polyethylene glycol (PEG).

	Proline (mmol mg^{-1}prot)		**µmol APX mg^{-1}prot·min**		**µmol CAT mg^{-1}prot·min**		**mg PHB mL^{-1}**	
	0%PEG	**40%PEG**	**0%PEG**	**40%PEG**	**0%PEG**	**40%PEG**	**0%PEG**	**40%PEG**
B. N2	0.12 a	0.3 ab	11760 c	586 a	606 b	46 a	0.33 c	0.38 d
B. N9	0.13 a	0.4 ab	277 a	261 a	32 a	26 a	0.31 c	0.45 e
Sp. N5	0.21 b	1.4 c	766 a	864 a	77 a	120 a	0.42 de	0.02 a
Ps. N1	0.14 a	2.5 d	795 a	7552 b	39 a	504 b	0.02 a	0.39 d
E. N10	0.15 a	0.3 ab	238 a	217 a	22 a	2 a	0.01 a	0.08 b

Within parameters values with different letters are significantly different ($p \leq 0.05$) as determined by Duncan´s multiple-range test (n=3).

In Lactuca sativa

The inoculation of autochthonous *Bacillus* increased shoot dry weight of *Lactuca sativa* in by 55% (B. N2) and by 27% [(B. N9) non-significant] compared with the uninoculated control (Table 4). The *Enterobacter* sp. also increased shoot biomass by 33% (E. N10), unlike of *P. koreensis* (Ps. N1) that decreased plant shoot growth. Regarding the bacterial effectiveness for root dry weight we observed an increasing effect of a mean of 126.3% in plants inoculated by the two *Bacillus* species. *Enterobacter* sp. increased by 158% this value and *S. paucimobilis* by 52.6% and no change in root biomass in plants inoculated with *P. koreensis* was found (Table 4).

Bacterial inocula did not change shoot water content (WC). The stomatal conductance (SC) was lower in plants inoculated with two *Bacillus* sp., *Enterobacter* sp. and *S. paucimobilis* that decreased this value by 35.9% (B. N2); 29.1% (B. N9); 28.8% (E. N10) and by 25.1% (Sp. N5) compared with non-inoculated controls. The *P. koreensis* did not significantly change stomatal conductance. The photosynthetic efficiency (PE) was not significantly different between non-inoculated control and inoculated plants (Table 4).

Table 4. Effect of autochthonous bacterial strains on shoot and root dry weight (g), water content (WC), stomatal conductance (SC) and photosynthetic efficiency (PE) in *Lactuca sativa* grown under drought conditions.

	Shoot dry weight (g)	Root dry weight (g)	WC (%)	SC (mmol m^{-2} s^{-1})	PE (Fv/Fm)
(-)	0.65 b	0.19 a	80.55 b	87.75 b	0.54 ab
B. N2	1.01 d	0.42 c	79.46 ab	56.25 a	0.59 b
B. N9	0.83 bc	0.44 bcd	80.07 b	62.25 a	0.52 a
Sp. N5	0.88 bc	0.29 b	80.74 b	65.75 a	0.61 b
Ps. N1	0.45 a	0.17 a	83.60 c	102.75 b	0.58 b
E. N10	0.87 c	0.49 d	82.37 bc	62.50 a	0.55 a

Within parameters values with different letters are significantly different ($p \leq 0.05$) as determined by Duncan´s multiple-range test (n=4).

The macronutrients content in shoot of *L. sativa* resulted increased in general by the inoculation of *B. thuringiensis* (B.N2) (Table 5). Bacterial inoculation did not significantly enhanced in P content. The inoculation of *B. thuringiensis* (B. N2) increased K content by 50%, Ca by 61%, as well as Mg by 53%. This was the most efficient bacterium increasing the uptake of these nutrients.

As well, the micronutrient content in shoot of *L. sativa* resulted more increased by the inoculation of *B. thuringiensis* (B. N2) (Table 6), that increased Fe (by 74.7%), Mn (by 46.4%) and Zn (by 125.4%). Not important changes were found in the uptake of these nutrients in inoculated plants with the rest of bacterial strains. *S. paucimobilis* and *Enterobacter* sp. increased Zn content by 50% and 103% respectively, compared with uninoculated control (Table 6).

Table 5. Effect of autochthonous bacterial strains on P, K, Ca and Mg shoot content in *Lactuca sativa* grown under drought conditions.

	P (mg plant^{-1})	**K** (mg plant^{-1})	**Ca** (mg plant^{-1})	**Mg** (mg plant^{-1})
(-)	0.56 ab	13.88 ab	4.86 ab	1.33 a
B. N2	0.93 b	20.83 c	7.82 c	2.04 b
B. N9	0.81 b	14.54 ab	5.52 abc	1.54 ab
Sp. N5	0.78 b	16.22 ab	5.84 b	1.67 ab
Ps. N1	0.48 a	11.61 a	4.05 a	1.21 a
E. N10	0.92 b	14.98 b	5.75 b	1.43 a

Values in the same column with different letters are significantly different ($p \leq 0.05$) as determined by Duncan´s multiple-range test (n=3).

Table 6. Effect of autochthonous bacterial strains on Fe, Mn, Cu and Zn shoot content in *Lactuca sativa* grown under drought conditions.

	Fe (µg plant^{-1})	**Mn** (µg plant^{-1})	**Cu** (µg plant^{-1})	**Zn** (µg plant^{-1})
(-)	37.25 a	43.09 ab	1.72 ab	17.43 a
B. N2	65.09 b	63.07 c	2.58 bc	39.29 bc
B. N9	42.95 ab	45.00 ab	1.98 ab	32.21 bc
Sp. N5	40.39 a	42.93 a	2.20 b	26.16 b
Ps. N1	69.88 ab	31.66 a	1.40 a	22.56 ab
E. N10	41.09 a	75.07 bcd	2.26 b	35.37 bc

Values in the same column with different letters are significantly different ($p \leq 0.05$) as determined by Duncan´s multiple-range test (n=3).

Regarding antioxidant enzymes activities in shoot of *L. sativa* (Table 7), was observed that SOD activity was the highest in E. N10 (increased by 68% compared with uninoculated control). Both CAT and APX activities were higher in plants colonized by this bacterial strain that increased CAT by 76% and APX by 398% (Table 7). Similarly the two *Bacillus* species and *P. koreensis* increased APX (B. N2 by 216%, B. N9 by 281% and Ps. N1 by 192%) compared with uninoculated control, CAT activity was also increased by B. N9 strain in by 63% (Table 7).

Table 7. Effect of autochthonous bacterial strains on shoot ntioxidant enzymatic activities [Superoxide dismutase (SOD); Catalase (CAT); Ascorbate peroxidase (APX) and Glutathione reductase (GR)] in *Lactuca sativa* under drought conditions.

	U SOD mg^{-1}protein	µmol CAT mg^{-1}protein·min	µmol APX mg^{-1}protein·min	µmoles GR mg^{-1}protein·min
(-)	0.82 b	5.48 ab	62.05 a	10.57 ab
B. N2	0.77 abc	4.88 ab	196.24 b	15.21 b
B. N9	0.97 bc	8.95 c	236.72 b	8.13 ab
Sp. N5	0.56 a	4.16 a	99.20 a	8.54 ab
Ps. N1	0.79 b	3.92 a	181.50 b	12.22 b
E. N10	1.38 d	9.63 c	309.33 bc	11.70 ab

Values in the same column with different letters are significantly different ($p \leq 0.05$) as determined by Duncan´s multiple-range test (n=3).

4. Discussion

The isolation of the five most abundant rhizobacteria was carried out. Subsequently they were screened *in vitro* to evaluate their plant growth promoting (PGP) activities and their capacity to resist the osmotic stress conditions caused by the application of 40% PEG. The osmotic stresses affect the growth and functional development of bacteria in the environment. Regarding the native bacterial strains selected we check that *B. thuringiensis* (B. N2) and *Enterobacter* sp. (E. N10) were bacterial strains less affected by osmotic stress. Given the ability of bacteria to synthethize compatible solutes (amino acids, sugars, or derivatives) they can act as osmolytes and help to these organisms to survive under extreme osmotic stresses (da Costa et al., 1998; Parida and Das, 2005). Some strains exhibited more than one PGP activity involved in promoting plant growth directly, indirectly or synergistically (Stefan et al., 2013). Specific bacterial traits, such as nitrogen fixation, phosphate solubilization and IAA synthesis, have exhibited an influence on plant growth by increasing nutrient availability and by influencing plant development (Glick, 2010).

Regarding the osmotic stress tolerance abilities of these bacteria, *P. koreensis* (Ps. N1) was one of the most sensitive bacterial strains to the osmotic stress applied and concomitantly also produced the highest of proline amount and increased antioxidant enzymatic activity (APX and CAT) under such osmotic stress conditions. Proline accumulation in bacterial cells not only has an osmolyte function but also maintains the redox balance and radical scavenging (Szabados

and Savoure, 2010). Thus, it could contribute to the scavenging of free radicals produced by the stress applied conditions in addition to its main role as an osmoprotectant under water-deficit (Alia et al., 2001; Kaul et al., 2008; Azcón et al., 2010). Similarly, *S. paucimobilis* and *P. koreensis* cells highly sensitive to 40% PEG, could use the high proline production under stress (at 40% PEG) for osmotic cellular adaptation (Marulanda et al., 2009). In contrast, *B. thuringiensis* (B. N2) strains decreased significantly the APX and CAT activities under the stress conditions tested. These antioxidants bacterial activities play an important role facilitating the removal of free radicals (Wang et al., 2007). Perhaps in these bacteria (two *Bacillus* sp. and *Enterobacter* sp.) antioxidant activities are not required or were compensated by the contribution of high PHB and/or ACC production in alleviating the cell osmotic stress. Ps. N1 increased the amounts of PHB and α-ketobutyrate (ACC-deaminase) production under such osmotic stress conditions. As an intracellular energy and carbon storage compound, the PHB is produced by bacteria when they are subjected to stress as a mechanisms that favors their establishment, survival and competition in competitive environments (Okon and Itzigsohn, 1992). Ayub et al., (2004) suggested the relationship between PHB accumulation and high stress resistance as was observed in B. N2 under 40% PEG. Bacterial ACC-deaminase converts the ACC to ammonia and α-ketobutyrate, thereby lowering ethylene levels in inoculated plants (Glick et al., 1998). The lowering of ethylene levels is essential when plants are exposed to environmental stresses as drought (Glick, 2004). ACC deaminase-expressing bacteria would be significantly involved in decreased the detrimental effects when plants were subject an ethylene-causing environmental stresses. Bacterial that express the enzyme ACC-deaminase facilitate the rooting of young seedlings (Glick et al., 1998). In this study we observed that the two *Bacillus* sp. and *P. koreensis* enhanced the ACC-deaminase accumulation under stress conditions.

The effectiveness of these bacterial strains in alleviating drought stress was evaluated on lettuce plants. *B. thuringiensis* and *Enterobacter* sp. significantly increased plant biomass (shoot and root), unlike of *P. koreensis* that decreased plant growth. Under stress conditions, bacteria in the rhizosphere may enhance the plant growth by different mechanisms such as by optimizing the supply of nutrients, solubilization of inorganic phosphorus, the synthesis of phytohormones as IAA or by ACC-deaminase production. Typically a bacterium directly affects the plant growth and development by using one or more of these mechanisms (Gamalero et al., 2008). The reduction of photosynthethic activity is one of the major biochemical and physiological responses to drought stress due to several factors such as stomatal closure and reduction of photosynthetic enzyme efficacy (Giardi et al., 1996). Stomatal closure is elicited not only by water stress but also in response to plant-microbe interactions (Frommel et al., 1991). The effect of applied bacterial inoculants regarding leaf stomatal conductance (SC), demonstrated that this value was reduced by inoculation bacterial particularly when inoculated with the two *Bacillus*

species and especifically with *B. thuringiensis* (B. N2). The leaf water content (WC) was increased by some bacterial treatments (Ps. N1). Nevertheless, couriously WC having similar stomatal conductance to uninoculated treatment. In contrast, bacterial strains that lowed SC did not increase WC.

In this study most of the bacterial inoculants used potentially improve the content of some essential nutrients in plant. In particular, the inoculation of *B. thuringiensis* (B. N2) increased phosphorus and potassium content in shoot of *L. sativa* by 66% and 50% respectively over the non-inoculated controls. These nutrients are considered as one of the key features of osmotic stress tolerance (Shabala and Cuin, 2008). In addition, an increase in the content of nutrients as Ca^{2+} and Mg^{2+} mediated by bacterial inoculation of *B. thuringiensis* (B. N2), was also observed, this may be explained by an increase in mineral availability mediated by the bacterial metabolism (e.g. releasing of organic acids) (Rojas-Tapias et al., 2012). The Ca is important in membrane protection and Mg modulates ionic currents across the chloroplasts and vacuole membranes (regulating stomatal opening and ion balance in cells) under dry conditions (Parida and Jha, 2013). The enhancement of Mg content in inoculated plants with B. N2 suggests that the functioning of photosynthetic apparatus was not affected by drought in bacterial colonized *L. sativa* but drought lends to severe damage to membrane integrity in many plants (Silva et al., 2010). PGPR strains that are capable of IAA production exhibited significantly enhanced P, K, Ca and Mg uptake (Farzana et al., 2005). But this relationship is not those observed. Plant physiology as values of the stomatal conductance and nutrients as K and Ca content (higher in plants colonized *B. thuringiensis* (B. N2) strains) are important physiological and nutritional values to adapt plants to drought since stomatal closure preserves water lost.

The microbial inoculants increased Zn content in shoot of *L. sativa* from to 125% in B. N2 to 50% in Sp. N5, there studies about certain bacteria that have shown increase heavy metal mobilization by the secretion of low-molecular-mass organic acids by endophytic diazotroph *Gluconacetobacter diazotrophicus*, which dissolves various Zn sources such as ZnO, $ZnCO_3$, or $Zn_3(PO_4)_2$, thus making Zn available for plant uptake (Saravanan et al., 2007). The protective role of Zn may be an important response in drought tolerance not only simply affecting the plant-water relationship and dry matter accumulation but also changes in antioxidant balance during drought stress (Upadhyaya et al., 2013). Zn is an essential functional component of thousands of proteins and approximately 100 enzymes require Zn as a cofactor. Roots of all plant species can take up Zn, Ca, and Mg present in their cationic forms in the rhizosphere, although soil properties and the intensity of crop harvesting determine the phytoavailability of these elements (White and Broadley, 2009). In particular, inoculation of *B. thuringiensis* (B. N2) highly increased these nutrients uptake in *L. sativa*.

Plants must balance uptake, utilization and storage of mineral elements in order to maintain proper ion homeostasis, and this can be negatively affected by adverse conditions as drought. Under some conditions, electrons and excitation energy not used in photosynthesis if it is depressed can be channeled to molecular O^{2-} and there is an overproduction of reactive oxygen species (ROS) in cell compartments. The presence of ROS can cause cellular damage through oxidation of lipids and proteins, chlorophyll bleaching, damage to nucleic acids, and ultimately leading to cell death (Apel and Hirt 2004). Maintenance of redox status requires a strict balance between ROS production and detoxification, and to protect against the toxic effect of ROS, cells have developed a complex antioxidant system that can be enzymatic, such as superoxide dismutase (SOD), which is the first line of defense against ROS, catalase (CAT) activity that prevents dangerous radicals formation, and ascorbate peroxidase (APX), the enzyme involved in the Halliwell-Asada cycle, as well as antioxidant compounds such as ascorbate that detoxifies a large number of free radicals, hence minimizing oxidative damage to many enzymatic activities and preventing photo-oxidation (Foyer and Noctor 2005, Blasco et al., 2011a,b). The SOD pattern is constituted by three isoenzymes (CuZn-SOD, Fe-SOD and Mn-SOD), drought stress increases the activity of all isoenzymes mainly under severe drought conditions (Talbi et al., 2015). The application of PGPR may also assist growth by alleviating the negative effects of drought by promoting the accumulation of antioxidant enzyme activities and decreasing ROS such as H_2O_2, O^{2-} and OH^- in response to water stress (Güneş et al., 2014). *L. sativa* plants inoculated with *Enterobacter* sp. (E. N10) showed increased producing of antioxidants activities such as SOD, CAT and APX and *Bacillus* species such as B. N2 increased APX activity and B. N9 increased APX and CAT activity. But plants inoculated with *B. thuringiensis* (B.N2) showed APX activity as the main resource to maintain osmotic balance against such drought conditions besides of that levels of this antioxidant activity was low with respect those bacterial strains that promote of growth of *L. sativa*, then this verifies that inoculated plants with B.N2 were much less affected by drought conditions, so they are more drought tolerant.

In conclusion, this study was presented as helps to have a better understanding for the selection of certain rhizosphere bacteria that go unnoticed and have a great importance its application as inocula in improving the plant growth and nutrient of plants growing in degraded areas. The water limitation and osmotic stress negatively affect plant growth but the bacterial inoculation was able to attenuate these detrimental effects by varied molecular, physiological and biochemical mechanisms. This study reveals that the tested autochthonous PGPR species were tolerant to osmotic stress (40% PEG), the bacterial autochthonous strains that belong a *Bacillus* and *Enterobacter* genus were of great resistance at osmotic stress because they are bacterial strains that are predisposed to adapt to unfavorable conditions.

As well, it is important, from a practical point of view, to know that *B. thuringiensis* was able to survive and to multiply to reach a sufficient population express himself activities under stress conditions. The water limitation and osmotic stress negatively affect plant growth but the *B. thuringiensis* inoculation was able to attenuated these detrimental effects and consequently may improve growth, nutrient uptake and the physiological quality of plants and thereby can help plants of *L. sativa* in the osmoregulation processes and in improving homeostatic mechanisms upon stress challenge (Dimkpa et al., 2009; Miller et al., 2010). However, further research studies are required to establish the main processes by which these native bacterial strains isolates of semiarid areas and in particularly *B. thuringiensis* improve plants performance under drought conditions.

Acknowledgments

E. Armada was financed by Ministry of Science and Innovation (Spain). This work was carried out in the framework of the project reference AGL2009-12530-C02-02. We thank the Instrumentation Service (EEZ-CSIC) for the plant analysis and bacterial sequencing.

References

Aebi H. (1984). Catalase *in vitro*. Method. Enzymol. 105, 121-126.

Ahemad M. (2012). Implications of bacterial resistance against heavy metals in bioremediation: a review. IIOABJ. 3, 39-46.

Ahemad M., Malik A. (2011). Bioaccumulation of heavy metals by zinc resistant bacteria isolated from agricultural soils irrigated with wastewater. J. Bacteriol. 2, 12-21.

Alia Mohanty P., Matysik J. (2001). Effect of proline on the production of singlet oxygen. Amino Acids. 21, 195-200.

Amako K., Chen GX., Asada K. (1994). Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol. 35, 497-504.

Apel K., Hirt H. (2004). Reactive oxygen species: metabolism, oxidative stress and signal transduction. Annu. Rev. Plant Biol. 55, 373-399.

Appuhn A., Joergensen RG. (2006). Microbial colonisation of roots as a function of plant species. Soil Biol. Biochem. 38, 1040-1051.

Armada E., Portela G., Roldán A., Azcón R. (2014a). Combined use of beneficial soil microorganisms and agrowaste residue to cope with palnt water limitation under semiarid conditions. Geoderma. 640-648.

Armada E., Roldán A., Azcón R. (2014b). Differential activity of autochthonous bacteria in controlling drought stress in native *Lavandula* and *Salvia* plants species under drought conditions in natural arid soil. Microb. Ecol. 67, 410-420.

Aroca R., Irigoyen JJ., Sánchez-Díaz M. (2003). Drought enhances maize chilling tolerance. II. Photosynthetic traits and protective mechanisms against oxidative stress. Physiol. Plant. 117, 540-549

Ayub ND., Pettinari MJ., Ruiz JA., Lopez NI. (2004). A polyhydroxybutyrate-producing *Pseudomonas* sp isolated from antarctic environments with high stress resistance. Curr. Microbiol. 49, 170-174.

Azcón R., Perálvarez MC., Roldán A., Barea JM. (2010). Arbuscular mycorrhizal fungi, *Bacillus cereus* and *Candida parapsilosis* from a multicontaminated soil alleviate metal toxicity in plants. Microb. Ecol. 59, 668-677.

Azcón, R., Medina, A., Aroca, R., Ruíz-Lozano, J.M. (2013). Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast interactions, in: de Bruijn, F.J. (Ed.), Molecular Microbial Ecology of the Rhizosphere. John Wiley & Sons, Hoboken, New Jersey, USA, pp. 991-1002.

Bais HP., Weir TL., Perry LG., Gilroy S., Vivanco JM. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 233-266.

Barea JM., Azcón R., Azcón-Aguilar C. (2002). Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 81, 343-351.

Barea JM., Pozo MJ., Azcón R., Azcón-Aguilar C. (2005). Microbial co-operation in the rhizosphere. J. Exp. Bot. 56, 1761-1778.

Barriuso J., Solano BR., Lucas JA., Lobo AP., Villaraco AG., Manero FJG. (2008). Ecology, genetic diversity and screening strategies of plant growth promoting rhizobacteria (PGPR). Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Edited by AhmadI, Pichtel J, Hayat S, 1-17.

Bashan Y., de-Bashan LE. (2005). Bacteria Plant Growth-Promoting. In: Hillel, D. (Ed.), Encyclopedia of Soils in the Environment. Elsevier, Oxford. 103-115.

Bashan Y., de-Bashan LE. (2010). How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. Adv. Agron. 77-136.

Bates LS., Waldren RP., Teare ID. (1973). Rapid determination of free proline for water-stress studies. Plant Soil. 39, 205-207.

Beneduzi A., Peres D., da Costa PB., Bodanese Zanettini MH., Passaglia LMP. (2008). Genetic and phenotypic diversity of plant-growth-promoting bacilli isolated from wheat fields in southern Brazil, Res. Microbiol. 159, 244-250.

Blasco B., Rios JJ., Leyva R., Cervilla LM., Sanchez-Rodriguez E., Rubio-Wilhelmi MM. (2011a). Does iodine biofortification affect oxidative metabolism in lettuce plants?. Biol. Trace Elem. Res. 142, 831-842.

Blasco B., Rios JR., Cervilla LM., Sanchez-Rodriguez E., Rubio-Wilhelmi MM., Rosales MA. (2011b). Iodine application affects nitrogen-use efficiency of lettuce plants (*Lactuca sativa* L.) Acta Agric. Scand. Sect. B Soil Plant Sci. 61, 378-383.

Bradford MM. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Burd GI., Dixon DG., Glick BR. (2000). Plant growth promoting bacteria that decreased heavy metal toxicity in plants. Can. J. Microbiol. 46, 237-245.

Çakmakçı R., Dönmez MF., Aydin A., Sahin F. (2006). Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biol. Biochem. 38, 1482-1487.

Carlberg I., Mannervik B. (1985). Glutathione reductase. Method. Enzymol. 113, 484-489.

Castellanos T., Dohrmann AB., Imfeld G., Baumgarte S., Tebbe CC. (2009). Search of environmental descriptors to explain the variability of the bacterial diversity from maize rhizospheres across a regional scale. Eur. J. Soil Biol. 45, 383-393.

Costa R., Götz M., Mrotzek N., Lottmann J., Berg G., Smalla K. (2006). Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiol. Ecol. 56, 236-249.

da Costa MS., Santos H., Galinski EA. (1998). An overview of the role and diversity of compatible solutes in Bacteria and Archaea. Adv. Biochem. Eng. Biotechnol. 61, 117-153.

Dimkpa C., Weinand T., Asch F. (2009). Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ. 32, 1682-1694.

Döbereiner J., Day JM. (1976). Associative symbiosis in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. In Proceedings of the First International Symposium on Nitrogen Fixation. Edited by W.E. Newton and C.J. Nyman. Washington State University Press, Pullman, Wash. 518-538.

Duncan DB. (1955). Multiple range and multiple F tests. Biometrics 11, 1-42.

Edi-Premono M., Moawad AM., Vlek PLG. (1996). Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere Indonesian. J. Crop Sci. 11, 13-23.

Farzana Y., Radizah O. (2005). Influence of rhizobacterial inoculation on growth of the sweet poptato cultivar. Am. J. Biochem. Biotechnol. 1, 176-179.

Forchetti G., Masciarelli O., Alemano S., Alvarez D., Abdala G. (2007). Endophytic bacteria in sunflower (*Helianthus annuus* L.): isolation, characterization, and production of jasmonates and abscisic acid in culture mendium. Appl. Microbiol. Biotechnol. 76,1145-1152.

Foyer CH., Noctor G. (2005). Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28, 1056-1071.

Frommel MI., Nowak J., Lazarovits G. (1991). Growth enhancement and developmental modifications of in vitro grown potato (*Solanum tuberosum spp. tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. Plant Physiol. 96, 928-936.

Gamalero E., Berta G., Massa N., Glick BR., Lingua G. (2008). Synergistic interactions between the ACC deaminase-producing bacterium *Pseudomonas putida* UW4 and the AM fungus *Gigaspora rosea* positively affect cucumber plant growth. FEMS Microbiol. Ecol. 64, 459-467.

Giardi MT., Cona A., Geiken B., Kučera T., Masojídek J., Mattoo AK. (1996). Long-term drought stress induces structural and functional reorganization of photosystem II. Planta 199, 118-125.

Glick BR. (1995). The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41, 109-117.

Glick BR. (2004). Bacterial ACC deaminase and the alleviation of plant stress. Adv. Appl. Microbiol. 56, 291-312.

Glick BR. (2010). Using soil bacteria to facilitate phytoremediation. Biotechnol. Adv. 28, 367-374.

Glick BR., Penrose DM., Li JP. (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190, 63-68.

Glick BR., Todorovic B., Czarny J., Cheng Z., Duan J., McConkey B. (2007). Promotion of plant growth by bacterial ACC deaminase. Crit. Rev. Plant Sci. 26, 227-242.

Gordon SA., Paleg LG. (1957). Observations on the quantitative determination of indole acetic acid. Physiol. Plant. 10, 39-47.

Güneş A., Turan M., Gulluce M., Sahin F. (2014). Nutritional content analysis of plant growth-promoting rhizobacteria species. Eur. J. Soil Biol. 60, 88-97.

Hariprasad P., Niranjana SR. (2009). Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato. Plant Soil 316, 13-24.

Hayat R., Ali S., Amara U., Khalid R., Ahmed I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. Ann. Microbiol. 60, 579–598.

Honma M., Shimomura T. (1978). Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agric. Biol. Chem. 42, 1825-1831.

Katznelson H., Bose B. (1959). Metabolic activity and phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere, and non-rhizosphere soil. Can. J. Microbiol. 5, 79-85.

Kaul S., Sharma SS., Mehta IK. (2008). Free radical scavenging potential of L-proline: evidence from in vitro assays. Amino Acids. 34, 315-320.

Kessler B., de Lorenzo V., Timmis KN. (1992). A general system to integrate LacZ fusions into the chromosome in the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. 233, 293-301.

Law JH., Slepecky RA. (1961). Assay of poly β-hydroxy-butyric acid. J. Bacteriol. 82, 32-36.

Lee, IY., Chang, HN., Park, YH. (1995). A simple method for recovery of microbial poly-beta-hydroxybutyrate by alkaline-solution treatment. J. Microbiol. Biotechnol. 5, 238-240.

Marschner P., Yang CH., Lieberei R., Crowley DE. (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. Soil Biol. Biochem. 33, 1437-1445.

Marulanda A., Barea JM., Azcón R. (2009). Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments. Mechanisms related to bacterial effectiveness. J. Plant Growth Regul. 28, 115-124.

Marulanda A., Porcel R., Barea JM., Azcón R. (2007). Drought tolerance and antioxidant activities in lavender plants colonized by native drought-tolerant or drought-sensitive *Glomus* species. Microb. Ecol. 54, 543-552.

Miller G., Suzuki N., Ciftci-Yilmaz S., Mittler R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 33, 453-467.

Okon Y., Itzigsohn R. (1992). Poly-beta-hydroxybutyrate metabolism in *Azospirillum brasilense* and the ecological role of PHB in the rhizosphere. FEMS Microbiol. Lett. 103, 131-139.

Oxborough K., Baker NR. (1997). Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components - calculation of qP and *Fv'/Fm'* without measuring *Fo'*. Photosynth. Res. 54, 135-142.

Parida AK., Das AB. (2005). Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60, 324-349.

Parida AK., Jha B. (2013). Physiological and biochemical responses reveal the drought tolerance efficacy of the halophyte *Salicornia brachiata*.J. Plant Growth Regul. 32, 342-352.

Park M., Kim C., Yang J., Lee H., Shin W., Kim S., Sa T. (2005). Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. Microbiol. Res. 160, 127-133.

Penrose DM., Glick BR. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol. Plant. 118, 10-15.

Pikovskaya RI. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologya 17, 362-370.

Rajkumar M., Ae N., Prasad MNV., Freitas H. (2010). Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends Biotechnol. 28, 142–149.

Ramsay, JA., Berger, E., Voyer, R., Chavarie, C., Ramsay, BA. (1994). Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnol. Tech. 8, 589-594.

Rojas-Tapias D., Moreno-Galván A., Pardo-Díaz S., Obando M., Rivera D., Bonilla R. (2012). Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (*Zea mays*). Appl. Soil Ecol. 61, 264-272.

Saravanan VS., Madhaiyan M., Thangaraju M. (2007). Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium *Gluconacetobacter diazotrophicus*. Chemosphere. 66, 1794-1798.

Schwyn B., Neilands JB. (1987). Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 47-56.

Shabala S., Cuin TA. (2008). Potassium transport and plant salt tolerance. Physiol. Plant. 133, 651-669.

Silva Ev., Ferreira-Silva SL., Fontenele AV., Ribeiro RV., Viégas RA., Silveira JAG. (2010). Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in *Jatropha curcas* plants. J. Plant Physiol. 167, 1157-1164.

Stefan M., Munteanu N., Stoleru V., Mihasan M., Hritcu L. (2013). Seed inoculation with plant growth promoting rhizobacteria enhances photosynthesis and yield of runner bean (*Phaseolus coccineus* L.). Sci. Hortic. 151, 22-29.

Szabados L., Savoure A. (2010). Proline: a multifunctional amino acid. Trends Plant Sci. 15, 89-97.

Talbi S., Romero-Puertas MC., Hernández A., Terrón L., Ferchichi A., Sandalio LM. (2015). Drought tolerance in a Saharian plant *Oudneya africana*: role of antioxidant defences. Environ. Exp. Bot. 111(0), 114-126.

Upadhyaya H., Dutta BK., Panda SK. (2013). Zinc modulates drought-induced biochemical damages in tea [*Camellia sinensis* (L) O Kuntze]. J. Agric. Food Chem. 61(27), 6660-6670.

Vessey JK. (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571-586.

Wang Y., Wang H., Yang, CH., Wang Q., Mei R. (2007). Two distinct manganese-containing superoxide dismutase genes in *Bacillus cereus*: their physiological characterizations and roles in surviving in wheat rhizosphere. FEMS Microbiol. Lett. 272, 206-213.

White PG., Broadley MR. (2009). Biofortification of crops with seven mineral elements often lacking in human diets-iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytol. 182, 49-84.

Wöhler I. (1997). Auxin-indole derivatives in soils determined by a colorimetric method and by high performance liquid chromatography. Microbiol. Res. 152, 399-405.

CHAPTER 2

CHAPTER 2.1

Characterization and management of autochthonous bacterial strains from semiarid soils of Spain and their interactions with fermented agrowastes to improve drought tolerance in native shrub species

Elisabeth Armada, José-Miguel Barea, Paula Castillo, Antonio Roldán, Rosario Azcón

Applied Soil Ecology (Aceptado)

1. Introduction

The re-establishment of a plant cover based on autochthonous plant species, adapted to the local environmental conditions, constitutes the most effective strategy for reclaiming degraded areas in semiarid Mediterranean environments (Vallejo et al., 1999). The success of re-vegetation programmes in semiarid areas is based on the use of technologies which benefit plant establishment and improve plant drought tolerance. As plants depend on their natural protection systems, including the help from microbial activities involved in stress adaptation, managing of plant associated microbial communities may be one strategy for attenuating the negative effect of detrimental factors such as drought (Azcón et al., 2013; Dimkpa et al., 2009; Pozo et al., 2015).

Plant growth promoting rhizobacteria (PGPR) play important roles in aiding to solve environmental problems and thus can help plant establishment and growth by several direct and indirect mechanisms (Kasim et al., 2013), this leads to increase tolerance of plants to stress situations as those caused by water shortage (Naveed et al., 2014). In fact, PGPR have been shown to affect the water balance of both well-watered and stressed plants (Kohler et al., 2008). Indeed, physiological variables such as stomatal conductance, transpiration rate and leaf water potential are generally affected by bacterial inoculation under water limited conditions (Benabdellah et al., 2011). Environmental stress factors affecting semiarid ecosystem decreased the diversity and density of microbial populations but microbial propagules do not completely disappear which is an indication of stress adaptation (Azcón et al., 2013; Barea et al., 2011). Drought adapted and tolerant microbial ecotypes are the best candidates to be used as inoculants

in reforestation programs under semiarid, water limited conditions (Alguacil et al., 2003; Caravaca et al., 2002).

The application of PGPR for the ecological restoration under natural soil conditions has been little explored. In this respect, the application of organic amendments to the soil, prior to the inoculation of beneficial microorganisms, as PGPR, might be recommended. As previously reported organic amendments are able to increase soil microbiota activity, particularly in degraded soils under semiarid conditions (Medina et al., 2004). The beneficial effects of organic amendments include provision of plant nutrients, increased humus content and thereby increased water–holding capacity, improved soil structure, and increased microbial activity (Caravaca et al., 2002). The extractions of sugar from the sugar beet produced agrowastes, but these products can only be used as organic amendment after biological transformation processes. In this context, the application of fermented agrowaste with microbiologically-solubilized rock-phosphate has been assayed for improving plant performance under stress conditions (Medina et al., 2004). Fermented agrowaste can be used as energy sources for heterotrophic microorganims such as PGPR as suggested by Bashan and Holguin (1998).

Accordingly, this investigation aims for the isolation, identification and characterization of autochthonous bacteria from semiarid soil (Murcia province of Spain) for their drought tolerant capacity and to assess their potential to act as PGPR on autochthonous shrubs. The use of drought-tolerant shrubs in semi-arid regions is one of the ways to conserve soil. As previously indicated PGPR can promote plant growth through different mechanisms in which biostimulation and/or biofertilization are involved (Azcón et al., 2013). As biofertilizer, PGPR increase the uptake of nutrients (P from phosphate solubilization and N from N_2-fixation) and as biostimulator by the production and/or modulation of phytohormones [indole acetic acid (IAA), abcisic acid (ABA), salicilic acid (SA), jasmonic acid (JA) and others] affecting plant physiology, root architecture and plant resistance to stress factors (Zahir et al., 2004). The bacterial production of hormones-like compounds has been shown to play an important role in ameliorating effects of drought and other stress factors (Glick, 2012; Groppa et al., 2012).

To evaluate PGPR abilities and the drought resistance capacity of these autochthonous bacterial isolates we determined variables related with plant biostimulation and also with cellular drought tolerance as production of proline, poly-ß-hydroxybutyrate (PHB), antioxidant ascorbate peroxidase (APX) and catalase (CAT) enzymes, and 1–aminocyclopropane–1–carboxylate (ACC) deaminase. The potential growth of bacterial cells under non-stress and drought stress conditions was also assessed. Drought is connected with the accumulation of ROS causing severe cell oxidative damage. Thus, a decrease in the oxidative stress in cells suggests lower stress symptoms and resulted important for cells survival under drought. Antioxidant enzymes superoxide dismutase (SODs), CATs and APXs are widely

distributed in aerobic bacteria but there are few studies in relation to the ability of bacteria to resist drought stress. Proline could contribute to the scavenging of free radicals produced by stress conditions in addition to its main role as an osmoprotectant under water-deficit (Azcón et al., 2010). The PHB is produced by bacteria when they are subjected to stress as a mechanism that favors their establishment and survival (Okon and Itzigsohn, 1992). In this context, Ayub et al (2004) suggested the relationship between PHB accumulation and high stress resistance. Bacteria are able of ACC deaminase production, the immediate precursor of ethylene in higher plants, and its regulation has been described as the major mechanism by which bacteria exert beneficial effects on plants under abiotic stress conditions (Saleem et al., 2007). Particularly, we hypothesized that drought resistance capacity of bacteria can be ascribed, at least partially, to the proline and antioxidative enzyme metabolism. Thus, we expected that the level of drought adaptation capacity of bacteria ought to be related with the oxidative stress attenuation.

Considering these premises, we postulate that the inoculation of autochthonous shrub plants (*Thymus vulgaris*, *Santolina chamaecyparissus, Lavandula dentata* and *Salvia officinalis*) with autochthonous drought resistant bacteria, having PGPR traits, can confer drought tolerance to these plants improving nutrition and altering physiological parameters. PGPR abilities and related processes are regulated in general by activities which confer resistance and intrinsic stress tolerance of both bacteria and plants. Accordingly, the objective of the present study was to isolate and characterize drought tolerant autochthonous bacterial strains, afterthought analyze their effects, in comparison with a reference strain (also drought tolerant) from our culture collection, on growth, nutrition and drought tolerance markers of four autochthonous shrubs and their modulation by the application of a fermented agrowastes (compost). Additionally, autochthonous bacteria can positively interact with native arbuscular mycorrhizal (AM) fungi, existing in the natural soil, thus AM development was also evaluated since such microbial interactions may affect plant drought tolerance. Some bacteria have been named mycorrhiza helper bacteria for their ability to promote mycelia growth and mycorrhiza formation (Frey-Klett et al., 2007).

2. Material and Methods

The experiment I consisted in the isolation of autochthonous bacteria from the rhizosphere of autochthonous shrubs (four) from the semiarid environment. The soil used was located in the Natural Ecological Park “Vicente Blanes” in the Province of Murcia (Southeast Spain). This area suffers drought and low nutrient availability and desertification processes. The soil in the experimental area is a Typic Torriorthent (SSS, 2006) very little developed with a silty-clay texture that facilitates the degradation of soil structure, and low organic matter

content. The vegetation in the zone was predominated by *T. vulgaris*, *S. chamaecyparissus, L. dentata* and *S. officinalis* growing with a patchy distribution. The climate in this semiarid Mediterranean zone is a mean annual temperature of 20 ºC and rainfall of 250 mm. The main soil characteristics are: organic carbon 0.94%, total N 0.22%, P 1.36 mg kg^{-1} (Olsen test), pH 8.9 and an electric conductivity of 1.55 dS m^{-1}.

Supposedly, isolated bacteria were adapted to drought and only were selected the most abundant and representative bacteria. Later they were identified by molecular techniques. We tested whether these autochthonous isolates actually were drought tolerant bacteria along with a drought-tolerant *Bacillus megaterium* strain (Accession CECRIbio 04 similarity 98%) from a culture collection selected in semiarid zone in previous experiments (Marulanda et al., 2006; 2009). The autochthonous bacterial abilities to cope with drought and their functional traits under osmotic stress conditions were analyzed in the experiment. In a subsequent bioassay (Experiment II), we evaluated the effect of these selected bacteria on the four most representative autochthonous shrub species growing in a soil under drought conditions. The treatments used in this Experiment II were: Three autochthonous bacteria and one from collection were inoculated in presence or absence of fermented agrowaste in each one of selected shrub. Plants without fermented agrowaste or bacteria were also assayed as controls. Each treatment was replicated five times a total of 50 pots per plant. The experiment consisted of a factorial block design (5 x 2) for each plant with five inoculations each with and without fermented agrowaste (total 10 treatments).

2.1. Experiment I

2.1.1. Isolation of bacteria autochthonous from a semiarid environment

The soil samples (three repetitions) for bacterial isolation were taken from the rhizosphere of (four) shrubs naturally growing in a Mediterranean semiarid soil from Murcia province (Spain). This soil was used as test soil for the greenhouse inoculation experiment (Experiment II).

Bacterial isolation was carried out following a conventional procedure: 1 g of homogenized rhizosphere soil was suspended in 9 mL of sterile water, to perform dilutions (10^{-2} to 10^{-4}), which were spread on Yeast Mannitol Agar (YMA), Potato Dextrose Agar (PDA), Luria-Bertani (LB) agar and incubated at 28 ºC for 48 h, to isolate bacteria. The most representative (abundant) colonies of different morphological appearances (the three most abundant cultivable types) were selected. Morphology and mobility of bacteria were examined by microscopy. In addition, *B. megaterium* from our collection was assayed as reference strain. It was previously selected as PGPR and drought tolerant strain from a similar semiarid soil. These three representative bacterial strains and *B. megaterium* were grown individually in 250-

mL flasks containing 50 mL of nutrients broth medium in shake culture for 48 h at 28 ºC for inocula preparation.

2.1.2. Molecular identification of the bacterial strains

Identification of isolated bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were collected, diluted, lysed and their DNA used as a template in the PCR reactions. All reactions were conducted in 25 µL volume containing PCR buffer 10X, 50 mM $MgCl_2$, 10 µM each primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), 5 U/µL of Taq polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with the following conditions: 5 min at 95 ºC, followed by 30 cycles of 45s at 95 ºC, 45s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. PCR products were analyzed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (NCBI) using BLAST program (Altschul et al., 1990).

2.1.3. Bacterial growth under increasing polyethylene glycol (PEG) levels in the growing medium

Autochthonous bacterial isolates and the *B. megaterium* used as a reference strain were grown at 28 ºC in an axenic medium (nutrient broth, 8 g L^{-1}) supplemented or not with increasing PEG concentrations (0%, 15%, 30% and 40%) to generate osmotic stress (equivalent to -1.02; -1.50; -3.60 and -3.99 MPa). This allows to test bacterial osmotic stress tolerance along the time, by estimating the number of viable cells, as cfu mL^{-1}. Number of viable cells was estimated after 4 and 6 days of growth following a conventional procedure: 1 mL of suspension was plated in agar nutrient broth medium. The bacterial growth was monitored by measuring optical densitiy at 600 nm. The four PEG treatments were replicated 3 times in the culture of each bacterial strain giving a total of 48 tubes.

2.1.4. Plant growth promoting bacterial activities growing without stress and with stress caused by application 40% polyethylene glycol (PEG) in the growing medium.

The four bacterial isolates were cultivated (three replicates) at 28 ºC in 100 mL of liquid nutrient medium for 48 h on a rotary shaker at 120 rpm supplemented or not with 40% of PEG (-3.99 MPa) in order to induce drought stress conditions. This level of PEG was selected in preliminary studies as the maximum PEG concentration supportable by bacterial strains.

The accumulation of proline was estimated by spectrophotometric analysis at 530 nm (Bates et al., 1973). The bacterial extracts react with ninhydrin and glacial acetic acid during 1 h at 100 ºC. The reaction stops by introducing the tubes in ice bath. The reaction mixture is

extracted with 2 mL of toluene, shaking vigorously for 20 seconds. A standard curve was prepared with known concentrations of proline.

Measurement of lipid peroxidation was done by the method based on the reaction of thiobarbituric acid (TBA) with reactive species derived from lipid peroxidation, particularly malondialdehyde (MDA). Detection of thiobarbituric acid reactive species (TBARS) was carried out by a colorimetric assay described by Buege and Aust (1978) with some modifications (Espindola et al., 2003). 50 mg of cells were resuspended in 500 µL of 50mM phosphate buffer (pH 6.0) containing 10% trichloroacetic acid (TCA), and 0.3 g glass beads were added. The samples were broken by three cycles of 1 min agitation on a vortex mixer followed by 1 min on ice. After centrifugation, supernatans were mixed with 0.1 mL of 0.1M EDTA and 0.6 mL of 1% (w/v) TBA in 0.05 M NaOH. The reaction mixture was incubated at 100 ºC for 15 min and then cooled on ice for 5 min. The absorbance was measured at 532 nm. Lipid peroxidation was expressed as µmoles of malondialdehyde g^{-1} of dry cell weight.

The method for the extraction of antioxidant enzymes in the microbial cells was described by Azcón et al. (2010). Bacterial cells were homogenized in a cold mortar with 4 ml 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, 8 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 6000 rpm for 15 min at 4ºC, and the supernatant was used for enzyme activity determination. Catalase (CAT) activity was measured as described by Aebi (1984), conducted in 2 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µL of enzyme extract. It was determined the consumption of H_2O_2 and followed by decrease in absorbance at 240 nm for 1 min [extinction coefficient (ε_{240}) of 39.6 mM^{-1} cm^{-1}]. Ascorbate peroxidase (APX) activity was measured in a 1 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide and 0.5 mM sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako et al., 1994). Total soluble protein amount was determined using Bradford method (1976), and bovine serum albumin as standard.

The poly-ß-hydroxybutyrate (PHB) production of the four bacterial strains on different osmotic concentrations (0% and 40% PEG) in N_2 deficient medium (pH 7) and incubated at 28 ºC for 72 h at 120 rpm was measured. PHB produced were extracted as described in the method of Ramsay et al (1994). The amount of PHB in the extracts was determined spectrophotometrically at 235 nm (Law and Slepecky, 1961; Lee et al., 1995). A standard curve was prepared to determine PHB in mg mL^{-1}.

The production of indole-3- acetic acid (IAA) by these bacteria was determined using the Salper's reagent (Gordon and Paleg, 1957). Three milliliters of fresh Salper's reagent (1mL 0.5

M $FeCl_3$ in 50 mL 37% $HClO_4$) was added to free-cell supernatant and kept in complete darkness for 30 minutes at room temperature, and the optical density at 535 nm was measured in each treatment (Wöhler, 1997). A standard curve was also prepared for IAA determination.

The activity of ACC deaminase enzyme in isolates was measured as described by Penrose and Glick (2003). The enzyme activity was assayed according to a modification of the method of Honma and Shimomura (1978) which measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase hydrolyses ACC. The quantity of μmol of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 1.0 mmol and 1.0 μmol. Protein concentration of cellular suspension in the toluenized cells was determined by the method of Bradford (1976).

To determine phosphate solubilization index (PSI), each bacterial culture was assayed on Pikovskaya agar plates (Pikovskaya, 1948) containing tricalcium phosphate ($Ca_3(PO_4)_2$) as insoluble phosphate source. Cells were grown overnight in LB medium, next they were washed twice with 0.9% NaCl and re-suspended in 0.9% NaCl to produce equal cell densities among all the isolates. Solutions were inoculated on the agar plates and incubated at 30 °C, and observed daily for 7 days for appearance of transparent "halos" (Katznelson and Bose, 1959). Experiments were performed in triplicate. Phosphorus solubilization index was measured using following formula (Edi-Premono et al., 1996):

PSI= (Colony diameter + Halo zone diameter) / Colony diameter

2.1.5. Hormones production by the bacterial strains growing without and with 15% of polyethylene glycol (PEG) in the growing medium

Bacterial strains were grown in LB medium with and without 15% PEG (-1.50 MPa) for four days to determine the production of these phytohormones. Treatments were replicated three times. The PEG concentration here used (15%) was selected because bacterial growth was quite considerable and it avoid problems in the detection of these hormones.

Bacterial culture medium (0.2 g) was homogenized in 5 mL ultrapure water and added with 20 μL of a mixture of internal standards containing, 50 ng [$^2H_6$]-ABA, 50 ng [$^2H_4$]-SA, 50 ng [$^2H_6$]-JA, and 50 ng [$^2H_5$]-OPDA (12-oxo phytodienoic acid). Centrifugation was performed at 5000 *g* for 15 min, the pellet was discarded, the pH of the supernatant was adjusted to 2.8 with acetic acid, and the supernatant was partitioned twice against an equal volume of diethyl ether (Durgbanshi et al., 2005). The aqueous phase was discarded, and the organic fraction was evaporated. The solid residue was resuspended in 1.5 mL methanol (MeOH) and filtered through a 0.22 μm cellulose acetate filter. The organic fraction was evaporated at 35 ºC in a

Speed Vac model SC110 (Savant Instruments Inc., New York, NY, USA) and resuspended in 50 µl 100% MeOH. A 5 µL aliquot of this solution was injected into the HPLC system.

HPLC analysis was performed using an Alliance 2695 (Separation Module, Waters, Milford, MA, USA) quaternary pump equipped with an auto-sampler. A Restek C18 (Restek, Bellefonte, PA, USA) column (2.1 x 100 mm, 5 µm) was used at 28 ºC with injected volume 5 µL. The binary solvent system used for the elution gradient consisted of 0.2% acetic acid in H_2O (solvent B) and MeOH (solvent A) at a constant flow rate of 200 µL min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied [t (min), % A]: (0, 40), (25, 80), with 7 min for re-equilibration. MS/MS was performed using a Micromass Quatro Ultima TM "*Pt*" double quadrupole mass spectrometer (Micromass, Manchester City, UK). All of the analyses were performed using a turbo ion spray source in negative ion mode with the following settings for SA, JA, ABA, and OPDA: capillary voltage -3000 V, energy cone 35 V, RF Lens1 (20), RF Lens2 (0.3), source temp 100 ºC, de-solvation temp 380 ºC, gas cone 100 l h^{-1}, gas de-solvation 70 l h^{-1}, collision (50), and multiplier (650). The MS/MS parameters were optimized in infusion experiments using individual standard solutions of SA, JA, ABA and OPDA at a concentration of 10 ng μL^{-1} diluted in mobile phase A/B (40:60, v/v). MS/MS product ions were produced by collision-activated dissociation of selected precursor ions in the collision cell of the mass spectrometer, and mass was analyzed using the second analyzer of the instrument. Quantification was performed in the multiple reaction monitoring (MRM) mode.

2.2. Experiment II

2.2.1. Fermentation agrowaste process

Aspergillus niger NB2 strain was used in this study. It was shown to produced organic acids, mainly citric acid when growing on complex substrates and to mineralize lignocellulosic materials (Vassilev et al., 1998) and solubilized the rock phosphate (RP) (Medina et al., 2006). Sugar beet waste a lignocellulosic material [cellulose (29%), hemicellulose (23%) and lignin (5%)], was ground in an electrical grinder to 1 mm fragments. It was mixed at a concentration of 10% with 50 mL Czapek's solution containing (g L^{-1} of distilled water): $FeSO_4$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $NaNO_3$, 3.0; sucrose, 30; K_2HPO_4, 1.0 and a final pH of 7.3 ± 0.2 for static fermentation in 250 mL Erlenmeyer flasks. Rock-phosphate at a concentration of 1.5 g L^{-1} was added. This medium was inoculated with 3 mL of *A. niger* spore suspension (1.2 x 10^6 spores). Static fermentation was performed at 28 ºC for 20 days. Result in a product that can be used as organic amendment in the soil/plant system.

2.2.2. Bacterial inoculation and plant growth conditions

The substrate used in this assay consisted in the target soil, previously described. It was screened (5mm), and mixed with sterile sand [5:2 (v/v)]. The capacity of pots was of 0.5 kg.

The fermented agrowaste was mixed at 2% (v/v) with the soil in half of the pots. Pots filled with 0.5 kg of the soil/sand mixture added or not with fermented agrowaste were stabilized for two weeks before to start the experiment. One millilitre of pure bacterial culture (10^8 cfu mL^{-1}) of each bacteria (*B. megaterium*, *Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) grown in LB medium for 48 h at 28 ºC, was applied to the appropriate pots. These treatments were replicated five times with a total of 200 pots placed in a random complete block designs.

Shrub seedlings were grown in 0.5 kg pots in a greenhouse under controlled conditions (18-24 °C, with a 18/6 light/dark period and 50% of relative humidity). A photoperiod of 16 h at a photosynthetic photon flux density (PPFD) of 400-700 μmol m^{-2} s^{-1} as measured with a light meter (model LI-188B; Licor Inc., Lincoln, NE, USA) was maintained during the experiment by supplementary light to compensate natural illumination.Water was supplied daily to maintain constant soil water close to field capacity (17% volumetric soil moisture) during 2 weeks after transplanting. After this time, and during a period of 1 year, these plants were allowed to dry until soil water content was 50% of field capacity. However, during the 24-h period comprised between each re-watering the soil water content was progressively decreasing until a minimum value of 30% of field capacity. Soil moisture was measured with an ML2 X ThetaProbe (AT Delta-T Devides Ltd, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moisture (Roth et al., 1992). This volumetric soil moisture is considered to be a normal environmental condition in dry Mediterranean areas. A completely random experimental design was adopted.

2.2.3. Plant biomass and nutrients content

One year after planting, plants were harvested (five replicates per each treatment). Dry biomass of roots and shoots (data non-shown) and nutrients concentrations were determined.

Shoot content (mg $plant^{-1}$) of P, K, Ca, Mg as well as of Zn, Fe, Mn and Cu (μg $plant^{-1}$) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Mineral analysis was carried out by the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura (CSIC) Murcia, Spain.

2.2.4. Stomatal conductance, photosynthetic efficiency and proline content and in shoot of *Lavandula dentata* and *Salvia officinalis*

Before harvest some physiological plants values as stomatal conductance and photosynthetic efficiency was measured, only in *L. dentata* and *S. officinalis*. In the two remaining plants (*T. vulgaris* and *S. chamaecyparissus*) because of its reduced number of leaves and small area was impossible to make the mentioned determinations.

Stomatal conductance was measured by using a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK).

Photosystem II efficiency was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll *a* fluorescence. FluorPen quantifies the quantum yield of photosystem II as the ratio between the actual fluorescence yield in the light-adapted state (F_v) and the maximum fluorescence yield in the light-adapted state (F_m), according to Oxborough and Baker (1997). Measurements of stomatal conductance and photosynthetic efficiency were taken in the 2nd youngest leaf of two different plants of each treatment.

The proline was extracted in 100 mM phosphate buffer (pH 7.8) from 0.5 g of fresh leaves, previously immersed in liquid N_2 and stored at -80 ºC according to Bates et al. (1973). Proline was estimated by spectrophotometric analysis at 520 nm using the ninhydrin reaction (Bates et al., 1973).

2.2.5. Percentage of arbuscular mycorrhizal (AM) fungal root colonization and glomalin production

Intraradical arbuscular mycorrhizal (AM) fungal colonization was assessed after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Philips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

The extraradical fungal development was determined as glomalin related soil protein (GRSP), operationally measured as Bradford-reactive soil protein (Rillig, 2004). It was recovered from soil according to the method described by Wright and Upadhyaya (1998) with minor modifications. For the easily extractable fraction of GRSP (EE-GRSP) samples of 1 g soil were subjected to extraction with 8 mL of 20 mM citrate pH 7.0, and autoclaving for 30 min at 121 °C.

Glomalin is a stable molecule with a high C content (until 50%) (Rillig, 2004) and acts in the soil aggregation (Wright and Upadhyaya, 1996).

2.3. Statistical analysis

Data from both experiments were analyzed using the SPSS 21 software package from Windows. For Experiment I, we used a one-way ANOVA, followed by Duncan's multiple-range test (Duncan, 1955) to find out significant differences at $p \leq 0.05$. For Experiment II, was based on a randomized complete factorial block design (5 × 2) for each plant species, consisting of 5 inoculations treatments with and without fermented agrowaste giving a total of 10 treatments. These treatments were analyzed with a general linear model ANOVA, followed by Duncan's multiple range test to find out significant differences at $p \leq 0.05$. Percentage values were arcsine-transformed before statistical analysis.

For the Pearson correlation analyses significant differences were determined at $p \leq 0.001$ in Experiment I and $p \leq 0.01$ in Experiment II.

3. Results

3.1. Experiment I

Autochthonous bacterial strains were identified as *Enterobacter* sp. (Accession NR 044977.1 similarity 99%), *Bacillus thuringiensis* (Accession NR 043403.1 similarity 98%) and *Bacillus* sp. (Accession NR 043403.1 similarity 91%). These bacteria were assayed under axenic conditions to evaluate their osmotic stress tolerance and their PGPR characteristics under stress situations.

The increasing levels of PEG in the growing medium specifically affected bacterial growth. *Enterobacter* sp. was the most stress tolerant bacteria showing the greatest growth under -3.6 and -3.9 MPa, whereas *B. megaterium* was the most sensitive to such conditions (Fig. 1). The PEG concentration is correlated with the growth of each bacterial species ($r = -0.75$; $p \leq 0.001$).

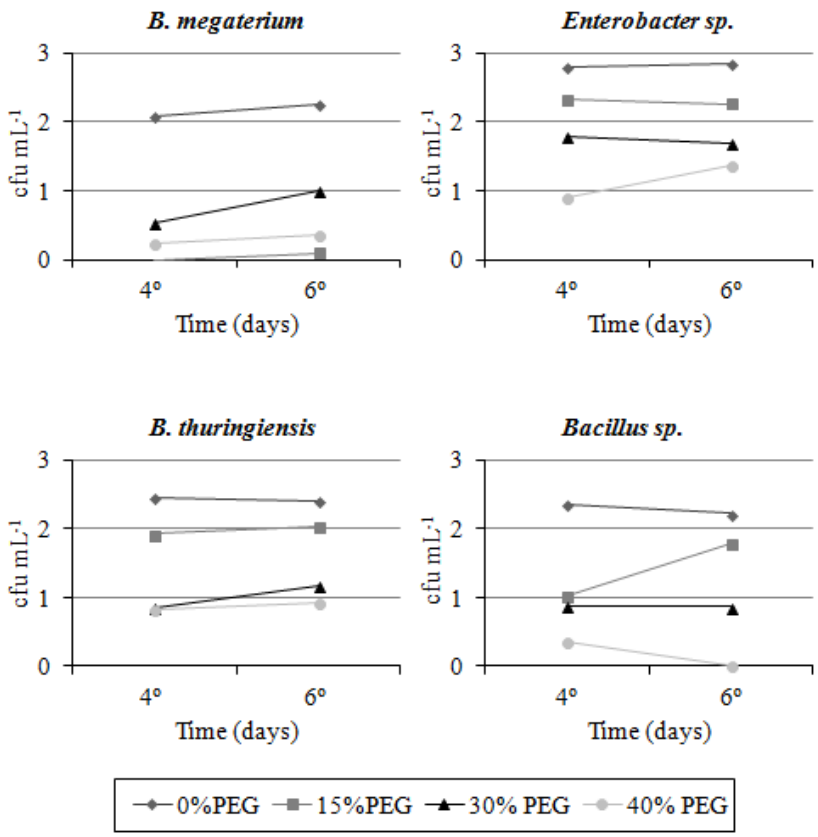


Figure 1. Viable cells (cfu mL^{-1}) of bacterial strains growing in axenic nutrient medium supplemented with increasing levels of PEG (equivalent to -1.02; -1.50; -3.60 and -3.99 MPa) at different time intervals (from 4 to 6 days).

Table 1 shows bacterial metabolic characteristics related to stress tolerance and/or their PGPR abilities under stress (40% PEG) and non stress conditions (0% PEG) in the growing medium. We observed that stressed bacterial cells accumulated more proline, particularly *Bacillus* sp. and *Enterobacter* sp., the two strains which were the lower proline producer in non-stress conditions. The oxidative lipid damage (MDA) increased with the stress particularly in *B. megaterium* and *Enterobacter* sp. and did not change in *Bacillus* sp. The CAT and APX antioxidant activities varied with the bacterial strain involved. *B. thuringiensis* showed the highest levels of antioxidants without stress and *Bacillus* sp. and *Enterobacter* sp. the lowest CAT and APX activities irrespective of stress conditions. With the application of stress factor increased both antioxidant activities in the allochthonous *B. megaterium*. Nevertheless the 40% PEG did not change (*Bacillus* sp. or *Enterobacter* sp.) or reduced (*B. thuringiensis*) these both antioxidant activities. The PHB accumulation was quite similar in the three *Bacillus* strains but under osmotic stress conditions only *B. thuringiensis* and *Bacillus* sp. increased but *B. megaterium* decreased the synthesis of this compound (Table 1).With regard to PGPR activities

the highest production of IAA was found in *Enterobacter* sp. non-stressed cells, but with the stress situation (40% PEG) IAA production decreased in this bacterial culture. In contrast, *B. megaterium* significantly increased IAA production under osmotic stress conditions by 4.5 folds, or did not change in *B. thuringiensis* and *Bacillus* sp. cultures (Table 1).

The stress conditions reduced ACC production in *B. megaterium* and *Enterobacter* sp. or increased this compound in *B. thuringiensis* and *Bacillus* sp. All target bacteria were able to produce other phytohormons such as SA, JA, ABA and OPDA under moderate stress (15% PEG) and non-stress conditions (Table 2). Without stress induction *Bacillus* sp. produced the highest amount of SA but stress greatly decreased the ability by this bacterium for the production of these compounds. The stress, in general, reduced JA production (having a significant correlation $r = -0.941$; $p \leq 0.001$), but enhanced OPDA production by most of the tested bacteria (except in the *Enterobacter* sp.). Nevertheless, ABA production was quite similar under non-stress and stress conditions in all bacteria tested. But no generalization can be given on SA production with regards stress effect (stimulating for *B. thuringiensis*, negative for *Bacillus* sp. and *Enterobacter* sp. and neutral for *B. megaterium*). The decreased SA amount under stress was only noted in the greatest SA producer as *Bacillus* sp. and *Enterobacter* sp. strains (Table 2). These results showed the complexity of mechanisms involved in the bacterial drought tolerance.

All the assayed bacteria showed PSI ability being this value highest in *B. megaterium* and *Enterobacter* sp. but the PSI was greatly depressed by the stress induction in *Enterobacter* sp. (Table 1).

In general, these drought tolerant bacteria highly reduced the levels of PGPR metabolites as ACC, IAA, and PSI by the osmotic stress, as well the antioxidant CAT activity.

Table 1. Proline, lipid peroxidation (MDA), antioxidant enzymatic [catalase (CAT) and ascorbate peroxidase (APX)] activities, poly-β-hydroxybutyrate (PHB), indolacetic acid (IAA), α-ketobutyrate (ACC) production and phosphorus solubilization index (PSI) by the reference *Bacillus megaterium* or autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) after four days of growth in axenic culture medium supplemented or not with 40% polyethylene glycol (PEG).

	[PEG]	mmol proline mg^{-1} prot	µmol MDA g^{-1} dry cell weight	µmol CAT mg^{-1} prot	µmol APX mg^{-1} prot	mg PHB mL^{-1}	µg IAA mg^{-1} prot	mmol α-ketobutyrate mg^{-1} prot	PSI
B. megaterium	0%	0.14 b	2.5 b	164 e	2,340 c	0.32 c	38.2 b	0.65 c	1.90 c
	40%	1.21 e	30.0 g	401 f	4,888 d	0.01 a	183.2 d	0.35 b	1.63 bc
B. thuringiensis	0%	0.05 a	6.7 d	22 b	238 a	0.01 a	110.0 c	0.37 b	2.06 c
	40%	1.10 d	20.5 f	2 a	217 a	0.08 b	53.0 b	0.22 a	1.00 a
Enterobacter sp.	0%	0.12 b	0.7 a	606 g	11,760 e	0.33 c	18.2 a	0.20 a	1.56 b
	40%	0.31 c	4.4 c	46 d	586 b	0.38 cd	13.0 a	0.41 b	1.37 b
Bacillus sp.	0%	0.05 a	10.0 c	32 c	277 a	0.31 c	10.0 a	0.41 b	1.00 a
	40%	1.50 f	10.2 c	26 b	261 a	0.45 d	10.3 a	1.09 d	1.00 a

Values within each column, having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

Table 2. Salicilic acid (SA), jasmonic acid (JA), abcisic acid (ABA) and 12-oxo phytodienoic (OPDA) produced by the reference *Bacillus megaterium* or autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus sp.*) growing four days in axenic culture medium supplemented or not with 15% polyethylene glycol (PEG).

	[PEG]	SA (pmol g^{-1})	JA (pmol g^{-1})	ABA (pmol g^{-1})	OPDA (pmol g^{-1})
B. megaterium	0%	1,494.5 a	525.9 cd	163.3 ab	1,577.7 a
	15%	1,351.0 a	186.1 b	152.8 a	3,813.5 c
Enterobacter sp.	0%	13,321.2 d	400.7 c	162.6 ab	2,700.3 b
	15%	3,498.0 b	108.3 a	153.2 a	1,891.3 ab
B. thuringiensis	0%	1,722.7 a	376.4 c	164.9 b	1,283.8 a
	15%	7,051.6 c	209.8 b	145.8 a	2,949.3 bc
Bacillus sp.	0%	17,220.5 e	428.3 c	148.8 a	3,176.8 b
	15%	3,091.8 b	145.8 b	150.5 a	8,648.9 d

Values within each column, having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

3.2. Experiment II

Plant biomass (shoot and root growth) were not significantly affected by the treatments applied (data not shown). Nevertheless, shoot nutrients content in the four shrub species grown with and without fermented agrowaste were affected, but differently, by the bacterial inocula. In soil without fermented agrowaste addition *B. thuringiensis* increased P content ranging between 11% in *S. chamaecyparissus* to 51% in *T. vulgaris*, and for K content the effect ranged between 28% in *S. chamaecyparissus* to 63% in *L. dentata*. Nutrient uptake was maximized by *B. thuringiensis* inoculation in most of the shrubs grown without fermented agrowaste, with the exception of *S. officinalis* (Table 3).

Table 3. P, K, Ca and Mg content (mg plant^{-1}) in four autochthonous plants (*Thymus vulgaris, Santolina chamaecyparissus, Lavandula dentata* and *Salvia officinalis*) non-inoculated (control) or inoculated with the reference *Bacillus megaterium* or autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) grown in an arid Mediterranean soil amended or not with fermented agrowaste (CO), under drought conditions.

	P		K		Ca		Mg	
	(-)	CO	(-)	CO	(-)	CO	(-)	CO
				Thymus vulgaris				
Control	0.6a	1.2d	7.1a	13.3d	6.0a	11.3c	1.7a	2.9d
B. megaterium	0.7b	0.9c	9.3b	11.2c	7.0a	7.7ab	2.0ab	2.2b
Enterobacter sp.	0.7b	0.9c	10.0b	10.9bc	8.7b	10.3b	2.2b	2.6c
B. thuringiensis	0.9c	1.1cd	10.4b	12.9d	7.9b	9.1b	2.2b	2.5c
Bacillus sp.	0.8c	1.2d	10.3b	10.5b	8.4b	9.9b	2.0b	2.8cd
				Santolina chamaecyparissus				
Control	0.9a	1.1b	10.1a	11.4b	8.4b	9.3b	1.0a	1.2b
B. megaterium	0.8a	2.3c	8.6a	18.9c	6.8b	15.2d	0.9a	2.4d
Enterobacter sp.	1.0b	1.0b	11.9b	8.4a	11.2c	5.2a	1.4b	0.8a
B. thuringiensis	1.0b	1.0b	12.9b	11.2b	11.9c	9.0b	1.4c	1.1b
Bacillus sp.	0.9a	0.9a	11.3b	11.0b	8.8b	8.9b	1.1a	1.1a
				Lavandula dentata				
Control	0.6ab	0.8b	13.5a	19.6bc	13.3b	10.9a	2.1b	1.9a
B. megaterium	0.5a	1.4c	18.4b	26.0d	15.3b	16.9c	2.4ab	2.7b
Enterobacter sp.	0.5a	1.3c	15.0a	26.1d	9.8a	16.3c	1.4a	2.9c
B. thuringiensis	0.6ab	0.9b	21.9c	19.1bc	16.8c	11.1a	2.9bc	1.6a
Bacillus sp.	0.6ab	0.9b	19.5b	20.4bc	15.4bc	13.0ab	2.6b	2.1ab
				Salvia officinalis				
Control	0.6a	0.8a	9.9a	8.7a	8.3a	7.3a	2.6a	2.7a
B. megaterium	0.8a	1.3b	9.9a	13.2b	12.2b	12.7b	3.2ab	3.5bc
Enterobacter sp.	0.7a	1.1ab	8.9a	12.9ab	10.8b	11.2b	2.7a	3.3ab
B. thuringiensis	0.8a	1.0ab	9.4a	10.3a	11.2b	12.0b	2.9ab	2.7a
Bacillus sp.	0.8a	1.1ab	10.6a	10.9a	16.9c	11.7b	3.3ab	3.2ab

Within each shrub species and each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

Table 4. Zn, Fe, Mn and Cu content (μg plant^{-1}) in four autochthonous plants (*Thymus vulgaris, Santolina, chamaecyparissus, Lavandula dentata* and *Salvia officinalis*) non-inoculated (control) or inoculated with the reference *Bacillus megaterium* or autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) grown in an arid Mediterranean soil amended or not with fermented agrowaste (CO), under drought conditions.

	Zn		Fe		Mn		Cu	
	(-)	CO	(-)	CO	(-)	CO	(-)	CO
				Thymus vulgaris				
Control	33.3 a	54.2 b	42.8 a	118.5 c	40.4 a	69.7 c	4.2 a	6.1 d
B. megaterium	46.7 b	45.4 b	60.9 ab	98.5 bc	50.3 ab	54.8 b	5.5 c	6.0 cd
Enterobacter sp.	53.8 b	57.1 b	80.1 b	120.5 c	62.8 b	61.3 b	4.9 b	6.6 d
B. thuringiensis	54.1 b	55.1 b	115.2 c	112.7 c	46.5 a	62.0 b	6.4 d	6.1 d
Bacillus sp.	45.8 b	50.8 b	119.2 c	147.1 d	50.7 a	61.8 b	5.5 c	6.9 d
				Santolina chamaecyparissus				
Control	60.9 c	62.3 cd	87.2 c	99.5 d	100.2 b	97.5 c	11.1 c	8.7 b
B. megaterium	53.6 b	87.1 de	70.0 b	123.7 e	74.0 a	198.8 d	9.7 b	12.3 d
Enterobacter sp.	72.8 d	40.9 a	77.8 b	27.9 a	127.7 c	79.0 a	12.5 d	5.2 a
B. thuringiensis	89.7 e	61.0 c	78.4 b	89.8 c	121.9 c	98.8 c	13.2 d	9.6 b
Bacillus sp.	78.0 d	60.4 c	77.7 b	89.4 c	98.9 c	97.6 c	12.4 d	9.4 b
				Lavandula dentata				
Control	38.5 b	36.6 b	104.2 b	85.2 b	13.4 a	17.9 b	5.2 b	6.3 c
B. megaterium	37.8 b	43.7 b	67.0 a	103.3 b	17.2 b	27.1 d	5.6 b	6.9 c
Enterobacter sp.	31.2 a	45.2 c	58.7 a	108.9 b	13.5 a	19.2 b	4.4 a	7.3 c
B. thuringiensis	47.4 c	46.0 c	100.2 b	79.9 b	20.6 c	16.0 b	7.2 c	5.8 b
Bacillus sp.	41.6 b	46.5 c	122.2 b	93.3 b	17.8 b	20.3 b	5.7 b	5.3 b
				Salvia officinalis				
Control	29.5 b	23.9 a	56.4 b	39.1 a	17.3 b	15.0 a	4.0 a	4.0 a
B. megaterium	26.4 a	29.4 b	80.9 c	108.3 cd	17.8 b	23.8 c	4.5 a	5.8 b
Enterobacter sp.	28.5 b	28.7 b	53.2 b	92.6 c	16.9 b	20.4 bc	4.4 a	4.8 a
B. thuringiensis	24.6 a	31.1 b	50.4 b	74.6 c	19.7 bc	20.9 bc	4.5 a	4.9 a
Bacillus sp.	30.6 b	25.8 a	57.2 b	54.8 b	24.0 c	19.8 bc	6.1 b	5.4 b

Within each shrub species and each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

Nutrient contents were significantly increased by the fermented agrowaste application in three of the four shrubs, excluding *S. officinalis*. Fermented agrowaste application had an important effect in increasing shoot P and K content in *T. vulgaris* by 100% (P) and by 87% (K) in comparison to plants grown without fermented agrowaste application. Nevertheless, shrubs grown with fermented agrowaste (excluding *T. vulgaris*) and inoculated with *B. megaterium* showed maximum P and K uptake. Such effects in the case of P were: 75% (*L. dentata*), 63% (*S. officinalis*) and 109% (*S. chamaecyparissus*). In the case of K were: 33% (*L. dentata*) and 66% (*S. chamaecyparissus*). A similar trend was observed for Ca and Mg contents in these three shrubs when *B. megaterium* was inoculated (Table 3). Micronutrient (Zn, Fe, Mn and Cu) content was also enhanced when these plants were inoculated with *B. megaterium* in fermented agrowaste amended soil (Table 4). Indeed, treatments involving *B. megaterium* inoculation and fermented agrowaste, dually applied, significantly enhanced nutrient uptake in most of the target plant species (Tables 3 and 4).

Physiological parameters related to drought tolerance as proline content, stomatal conductance and photosynthetic efficiency were measured only in *L. dentata* and *S. officinalis*, as in these species shoot biomass manipulation is easier. *S. officinalis* exhibited higher accumulation of proline in fresh leaves than *L. dentata* in absence of fermented agrowaste. In *L. dentata*, bacterial inoculation decreased proline accumulation, but *B. thuringiensis* plus fermented agrowaste caused a significant increase of proline in this plant species (Fig. 2A). Nevertheless, in *S. officinalis* (without fermented agrowaste) the opposite trends were found since whatever bacterial treatment did not either change or increase proline accumulation. In general, the fermented agrowaste reduced proline production in both plants but most of bacteria increased proline level in amended soil (Fig. 2A).

The stomatal conductance mainly depends on the plant used. In general, *S. officinalis* showed a higher stomatal conductance than *L. dentata*, this value was in *L. dentata* (without fermented agrowaste) by *B. thuringiensis* reduced and in both plants with by *B. megaterium* (with fermented agrowaste). Nevertheless, the bacterial inoculants could also influence this parameter (Fig 2B).

The photosynthetic efficiency of photosystem II (F_v/F_m) was less modified in *S. officinalis* than in *L. dentata* by the bacterial treatments and/or fermented agrowaste (Fig. 2C). This value dropped by the treatments applied (bacteria or fermented agrowaste) only in *L. dentata*. However, in fermented agrowaste amended soil it was increased by the bacteria inoculation. Again, *B. thuringiensis* decreased photosynthetic efficiency in *L. dentata* without fermented agrowaste in concordance with the reduction of stomatal conductance (Fig. 2B). However, the reduction of stomatal conductance in *L. dentata* caused by *B. megaterium* + fermented agrowaste (Fig. 2B) was not reflected in the photosynthetic efficiency (Fig. 2C).

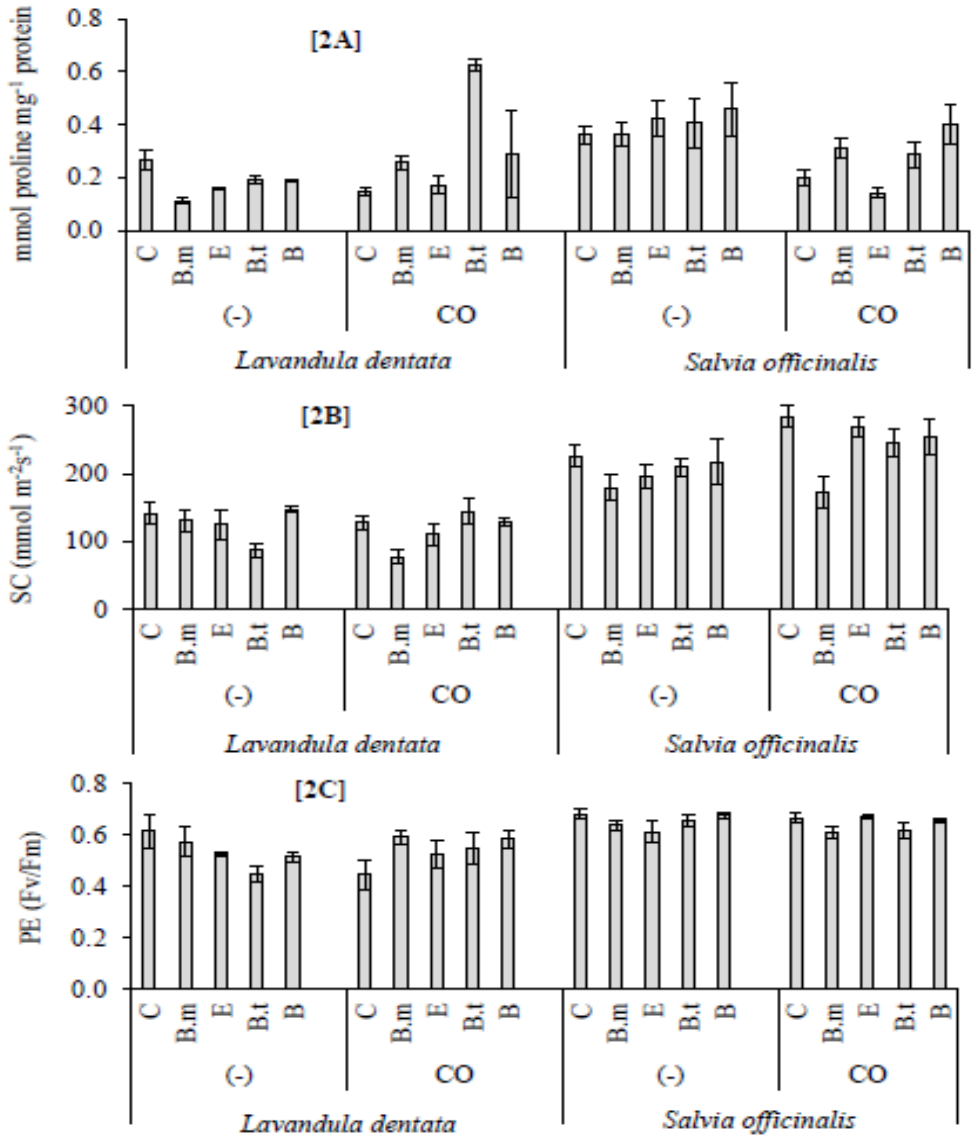


Figure 2. Proline accumulation [2A], Stomatal Conductance (SC) [2B] and Photosynthetic Efficiency (PE) [2C] in *Lavandula dentata* and *Salvia officinalis* non-inoculated (control) or inoculated with the reference Bacillus megaterium (Bm) or autochthonous bacterial strains (*Enterobacter* sp. (E); *Bacillus thuringiensis* (Bt) and *Bacillus* sp. (B)) grown in an arid Mediterranean soil amended or not with fermented agrowasste (CO), under drought conditions. Errors bars represented standard errors (n=3).

A wide range of natural AM colonization levels, (as a percentage of root length colonized), was found in the four shrub species (Fig 3). In control plants grown without fermented agrowaste the greatest AM colonization was observed for *T. vulgaris* and the lowest in *S. chamaecyparissus*. All bacterial inoculation treatment increased percentage of the mycorrhization in *S. chamaecyparissus* and *S. officinalis*, while this effect was only observed in *L. dentata* when inoculated with *Enterobacter* sp. The highest percentage of natural AM root colonization in each plant growing in soil without fermented agrowaste was reached in the

bacteria-inoculated plants, with the exception of *B. thuringiensis* and *Bacillus* sp. when inoculated in *T. vulgaris* (Fig 3).

In control plants grown with fermented agrowaste the greatest AM colonization was observed in *S. chamaecyparissus*. In general, bacterial inoculation resulted less effective in increasing AM colonization in presence of fermented agrowaste in the medium. Nevertheless, inoculation of *S. officinalis* with *B. megaterium*, *Enterobacter* sp. and *B. thuringiensis* enhanced AM colonization in presence of fermented agowaste (Fig. 3).

Values of glomalin, as GRSP, content reflect the amount of extraradical mycelium (Fig 3). The fermented agrowaste was effective in increasing GRSP in *L. dentata* and *S. officinalis* and the bacterial treatment did not affect this response variable. Correlations cannot be generalized since they are produced only significantly between fermented agrowaste and extra mycorrhizal (GRSP) development ($r = 0.546$; $p \leq 0.01$) but no in intra mycorrhizal ($r = -0.115$; $p \geq 0.05$) development.

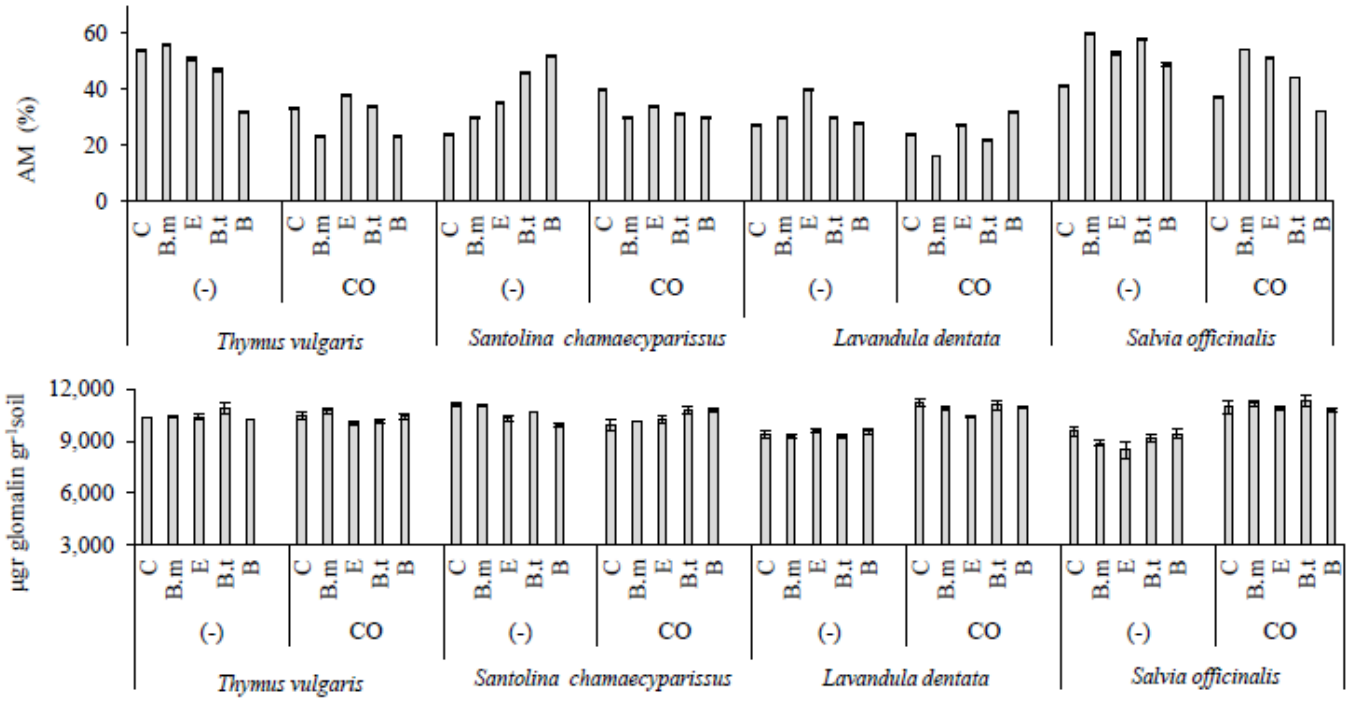


Figure 3. Percentage of root AM colonization and glomalin content in rhizosphere soil of four autochthonous shrubs (*Thymus vulgaris, Santolina chamaecyparissus, Lavandula dentata* and *Salvia officinalis*) non-inoculated (control) or inoculated with the reference *Bacillus megaterium* (Bm) or autochthonous bacterial strains (*Enterobacter* sp. (E); *Bacillus thuringiensis* (Bt) and *Bacillus* sp. (B)) grown in an arid Mediterranean soil amended or not with fermented agrowaste (CO), under drought conditions. Errors bars represent standard errors (n=3).

4. Discussion

The three *Bacillus* sp. and the *Enterobacter* sp. bacterial strains here selected were able to produce IAA and to solubilise phosphate (particularly *B. megaterium* and *Enterobacter* sp.) under non stress and stress conditions *in vitro*. Minaxi (2011) also reported multiple plant promoting traits in a *Bacillus* sp. isolated from semiarid crops. *Bacillus* was the most abundant genus in the rhizosphere of autochthonous drought-adapted target plants, probably because the ability of these bacteria to form spores allows a better survival under stress conditions (Marulanda et al., 2006). Nevertheless, *Enterobacter* sp. resulted in the most tolerant bacteria able to survive under 40% of PEG in the growing medium. Indeed, under the greatest osmotic stress assayed (40% PEG), shows a high level of proline and MDA but the lowest antioxidants activities (CAT and APX) for osmotic cellular adaptation, as previously found (Marulanda et al., 2009). Such bacterial activities may represent an important protection against water limitation. The intrinsic metabolic characteristics that the test bacterial strains shown in axenic culture under non-stress and stress conditions support that these bacteria can be candidates to facilitate revegetation of semi-arid areas. However, as many factors may affect the performance of inoculated bacteria under natural conditions (Bais et al., 2006), their applications must be first tested.

It has been shown that the exposure of bacterial cells to osmotic stress induce the production of reactive oxygen species (ROS) that disturb the metabolic balance of the cells and cause oxidative stress (Maksimovic et al., 2013). Here the antioxidants activities in the bacterial cultures did not correlated with the osmotic tolerance capacity since the two most tolerant bacteria, *B. thuringiensis* and *Enterobacter* sp., showed the highest CAT and APX (*B. thuringiensis*) and the lowest (*Enterobacter* sp.) activities. Antioxidant activities in stressed cells are highly variable depending of bacteria involved but these enzymes reflects the modified redox status of the stressed cells. Proline accumulation in cells not only has an osmolyte function but also maintains the redox balance and radical scavenging (Szabados and Savoure, 2010).

The production of ACC by the target bacteria was also evaluated because it is the precursor for ethylene synthesis in plant. Bacterial ACC deaminase converts the ACC to ammonia and α-ketobutyrate, thereby lowering ethylene levels in inoculated plants (Glick et al., 1998). The lowering of ethylene levels is essential when plants are exposed to environmental stressors as drought (Glick, 2004). *Bacillus* sp. was the most drought sensitive bacteria and it produced the highest ACC-deaminase and proline accumulation under stress conditions. Both compounds would account for the compensation of the bacterial lack of stress tolerance (40% of PEG addition). However, this bacterial strain changed very little the APX and CAT activities and lipid peroxidation (MDA) under the stress conditions tested. These antioxidant bacterial

activities play an important role facilitating the removal of free radicals (Wang et al., 2007). Perhaps in this bacterial strain the low reaction of these antioxidant activities were compensated by the contribution of high PHB and/or ACC deaminase production in alleviating cell osmotic stress. Nevertheless, the low survival of this bacterial strain under 40% PEG is contrasting with its abilities to synthesize these compounds.

Production of IAA-like compounds is common in *Bacillus* strains and this bacterial trait may improve root growth during the early plant growth stage. In addition, stomatal closure and transpiration reduction in response to water deficiency in plants may be induced by auxins. The participation of bacterial auxins in the responses to water stress was also observed by Havlova et al. (2008). Drought significantly increased IAA production in *B. megaterium*, as previously reported by Dobra et al. (2010), corroborating that IAA also plays an important role in the stress responses. In fact, this bacterium decreases stomatal conductance in *L. dentata* (with fermented agrowaste) and in *S. officinalis* (with and without fermented agrowaste).

The production of hormones, such as ABA, SA, OPDA and JA, was also tested for the target bacteria, because these signal molecules are the basis for important mechanisms to cope with osmotic stress and be considered as PGPR (Pozo et al., 2015). Particularly ABA is described as the primary chemical involved in acting as signal of osmotic stress (Schurr et al., 1992). In plants, ABA has been proposed to play a role in water transport via activation of aquaporins such as plasma membrane intrinsic proteins type 1 (PIP1) (Parent et al., 2009). Actually, ABA plays an important role in the stress signal transductions (Aroca et al., 2008). The bacterial strains here tested produced quite similar levels of ABA under moderate osmotic stress conditions. In addition to ABA, the hormone JA has been shown to protect cells from osmotic stressors (Pedranzani et al., 2003). The JA is considered as a growth regulator able to induce tolerance to stress conditions. Here, the highest producers of JA under non-stress conditions was *B. megaterium,* the most PEG-sensitive bacteria, while the lowest JA producers was *Enterobacter* sp. under stress conditions, the most PEG-tolerant bacteria. In this context, significant differences between *B. megaterium* and *Enterobacter* sp. in JA production were observed with and without PEG application. Nevertheless, the coordinated action of ABA and JA protected cells from the effects of stress. Observations by Brossa et al. (2011) indicate a relationship between JA and lipid peroxidation but in the here tested bacteria such relation was not observed. The SA synthesized by bacteria also plays an important role in osmotic stress tolerance (Gautam and Singh, 2009). This phenolic hormone is associated to abiotic stress responses and thus, it was determined in the target bacteria because of it signalling activity on the antioxidant defence system (Zhou, 1999). *Bacillus* sp. synthesized the highest amount of SA under whatever tested condition. All those hormones (ABA, SA and JA) synthesized by these

bacteria may play also an important role in mediating plant reactions to drought (Groppa et al., 2012).

Autochthonous bacteria here selected to be used as inoculants for the target shrubs produced different quantities of IAA, ACC-deaminase and PSI and also differed in their ability to produce antioxidants, proline, PHB, ABA, JA, OPDA and SA under stress and non-stress conditions. These physiological and biochemical bacterial traits did not totally explain the benefits obtained by inoculated plants under drought conditions.

In this study we demonstrated that both survival and nutrition of shrub plants were highly benefited by the autochthonous inoculated bacteria and/or fermented agrowaste application in soil affected by drought. The enhancement of nutrients uptake (mainly P and K) in inoculated plants added of fermented agrowaste may affect plant water relations and drought tolerance (Subramanian et al., 2006). In dry soils, the P availability is highly reduced since the decline in soil moisture results in an important lowering in the rate of nutrients diffusion in the soil solution, particularly those having a low diffusing rate, like P.

Fermented agrowaste amendment improves nutrient uptake in most of shrubs assayed (except in *S. officinalis*), as previously found in the legume shrub *Anthyllis cytosoides* (Medina et al., 2004). Along the fermentation process by *A. niger* can occur simultaneous activities such as the mineralization of the lignocellulosic agrowaste compounds, the biosynthesis of organic acids, as the tricarboxilic citric acid, and consequently rock phosphate solubilization (Vassilev et al., 1998). In addition, the fermented agrowaste added to the soil seems to be used as C source and energy for the inoculated bacteria which could lead to an enhancement of the beneficial bacterial activity resulting in increased functional traits that benefited the shrubs nutrition here tested. Particularly, inoculation of *B. megaterium* in fermented agrowaste soil enhanced nutrient uptake, such as P, K, Ca and Mg, by *S. chamaecyparissus*, *L. dentata* and *S. officinalis.*

In *L. dentata* and *S. officinalis* values of proline, stomatal conductance and photosynthetic efficiency were also analyzed. The improvement of such specific characteristics of these two shrubs could be considered as strategies to facilitate water stress tolerance by bacteria and/or fermented agrowaste applied. The stomatal conductance and nutrients as K and Ca content (higher in *L. dentata* than in *S. officinalis*) are important physiological and nutritional values to adapt plants to drought since stomatal closure preserves water lost.

Both under axenic and natural conditions we found that the stress factors applied did not suppress the PGPR abilities of the autochthonous drought-adapted bacteria which indicated their potential to be used as inoculants under such detrimental conditions. Due to their adaptability to stress the bacterial cells may improve its competitive advantage for coping with the stress

situation. Thus, the interest of microbial inoculations and their effectiveness increased under unfavourable environmental situations as it is drought (Belimov et al., 2009). However, since many mechanisms and factors may be involved in the adaptation and response to drought, the prevailing mechanisms for stress tolerance of the target bacteria and/or inoculated plants are difficult to be established. The PGPR ability of *B. megaterium* was related to endogenous ABA content in tomato plants (Porcel et al., 2014).

Nutrient acquisition of the target shrubs was differently affected by the inoculation with each one of the PGPR bacteria applied. This may be due to differences in the specific bacterial characteristics i.e. ability to produce hormones, to colonize roots (Li et al., 2000), to solubilise P or to hydrolyze ACC. However, it is not clear from these results the main bacterial activity involved in the positive effects and potential to minimize the deleterious effect of drought in these inoculated plants (Glick, 2012). The target bacteria, in general, safeguard the plants from the deleterious effects of drought by producing phytohormones, ACC deaminase, PHB, antioxidant enzymes and by increasing nutrients availability in different amount. But these microbial traits seem not always efficiently functioning under such stress conditions in each one of the shrubs. Thus, inoculation with PGPR induces different range of plant tolerance to abiotic stresses with an osmotic component like drought and in this effect may account the level of improvement of physiological and biochemical parameters related with water status (Kohler et al., 2009) and the characteristics of plant involved (Porcel et al., 2014). In fact, the effect of each bacteria on plant physiological values as leaf transpiration and photosynthetic efficiency (PE) cannot be generalized (Alguacil et al., 2009). Results related to a deficient nutrition caused by osmotic stress in non-inoculated plants could be induced by lower root, nutrients and water uptake capacity.

Since ROS produced by the stress situation are removed by several enzymatic systems, it is clear whether the enhancement of these activities in cells correlated with the stress severity (Koussevitzky et al., 2008). APX is the key antioxidant enzyme in the ascorbate/glutathione cycle (Orvar and Ellis, 1997). Enzymatic systems resulted here sensitive and indicative of the bacterial effectiveness in supporting drought impact in the stressed cells. The lowering in these activities in bacterial cells under water stress may be interpreted as a higher water retention and subsequent increased drought stress tolerance.

Proline is also an important compound involved in turgor maintenance. This osmolite is often synthesized by cells in response to stress factors mediating osmotic adjustment and the accumulation of this compounds increases cell resistance to water deficiency (Kishor et al., 2005). Bacterial inoculation and/or fermented agrowaste application decreased proline accumulation in *L. dentata*, which may reflect an increased drought tolerance.

In *L. dentata* the bacteria and the fermented agrowaste highly increased K^+ retention and it is considered as one of the key features of osmotic stress tolerance (Shabala and Cuin, 2008). Tolerant varieties are capable to better retain K^+ (Chen et al., 2007).

Nevertheless, it is very difficult to attribute the bacterial effectiveness to specific nutritional or physiological activities. The particular bacterial effectiveness on the performance and drought tolerance ability in the test plants depended on the plant species involved. It is important to assess whether tolerant mechanisms are not only transient but also long-term lasting.

Significant differences in the natural AM colonization level among the test plants were evident. In *T. vulgaris* plants showed the highest AM colonization levels, which were not affected by bacterial inoculation. In contrast, all bacteria increased the mycorrhization degree in *S. chamaecyparissus* and *S. officinalis*, thereby acting as mycorrhiza helper bacteria (Frey-Klett et al., 2007). Fermented agrowastes applications decreased the ratio of AM intra and extra-radical colonization in all plants, which suggest a particular stimulating effect of this amendment on the fungal mycelia developed in soil, in comparison with that developed inside the root. The extraradical mycelium size was quite similar irrespective of plant and the bacterial inoculum involved. Thus, significant increases of macro and micro nutrients uptake by *T. vulgaris*, *S. chamaecyparissus* and *L. dentata* inoculated with *B. thuringiensis* cannot be explained by an enlargement of the extraradical mycelium emerging from the root systems of those naturally AM-colonized plants.

Structural soil stability of the degraded test soil has been shown to be significantly improved by about a 79% by the addition of fermented agrowaste (Alguacil et al., 2003). The glomalin, present in the extraradical mycelia component, is a recalcitrant glycoprotein acting as a binding agent in the aggregation process (Lovelock et al., 2004). An improved soil structure means an increased water retention, nutrient uptake, drainage, aeration and root growth, which consequently determines an improvement of soil quality and fertility (Caravaca et al., 2002).

All the applied treatments resulted fundamental for the target shrubs species to reach their optimal nutritional and physiological traits under conditions which are characteristics of the natural semiarid Mediterranean drought conditions. The diverse bacterial activities and plant characteristics could explain the unpredictable effectiveness of inoculated bacteria. Detailed molecular and physiological studies will be helpful for understanding microbial and plant tolerance and adaptative processes that are yet poorly understood (Cappellari et al., 2013), and these are the subject of current research. In any case, further experiment under natural soil conditions should be conducted for a proper exploitation of stress-adapted PGPR in the restoration of degraded ecosystems. The selection of efficient bacterial strains with well-defined

mechanisms, consistent and reproducible activities under field conditions is very important to develop PGPR inocula.

Acknowledgments

E. Armada was financed by Ministry of Science and Innovation (Spain). This work was carried out in the framework of the project reference AGL2009-12530-C02-02.

References

Aebi, H., 1984. Catalase *in vitro*. Methods Enzymol. 105, 121-126.

Alguacil, M.M., Caravaca, F., Azcón, R., Pera, J., Díaz, G., Roldán, A., 2003. Improvements in soil quality and performance of mycorrhizal *Cistus albidus* L. seedlings resulting from addition of microbially treated sugar beet residue to a degraded semiarid Mediterranean soil. Soil Use Manage. 19, 277-283.

Alguacil, M.M., Díaz-Pereira, E., Caravaca, F., Fernández, D.A., Roldán, A., 2009. Increased diversity of arbuscular mycorrhizal fungi in a long-term field experiment via application of organic amendments to a semiarid degraded soil. Appl. Environ. Microbiol. 75, 4254-4263.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403-410.

Amako, K., Chen, G.X., Asada, K., 1994. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol 35, 497-504.

Aroca, R., Vernieri, P., Ruíz-Lozano, J.M., 2008. Mycorrhizal and non-mycorrhizal *Lactuca sativa* plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. J. Exp. Bot. 59, 2029-2041.

Ayub, N.D., Pettinari, M.J., Ruiz, J.A., Lopez, N.I., 2004. A polyhydroxybutyrate-producing *Pseudomonas* sp isolated from antarctic environments with high stress resistance. Curr. Microbiol. 49, 170-174.

Azcón, R., Medina, A., Aroca, R., Ruíz-Lozano, J.M., 2013. Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast interactions, in: de Bruijn, F.J. (Ed.), Molecular Microbial Ecology of the Rhizosphere. John Wiley & Sons, Hoboken, New Jersey, USA, pp. 991-1002.

Azcón, R., Perálvarez, M.C., Roldán, A., Barea, J.M., 2010. Arbuscular mycorrhizal fungi, *Bacillus cereus*, and *Candida parapsilosis* from a multicontaminated soil alleviate metal toxicity in plants. Microb. Ecol. 59, 668-677.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interations with plants and other organisms, Annu. Rev. Plant Biol., pp. 233-266.

Barea, J.M., Palenzuela, J., Cornejo, P., Sánchez-Castro, I., Navarro-Fernández, C., Lopéz-García, A., Estrada, B., Azcón, R., Ferrol, N., Azcón-Aguilar, C., 2011. Ecological and functional roles of mycorrhizas in semi-arid ecosystems of Southeast Spain. J. Arid Environ. 75, 1292-1301.

Bashan, Y., Holguin, G., 1998. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: Biocontrol-PGPB (Plant Growth-Promoting Bacteria) and PGPB. Soil Biol. Biochem. 30, 1225-1228.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205-207.

Belimov, A.A., Dodd, I.C., Hontzeas, N., Theobald, J.C., Safronova, V.I., Davies, W.J., 2009. Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase yield of plants grown in drying soil via both local and systemic hormone signalling. New Phytol. 181, 413-423.

Benabdellah, K., Abbas, Y., Abourouh, M., Aroca, R., Azcon, R., 2011. Influence of two bacterial isolates from degraded and non-degraded soils and arbuscular mycorrhizae fungi isolated from semi-arid zone on the growth of *Trifolium repens* under drought conditions: Mechanisms related to bacterial effectiveness. Eur. J. Soil Biol. 47, 303-309.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brossa, R., López-Carbonell, M., Jubany-Mari, T., Alegre, L., 2011. Interplay between abscisic acid and jasmonic acid and its role in water-oxidative stress in wild-type, ABA-deficient, JA-deficient, and ascorbate-deficient *Arabidopsis* Plants. J. Plant Growth Regul. 30, 322-333.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302-310.

Cappellari, L.R., Valeria Santoro, M., Nievas, F., Giordano, W., Banchio, E., 2013. Increase of secondary metabolite content in marigold by inoculation with plant growth-promoting rhizobacteria. Appl. Soil Ecol. 70, 16-22.

Caravaca, F., Barea, J.M., Figueroa, D., Roldán, A., 2002. Assessing the effectiveness of mycorrhizal inoculation and soil compost addition for enhancing reaforestation with *Olea europaea* subsp. *sylvestris* through changes in soil biological and physical parameters. Appl. Soil Ecol. 20, 107-118.

Chen, B.D., Zhu, Y.G., Duan, J., Xiao, X.Y., Smith, S.E., 2007. Effects of the arbuscular mycorrhizal fungus *Glomus mosseae* on growth and metal uptake by four plant species in copper mine tailings. Environ. Pollut. 147, 374-380.

Dimkpa, C., Weinand, T., Asch, F., 2009. Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ. 32, 1682-1694.

Dobra, J., Motyka, V., Dobrev, P., Malbeck, J., Prasil, I.T., Haisel, D., Gaudinova, A., Havlova, M., Gubis, J., Vankova, R., 2010. Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content. J. Plant Physiol. 167, 1360-1370.

Duncan, D.B., 1955. Multiple range and multiple F tests. Biometrics 11, 1-42.

Durgbanshi, A., Arbona, V., Pozo, O., Miersch, O., Sancho, J.V., Gomez-Cadenas, A., 2005. Simultaneous determination of multiple phytohormones in plant extracts by liquid chromatography-electrospray tandem mass spectrometry. J. Agric. Food Chem. 53, 8437-8442.

Edi-Premono, M., Moawad, A.M., Vlek, P.L.G., 1996. Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Indonesian J. Agric. Sci. 11, 13-23.

Espindola, A.D., Gomes, D.S., Panek, A.D., Eleutherio, E.C.A., 2003. The role of glutathione in yeast dehydration tolerance. Cryobiology 47, 236-241.

Frey-Klett, P., Garbaye, J., Tarkka, M., 2007. The mycorrhiza helper bacteria revisited. New Phytol. 176, 22-36.

Gautam, S., Singh, P.K., 2009. Salicylic acid-induced salinity tolerance in corn grown under NaCl stress. Acta Physiol. Plant. 31, 1185-1190.

Giovannetti, M., Mosse, B., 1980. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84, 489-500.

Glick, B.R., 2004. Bacterial ACC deaminase and the alleviation of plant stress. Adv. Appl. Microbiol. 56, 291-312.

Glick, B.R., 2012. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Hindawi Publishing Corporation Scientifica.

Glick, B.R., Penrose, D.M., Li, J.P., 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190, 63-68.

Gordon, S.A., Paleg, L.G., 1957. Observations on the quantitative determination of indoleacetic acid. Physiol. Plant. 10, 39-47.

Groppa, M.D., Benavides, M.P., Zawoznik, M.S., 2012. Root hydraulic conductance, aquaporins and plant growth promoting microorganisms: A revision. Appl. Soil Ecol. 61, 247-254.

Havlova, M., Dobrev, P.I., Motyka, V., Storchova, H., Libus, J., Dobra, J., Malbeck, J., Gaudinova, A., Vankova, R., 2008. The role of cytokinins in responses to water deficit in tobacco plants over-expressing *trans*-zeatin O-glucosyltransferase gene under *35S* or *SAG12* promoters. Plant Cell Environ. 31, 341-353.

Honma, M., Shimomura, T., 1978. Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agric. Biol. Chem. 42, 1825-1831.

Kasim, W.A., Osman, M.E., Omar, M.N., Abd El-Daim, I.A., Bejai, S., Meijer, J., 2013. Control of drought stress in wheat using plant-growth-promoting bacteria. J. Plant Growth Regul. 32, 122-130.

Katznelson, H., Bose, B., 1959. Metabolic activity and phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere, and non-rhizosphere soil. Can. J. Microbiol. 5, 79-&.

Kishor, P.B.K., Sangam, S., Amrutha, R.N., Laxmi, P.S., Naidu, K.R., Rao, K., Rao, S., Reddy, K.J., Theriappan, P., Sreenivasulu, N., 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. Curr. Sci. 88, 424-438.

Kohler, J., Hernández, J.A., Caravaca, F., Roldán, A., 2008. Plant-growth-promoting rhizobacteria and arbuscular mycorrhizal fungi modify alleviation biochemical mechanisms in water-stressed plants. Funct. Plant Biol. 35, 141-151.

Kohler, J., Hernández, J.A., Caravaca, F., Roldán, A., 2009. Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. Environ. Exp. Bot. 65, 245-252.

Koussevitzky, S., Suzuki, N., Huntington, S., Armijo, L., Sha, W., Cortes, D., Shulaev, V., Mittler, R., 2008. Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. J. Biol. Chem. 283, 34197-34203.

Law, J.H., Slepecky, R.A., 1961. Assay of poly-beta-hydroxybutyric acid. J. Bacteriol. 82, 33-&.

Lee, I.Y., Chang, H.N., Park, Y.H., 1995. A simple method for recovery of microbial poly-beta-hydroxybutyrate by alkaline-solution treatment. Journal of Microbiology and Biotechnology 5, 238-240.

Li, J.P., Ovakim, D.H., Charles, T.C., Glick, B.R., 2000. An ACC deaminase minus mutant of Enterobacter cloacae UW4 no longer promotes root elongation. Curr. Microbiol. 41, 101-105.

Lovelock, C.E., Wright, S.F., Clark, D.A., Ruess, R.W., 2004. Soil stocks of glomalin produced by arbuscular mycorrhizal fungi across a tropical rain forest landscape. J. Ecol. 92, 278-287.

Maksimovic, J.D., Zhang, J., Zeng, F., Zivanovic, B.D., Shabala, L., Zhou, M., Shabala, S., 2013. Linking oxidative and salinity stress tolerance in barley: can root antioxidant enzyme activity be used as a measure of stress tolerance? Plant Soil 365, 141-155.

Marulanda, A., Barea, J.M., Azcón, R., 2006. An indigenous drought-tolerant strain of *Glomus intraradices* associated with a native bacterium improves water transport and root development in *Retama sphaerocarpa*. Microb. Ecol. 52, 670-678.

Marulanda, A., Barea, J.M., Azcón, R., 2009. Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments. Mechanisms related to bacterial effectiveness. J. Plant Growth Regul. 28, 115-124.

Medina, A., Vassilev, N., Alguacil, M.M., Roldán, A., Azcón, R., 2004. Increased plant growth, nutrient uptake, and soil enzymatic activities in a desertified mediterranean soil amended with treated residues and inoculated with native mycorrhizal fungi and a plant growth-promoting yeast. Soil Sci. 169, 260-270.

Medina, A., Vassileva, M., Barea, J.M., Azcón, R., 2006. The growth-enhancement of clover by *Aspergillus*- treated sugar beet waste and *Glomus mosseae* inoculation in Zn contaminated soil. Appl. Soil Ecol. 33, 87-98.

Minaxi, J.S., 2011. Efficacy of rhizobacterial strains encapsulated in nontoxic biodegradable gel matrices to promote growth and yield of wheat plants. Appl. Soil Ecol. 48, 301-308.

Naveed, M., Hussain, M.B., Zahir, Z.A., Mitter, B., Sessitsch, A., 2014. Drought stress amelioration in wheat through inoculation with Burkholderia phytofirmans strain PsJN. Plant Growth Regul. 73, 121-131.

Okon, Y., Itzigsohn, R., 1992. Poly-beta-hydroxybutyrate metabolism in *Azospirillum brasilense* and the ecological role of PHB in the rhizosphere. FEMS Microbiol. Lett. 103, 131-139.

Orvar, B.L., Ellis, B.E., 1997. Transgenic tobacco plants expressing antisense RNA for cytosolic ascorbate peroxidase show increased susceptibility to ozone injury. Plant J. 11, 1297-1305.

Oxborough, K., Baker, N.R., 1997. Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components - calculation of qP and *Fv'/Fm'* without measuring *Fo'*. Photosynth. Res. 54, 135-142.

Parent, B., Hachez, C., Redondo, E., Simonneau, T., Chaumont, F., Tardieu, F., 2009. Drought and abscisic acid effects on aquaporin content translate into changes in hydraulic conductivity and leaf growth rate: A trans-scale approach. Plant Physiol. 149, 2000-2012.

Pedranzani, H., Racagni, G., Alemano, S., Miersch, O., Ramirez, I., Pena-Cortes, H., Taleisnik, E., Machado-Domenech, E., Abdala, G., 2003. Salt tolerant tomato plants show increased levels of jasmonic acid. Plant Growth Regul. 41, 149-158.

Penrose, D.M., Glick, B.R., 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol. Plant. 118, 10-15.

Phillips, J.M., Hayman, D.S., 1970. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 159-161.

Pikovskaya, R.I., 1948. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologya 17, 362-370.

Porcel, R., Zamarreno, A.M., García-Mina, J.M., Aroca, R., 2014. Involvement of plant endogenous ABA in *Bacillus megaterium* PGPR activity in tomato plants. BMC Plant Biol. 14.

Pozo, M.J., Lopez-Raez, J.A., Azcon-Aguilar, C., Garcia-Garrido, J.M., 2015. Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. New Phytol. 205, 1431-1436.

Ramsay, J.A., Berger, E., Voyer, R., Chavarie, C., Ramsay, B.A., 1994. Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnol. Tech. 8, 589-594.

Rillig, M.C., 2004. Arbuscular mycorrhizae, glomalin, and soil aggregation. Can. J. Soil Sci. 84, 355-363.

Roth, C.H., Malicki, M.A., Plagge, R., 1992. Empirical evaluation of the relationship between soil dielectric constant and volumetric water content as the basis for calibrating soil moisture measurements. J. Soil Sci. 43, 1-13.

Saleem, M., Arshad, M., Hussain, S., Bhatti, A.S., 2007. Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J. Ind. Microbiol. Biotechnol. 34, 635-648.

Schurr, U., Gollan, T., Schulze, E.D., 1992. Stomatal response to drying soil in relation to changes in the xylem sap composition of *Helianthus annuus*. 2. Stomatal sensitivity to abscisic-acid imported from the xylem sap. Plant Cell Environ. 15, 561-567.

Shabala, S., Cuin, T.A., 2008. Potassium transport and plant salt tolerance. Physiol. Plant. 133, 651-669.

SSS, 2006. Soil Survey Staff (SSS). "Keys to Soil Taxonomy" 10th ed. USDA. Natural Resources, Conservation Service, Washington DC.

Subramanian, K.S., Santhanakrishnan, P., Balasubramanian, P., 2006. Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress. Scientia Hortic. 107, 245-253.

Szabados, L., Savoure, A., 2010. Proline: a multifunctional amino acid. Trends Plant Sci. 15, 89-97.

Vallejo, V.R., Bautista, S., Cortina, J., 1999. Restoration for soil protection after disturbances, in: Trabaud, L. (Ed.), Life and Environment in the Mediterranean. Advances in Ecological Sciences. WIT Press, Wessex, pp. 301-343.

Vassilev, N., Vassileva, M., Azcón, R., Fenice, M., Federici, F., Barea, J.M., 1998. Fertilizing effect of microbially treated olive mill wastewater on *Trifolium* plants. Bioresour. Technol. 66, 133-137.

Wang, Y., Wang, H., Yang, C.-H., Wang, Q., Mei, R., 2007. Two distinct manganese-containing superoxide dismutase genes in Bacillus cereus: their physiological characterizations and roles in surviving in wheat rhizosphere. FEMS Microbiol. Lett. 272, 206-213.

Wöhler, I., 1997. Auxin-indole derivatives in soils determined by a colorimetric method and by high performance liquid chromatography. Microbiol. Res. 152, 399-405.

Wright, S.F., Upadhyaya, A., 1996. Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Sci. 161, 575-586.

Wright, S.F., Upadhyaya, A., 1998. A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 198, 97-107.

Zahir, Z.A., Arshad, M., Frankenberger, W.T., 2004. Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Adv. Agron. 81, 97-168.

Zhou, J.L., 1999. Zn biosorption by *Rhizopus arrhizus* and other fungi. Appl. Microbiol. Biotechnol. 51, 686-693.

CHAPTER 2.2

Differential activity of autochthonous bacteria in controlling drought stress in native *Lavandula* and *Salvia* plants species under drought conditions in natural arid soil

Elisabeth Armada, Antonio Roldán, Rosario Azcón

Microbial Ecology (2014) 67: 410-420

1. Introduction

Rhizosphere bacteria are ubiquitous soil inhabitants able to establish relations with plants. Bacteria assist the associated plants in the uptake of mineral nutrients and water and also they increase tolerance to environmental stresses [1]. Bacterial are usually the most numerous organisms which could be cultivable in soil with 10^6-10^9 viable cell by per cubic centimeter [2]. Nevertheless, much more research on the bacteria drought resistance is required to know mechanisms related to grow effect and adaptation to dry soils. Under such drought conditions the development of indigenous microbial community is limited or even inhibited. Thus, the application of plant growth promoting microorganisms (PGPM) has been suggested [3]. The ability of certain bacteria to attenuate detrimental stress effect in plants is previously reported [1, 4-5].

The physiological benefits of rhizosphere bacteria for the host plants are well known and they effectiveness is ecologically relevant particularly under detrimental conditions. The establishment of a plant cover based on autochthonous plant species is an effective strategy for restoring the Mediterranean semiarid degraded lands. In such areas, having low soil fertility and water deficiency, the establishment of plants is difficult and it requires to apply methods for improving the ability of these plant species to resist the drought environmental conditions [6]. Thus, to carry out successful reforestation programs, it is necessary to apply inoculation technologies which reinforce the limited microbial potential in these degraded areas [4, 7-8]. Regarding the competitiveness of autochthonous rhizosphere bacteria one efficient strategy contributing to the establishment of pre-selected beneficial microorganisms in these poor-infertile semiarid soils is through early bacterial establishment in the rhizosphere by inoculation at the seedling stage. Bacterial inoculation, selecting adapted and efficient specific microorganisms, has long been recognized as an interesting possibility to increase plant growth

[9]. Nevertheless, the plant growth responses to bacterial inoculation involve from bacterial strain to plant species and even ecotype and site specificity [4]. Authors reported that variable effects were determined depending on plant species, cultivar and environmental conditions [10].

Lavandula dentata and *Salvia officinalis* constitute important plants for revegetation programs in a semiarid Mediterranean area and to improve the plant establishment by the direct application of bacterial inocula may be a recommended practice. Previous results evidenced that selected bacteria help plants to grow under arid conditions by increasing nutrients supply and water stress tolerance [3].

The role of bacteria in growth, nutrition and drought tolerance under nutritional limited conditions is based on a range of physiological and cellular mechanisms [1]. In this regard, microorganisms are also able to reduce water stress by alleviating cellular oxidative damage produced in plants under drought conditions. In fact, the view nowadays is to consider ROS as an integrative part of cell signaling metabolism modulated by the cellular redox state loading to different responses related to programmed cell death, plant development or defense and gene expression [11]. The establishment of inocula in dry soils includes the activation of antioxidant metabolic pathways [12-13].

Arid environments determine the ability of organisms to proliferate is such habitat. The microbial ability to adapt to environmental changes is fundamental to the survival of these organisms and several mechanisms are responsible for the required adaptation. Remarkable similarities exist between plants and bacteria in their cellular responses to an osmotic stress [14]. Several organisms (microorganisms and plants) from different kingdoms are able to accumulate the same set of cellular compounds upon exposure to stress conditions. There are close parallelism in the mechanisms that plants and microorganisms use to regulate responses to environmental stresses. In fact, there are processes that enable organisms to cope with environmental changes or stress conditions and they determine the ability of organisms to live in particular environments.

This study reports information on the relevance of cells metabolic processes conducting to proline and indolacetic acid (IAA) microbial production in the growing medium along the time when this medium was added of increasing polyethylene glycol (PEG) to create an osmotic stress. The bacterial IAA productions are related to plant improvement effect and proline is accumulated in the cell under stress condition to protect cells against adverse effect of ROS and stabilizing proteins. This compound increases resistance to water deficiency by that it can be considered a good stress indicator. As well, previous studies [15] report mechanisms commonly involved in the plant growth-promoting activity of bacteria as is the production of

phytohormones and particularly IAA plays the most important role in plant growth promotion. Thus, it was selected as representative index of bacterial efficiency.

Plants and microorganisms living in semiarid soils are often adapted to such stress conditions and the applications of such organisms to establish vegetation cover in these areas in an attractive possibility to recover these soils. But plants and microorganisms are affected by these detrimental conditions which alter cells and metabolism reducing growth. Under drought conditions, the relative plant benefit from the microorganisms may be different according to the photosynthetic activity of the associated plant which affects microbial performance. However, adapted/tolerant bacteria can enhance plant growth and nutrition under drought conditions and several physiological mechanisms may enhance the plant resistance to water stress. In general, plants may increase drought tolerance by reducing stomatal conductance and evapotranspiration, by increasing the cellular osmolyte accumulations and by enhancing drought tolerance and/or avoidance strategies.

The aim of this study was to determine the effect of some autochthonous drought-adapted bacteria and one selected as drought tolerant from our collection on the growth, nutrition and physiological values of *L. dentata* and *S. officinalis*. Both are plants that naturally grown in semiarid soils and are drought resistant. To reach these objectives, we test the mechanisms of bacteria and plant drought resistance and their interactions in drought tolerance.

The specific objectives of this study were the following: (1) isolation and characterization of autochthonous bacteria from rhizospheres of autochthonous plants; (2) to assess in native *Lavandula* and *Salvia* plant species, under drought conditions, the growth promotion, nutrition and physiological and biochemical traits related to drought tolerance in both non-inoculated and inoculated plants; and (3) to determine the bacterial characteristics as growth, proline and IAA production under stress conditions. One reference strain of *Bacillus megaterium* drought-tolerant was used as reference to compare the particular activity among species of bacteria under drought stress conditions.

Values related to bacterial tolerance to osmotic stress as growth was determined along time with increasing levels of PEG in the culture medium. As well, proline and IAA produced were also evaluated in axenic culture under stress (15% PEG) conditions.

2. Materials and Methods

Independent experiments were carried out in the present study. One microcosm experiment (Experiment I) analyzed the effectiveness of three autochthonous or one of reference drought-adapted bacteria in improving plant growth, physiology, antioxidant activities and

nutrition as indexes of drought tolerance. In a second assay, we determine changes on maintenance of growth of the bacterial cells in axenic culture medium under increasing osmotic stress conditions (by PEG application) and their abilities for proline and IAA production under such stress conditions. These autochthonous bacteria were also identified using molecular methods.

2.1. Pot experiment for plant growth

The plants used in the microcosm experiments under greenhouse conditions were *L. dentata* and *S. officinalis*. Both are low-growing shrubs widely distributed in the Mediterranean area selected. They are well adapted to the water stress conditions of this zone and, therefore, potentially could be used in the reforestation of semiarid disturbed lands. In this bioassay we tested the effect of three autochthonous drought-tolerant bacteria and *Bacillus megaterium* (used as reference, drought-adapted strain) on these two native shrubs. The plant biomass, nutrition, stomatal conductance, antioxidant (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX)) activities, proline accumulation and mycorrhizal intra- and extraradical colonization were evaluated. These values were determined after 1 year of plant growth in a natural soil under drought. Five replicated by the Mediterranean test soil used in this greenhouse experiment was air-dried, sieved to less than 2 mm, and mixed with quartz sand (< 1 mm) at a soil/sand proportion (5:2, v/v). The test soil came from Molina de Segura (province of Murcia, Spain). Pots were filled with 500 g of the soil/sand (5:2, v/v) mixture. The main soil characteristics were pH 8.90, P value 1.36 μg/g by Olsen test, organic carbon 0.94%, total N 0.22%, and on electric conductivity of 1.55.

2.1.1. ***Bacteria isolation and identification***

The autochthonous bacteria, identified as Enterobacter sp., Bacillus thuringiensis, and Bacillus sp. were isolated from the semiarid experimental soil from the Murcia province (Spain). This area suffers from drought and low nutrients availability and as a result desertification. They were the most abundant bacterial types in such arid soil exhibiting different colony morphology and were isolated from the above-mentioned soil (a mixture of rhizospheres from several autochthonous plant species). For that following serial soil dilutions, 1 g of homogeneized soil was suspended in 100 mL of sterile water (dilution 102) and this suspension was further diluted to reach dilution 104 to 106. These suspensions (104 to 106 sown in agar nutrient broth medium, 8g L-1) and cultivated for 48 h at 28 ºC. The abundance of those dominant colony forms, preliminarily referred as strains A, B or C, were (as colony-forming units per milliliter counts (cfu mL^{-1})) 120.10^4 (A), 85.10^4 (B) and 145.10^4 (C). They were independently grown in 250 mL flasks containing 50 mL of nutrients broth (8g L^{-1})

medium in shake culture for 48 h at 28 ºC. These bacteria isolates were cleaned and maintained suitable for the further in vitro and microcosm applications.

One milliter of pure bacterial culture (10^8 cfu mL^{-1}) grown in nutrient broth medium for 24-48 h at 28 ºC of temperature was applied to the appropriate pots at sowing time just below to plant seedlings, and 15 days later the bacterial culture (1mL, 10^8 cfu mL^{-1}) was applied around the plant on the soil.

Identification of bacteria isolates was done by sequencing the 16S rDNA gene. Bacterial cells extracted, diluted, lysed, and directly used as a template in the PCR reactions. All reactions were conducted 25 µl volume containing PCR buffer 10X, 50 mM $MgCl_2$, 10 µM each primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), 5U/µl of *Taq* polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with following conditions: 5 min at 95 ºC, followed by 30 cycles of 45s at 95 ºC, 45s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. The products of PCR were analyzed by 1% agarose gel electrophoresis. Extraction of DNA bacterial used QIAquick Gel extraction kit (QUIAGEN). Each sequence was compared with the database of 16S rRNA, the NCBI/BLAST. Autochthonous bacterial strains were identified as *B. thuringiensis* (98%), *Bacillus* sp. (91%) and *Enterobacter* sp. (99%).

2.1.2. *Plant growth conditions*

These plants were grown for 1 year in pots containing a mixture of natural soil and quartz sand (5v/2v) under greenhouse conditions (temperature ranging from 19 to 25 ºC, 16/8 light/dark photoperiod and a relative humidity of 50-70%). A photosynthetic photon flux density of 400-700 µmol m^{-1} s^{-2} was applied as supplementary light. Plants were grown along the experiment under drought conditions by keeping soil water capacity to 50% each day after water application but water level decreased along day to nearly 30% water capacity to the next water application.

2.1.3. *Measurements*

One year after planting, plants were harvested (five replicated per each treatment). Dry biomass of roots and shoots, nutrients concentrations and mycorrhizal infection were determined.

Shoot concentrations (in milligram per gram) of P, K, Ca, Mg as well as of Zn, Fe, Cu and (in microgram per gram) were determined from five different replicates per treatment after by flame photometry and colorimetry, respectively (Analytical Service of the "Centro de Edafología y Biología Aplicada del Segura" CSIC, Murcia, Spain)

Before harvest, some physiological plants values as stomatal conductance was measured (see below).

2.1.4. *Stomatal conductance*

Stomatal conductance was measured 2 h after the light was turned on by using a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the second youngest leaf from two different plants from each treatment.

2.1.5. *Root colonization*

Roots were carefully washed and stained. The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples [16], using the gridline intersect method [17] where the root sample was spread out evenly in dishes that had gridlines marked on the bottom to form 1.27 cm squares. Vertical and horizontal gridlines were scanned under a dissecting microscope at 40 to 100 x magnification. The absence or presence of AM colonization was recorded at each point where a root intersected a line and at least 100 gridline intersects were tallied as the authors recommended.

The mycorrhizal extraradical mycelium was evaluated following the methodology proposed [18] which measured easily extractable protein.

2.1.6. *Antioxidant enzymatic activities*

Regarding method for the extraction of enzymes, plant cells were homogenized [19] in a cold mortar with 4 mL 100 mM phosphate buffer (pH 7.2) containing 60 mM KH_2PO_4, 40 mM K_2HPO_4, 0.1 mM DTPA, and 1 % (w/v) PVPP. The homogenate was centrifuged at 18,000 *g* for 10 min at 4 ºC, and the supernatant was used for enzyme activity determination. Total SOD activity (EC 1.15.1.1) [20] was measured on the basis of SOD's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 ºC. CAT activity (EC 1.11.1.6) was measured as described [21]. Consumption of H_2O_2 (extinction coefficient of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min was monitored. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 100 µL of enzyme extract in a 2 mL volume. APX activity (EC 1.11.1.11) was measured in a 1-mL reaction volume containing 80 mM M potassium phosphate buffer (pH 7.0), 2.5 mM hydrogen peroxide and 1 M sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate [22]. GR activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH [23]. The reaction mixture (1

mL) contained 50mM Tris buffer, 3 mM $MgCl_2$, 1 mM oxidized glutathione, 50 µl enzyme extract, and 0.3 mM NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in micromole NADPH oxidized per gram fresh weight per minute, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH (ε_{340}=6.22 mM^{-1} cm^{-1}). Total soluble protein amount was determined using the Bradford method [24] and BSA as standard.

2.1.7. *Shoot proline content*

Free proline was extracted from 0.5 g of fresh leaves [25]. The methanolic phase was used for quantification of proline content. Proline was estimated by spectrophotometric analysis at 530 nm of the ninhydrin reaction [26].

2.2. In vitro experiment to determine microbial characteristics

2.2.1. *Bacterial growth under increasing PEG levels in the culture medium*

The bacterial isolates were checked in an additional in vitro experiment for testing the drought tolerance abilities to a reference strain. For that, the growth of drought-tolerant autochthonous bacteria under increasing PEG levels were assayed in comparison to a reference *B. megaterium* strain from our collection. The bacterial strains were cultivated at 28 ºC in nutrient medium supplemented with 0, 15, 30 and 40% of PEG. These treatments were replicated three times.

The number of viable cells was estimated along the time, from 3 to 6 days.

2.2.2. *Production of IAA and Proline by the Bacteria under 15% PEG along time (5, 6 and 7 days)*

The production of IAA by the bacteria was determined using the Salper reagent [27]. Three millilitres of fresh Salper reagent were added to free-cell supernatant and kept in complete darkness for 30 min, and the optical density at 535 nm was measured in each treatment. A standard curve was prepared for IAA (Sigma, USA). The proline was estimated by spectrophotometric analysis at 530 nm [26].

2.3. Statistical analyses

The data results of both experiments were subjected to analysis of variance (ANOVA), Duncan's multiple-range test [28]. Percentage values were arc sine-transformed before statistical analysis.

3. Results

3.1. Differential bacterial effects on plant growth responses mycorrhizal colonization and plant nutrition

As results show, the inoculations of these bacteria resulted effective for plant growth and nutrition under the drought conditions along the experimented period here used (1 year). Nevertheless, responses of *L. dentata* and *S. officinalis* to the native and reference bacterial strains inoculated resulted different. In *L. dentata*, the autochthonous *B. thuringiensis* clearly caused the highest beneficial effect on shoot and root growth (Table 1). Nevertheless, *Enterobacter* did not affect *L. dentata* biomass. In the case of *S. officinalis*, the plant reaction to the bacteria applied was different and less relevant than in *L. dentata*. In fact, all of the inoculated bacteria enhanced *S. officinalis* growth but non-significant differences *S. officinalis* plants on shoot growth between the inoculated with each one of the four bacteria were observed (Table 1). In *S. officinalis*, the bacterial inoculation increased particularly root development being *B. megaterium* and *B. thuringiensis* the most effective strains in increasing this value by 53 and 43%, respectively, over controls plants. These bacteria also significantly increase total AM colonization in both plants (Table 1).

Table 1 Effect of autochthonous bacterial strains (*Enterobacter* sp., *B. thuringiensis*, *Bacillus* sp.) and the reference *B. megaterium* on shoot and root growth (in milligram) and total AM colonization in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions.

	Shoot dry weight (mg)	Root dry weight (mg)	Shoot/root ratio	Total AM colonization
Lavandula dentata				
Control	650 a	360 a	1.80 ab	97 a
B. megaterium	970 b	460 b	1.50 a	138 b
Enterobacter sp.	680 a	420 ab	1.62 a	168 b
B. thuringiensis	1090 c	510 c	2.14 b	153 b
Bacillus sp.	860 b	410 ab	2.10 b	115 a
Salvia officinalis				
Control	510 a	790 a	0.64 b	324 a
B. megaterium	670 b	1210 b	0.55 a	726 c
Enterobacter sp.	620 b	880 a	0.70 b	466 b
B. thuringiensis	620 b	1130 b	0.55 a	655 c
Bacillus sp.	650 b	970 ab	0.67 b	475 b

Within each plant and value means followed by the same letter are not significantly different ($p \leq 0.05$) after ANOVA and Duncan tests.

The bacterial inoculation of each bacteria increased the mycorrhizal potential of the natural soil particularly in *S. officinalis* (Table 1). Nevertheless, the mycorrhizal frequency, arbuscules production (a % and A %), and the extraradical mycorrhizal mycelium, estimated as glomalin content, in rhizosphere soil was not affected by the bacterial treatments (data not shown). Shoot/root ratio was greater in *L. dentata* than in *S. officinalis* (Table 1).

In *S. officinalis*, inoculated bacteria did not increase K uptake but in *L. dentata* a big enhancement in K content was found in plants inoculated by bacteria (except *B. megaterium*) particularly by *B. thuringiensis* that increased this nutrient by 63% (Table 2). Similarly, the highest bacterial effect on Ca and Mg content was determined in this plant associated to *B. thuringiensis*. Nevertheless, in both shrubs plants nonsignificant differences in P content were found as result of the bacterial inoculation (Table 2). Concerning to the microelements acquisition, different trends were also observed in these both plants as affected by the bacteria inoculated. In *L. dentata*, *B. thuringiensis* enhanced Zn, Mn and Cu by 23, 54 and 39% respectively. This bacterium did not increased any of these micronutrients in *S. officinalis* (Table 3).

Table 2 Effect of autochthonous bacterial strains (*Enterobacter* sp., *B. thuringiensis*, *Bacillus* sp.) and the reference *B. megaterium* on P, K, Ca and Mg shoot acquisition (milligram/plant) inoculated in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean under drought conditions.

	P	K	Ca	Mg
Lavandula dentata				
Control	0.624 ab	13.512 a	13.297 b	2.143 ab
B. megaterium	0.542 a	18.353 b	15.270 b	2.360 ab
Enterobacter sp.	0.502 a	15.026 a	9.831 a	1.449 a
B. thuringiensis	0.644 b	21.967 c	16.833 c	2.911 c
Bacillus sp.	0.642 b	19.478 b	15.449 bc	2.563 bc
Salvia officinalis				
Control	0.635 a	9.874 a	8.288 a	2.557 a
B. megaterium	0.757 a	9.953 a	12.191 b	3.228 a
Enterobacter sp.	0.710 a	8.903 a	10.797 b	2.703 a
B. thuringiensis	0.779 a	9.416 a	11.196 b	2.876 a
Bacillus sp.	0.765 a	10.652 a	16.918 c	3.304 a

Within each plant and value means followed by the same letter are not significantly different ($p \leq 0.05$) after ANOVA and Duncan tests.

Table 3 Effect of autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis, Bacillus* sp.) and the reference *B. megaterium* on Zn, Fe, Mn and Cu content (microgram/plant) inoculated in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions.

	Zn	Fe	Mn	Cu
Lavandula dentata				
Control	38.504 b	104.247 b	13.409 a	5.189 b
B. megaterium	37.806 b	67.026 a	17.192 b	5.628 b
Enterobacter sp.	31.227 a	58.727 a	13.481 a	4.413 a
B. thuringiensis	47.391 c	100.209 b	20.599 c	7.226 c
Bacillus sp.	41.621 b	122.200 b	17.753 b	5.678 b
Salvia officinalis				
Control	29.495 b	56.409 b	17.300 b	3.981 a
B. megaterium	26.385 a	80.935 c	17.810 b	4.492 a
Enterobacter sp.	28.464 b	53.168 b	16.894 b	4.381 a
B. thuringiensis	24.600 a	50.378 b	19.657 bc	4.531 a
Bacillus sp.	30.573 b	57.186 b	23.974 c	6.080 b

Within each plant and value means followed by the same letter are not significantly different ($p \leq 0.05$) after ANOVA and Duncan tests.

3.2. Differential bacterial effects on plant physiological and antioxidant responses

As Fig. 1 shows, *B. thuringiensis* highly depressed stomatal conductance in *L. dentata* but such bacterial effect was not observed in *S. officinalis*. In *S. officinalis* the most active bacteria in decreasing such value was *B. megaterium* (Fig. 1).

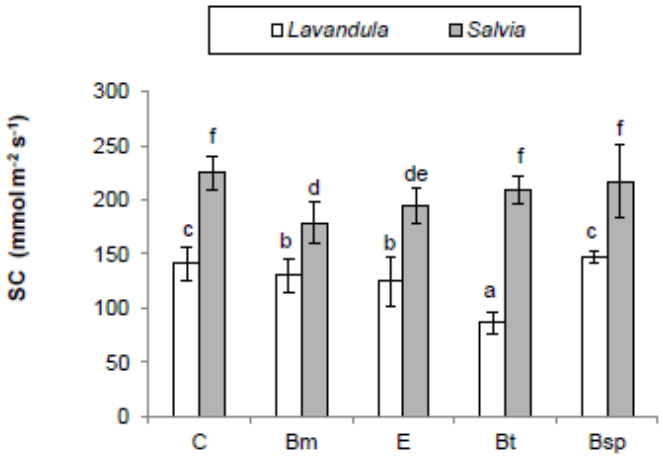


Fig. 1 Effect of autochthonous bacterial strains *Enterobacter* sp. (E), *B. thuringiensis* (Bt) and *Bacillus* sp. (Bsp) and the reference *B. megaterium* (Bm) on stomatal conductance (SC) in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions. Means followed by the same letter are not significantly different ($p \leq 0.05$) after ANOVA and Duncan tests.

Regarding the antioxidant activities (Fig. 2), we can observe that in *S. officinalis*, in different way than in *L. dentata,* the antioxidant APX, GR activities, and proline did not change or were increased as affected by the bacterial inoculations particularly by the native strains. In *L. dentata*, the opposite bacterial effects were four on these values. GR activity and proline were highly decreased in *L. dentata* by whatever bacteria inoculated.

The higher APX and GR activities were determined in non-inoculated *L. dentata* while the highest proline accumulation was determined in *S. officinalis* plants. As well, proline as GR and APX activities highly decreased in *L. dentata* by the bacteria applied. In *S. officinalis*, the bacterial inocula did not down regulated any of these activities as in *L. dentata* did. The similar SOD activity here observed in these plants and the lack of change as results of whatever bacterial inoculation indicates the lower value of this enzymatic activity as drought stress index in these plants (Fig. 2).

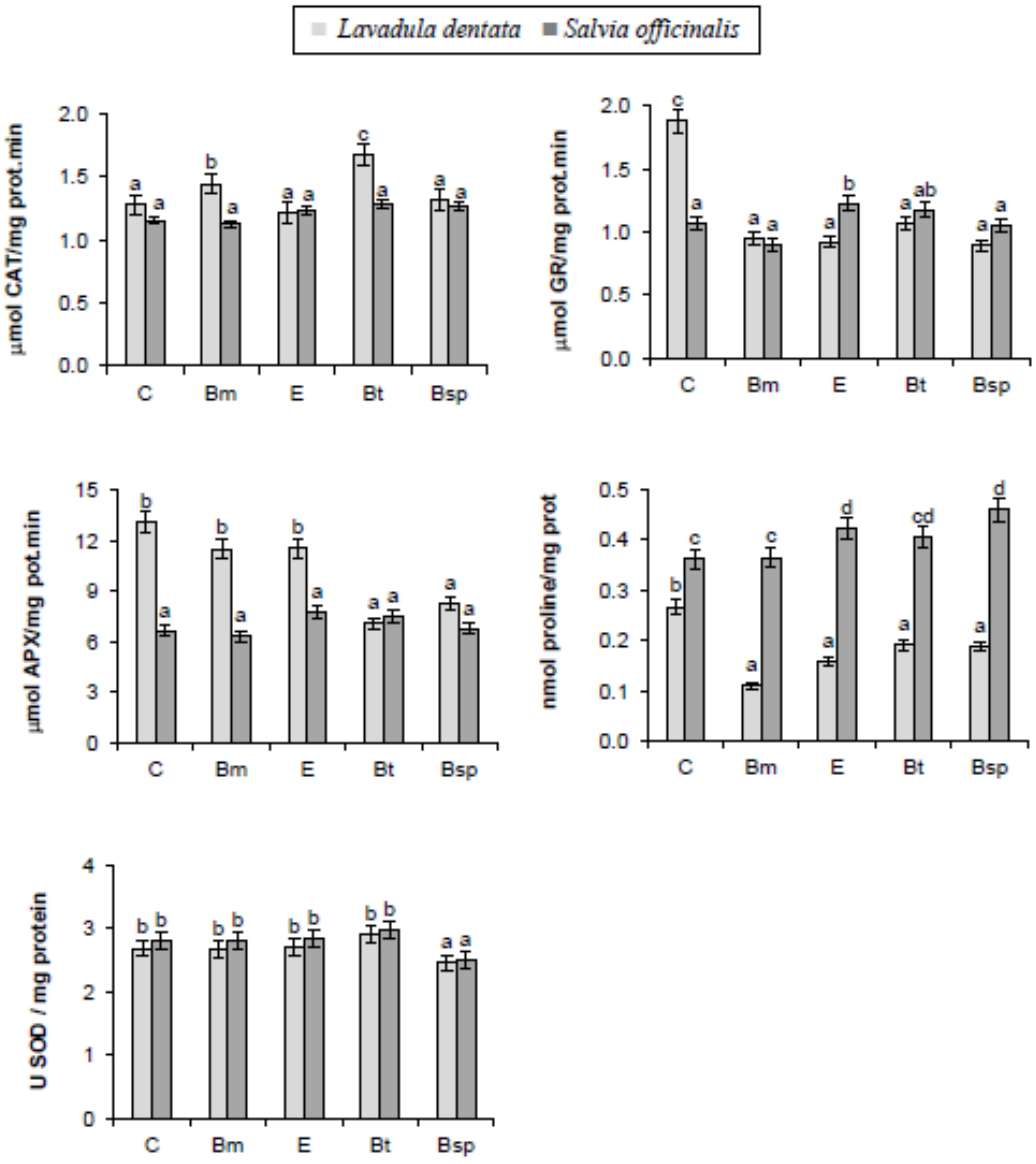


Fig. 2 Effect of autochthonous bacterial strains *Enterobacter* sp. (E), *B. thuringiensis* (Bt) and *Bacillus* sp. (Bsp) and the reference *B. megaterium* (Bm) on CAT, APX, GR and SOD antioxidant activities in shoot and proline accumulation in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions. Means followed by the same letter are not significantly different ($p \leq 0.05$) after ANOVA and Duncan tests.

3.3. Bacterial growth and response under drought conditions

With regard to the bacterial growth under increasing PEG levels in the axenic medium, we tested that *Enterobacter* exhibited the highest growth (cfu) which is indication of tolerance to the stress caused by highest levels of PEG. In contrast, the reference strain *B. megaterium* resulted the most sensitive to whatever PEG level in the growing medium since all of the PEG concentrations used highly reduced the bacterial growth (Fig. 3).

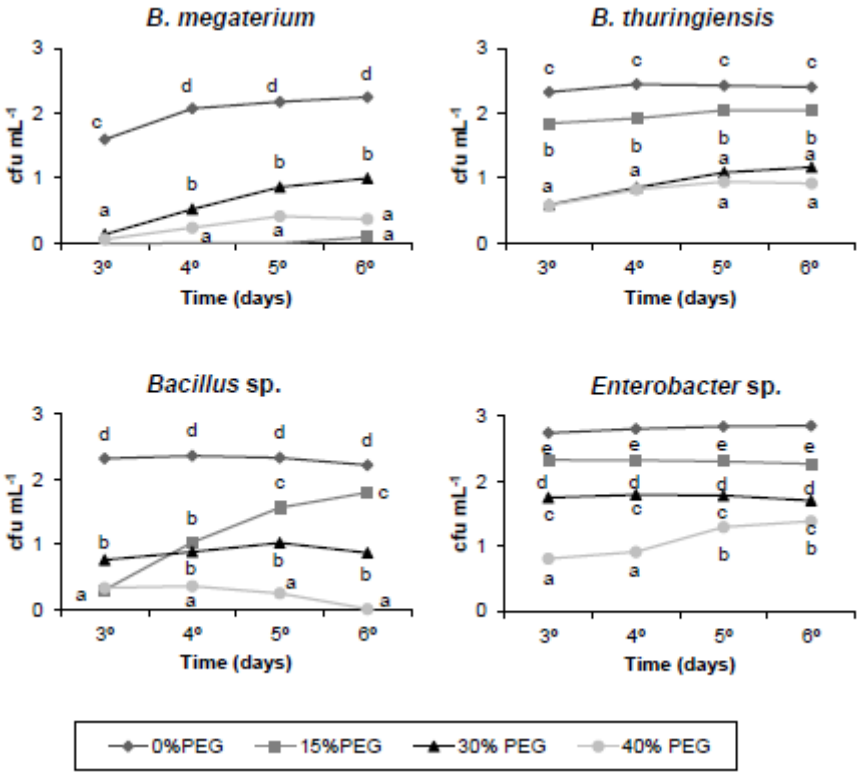


Fig. 3 Viable cells (cfu per milliliter) of bacterial strains growing in axenic nutrient medium supplemented with increasing levels of PEG at different time intervals (from 3 to 6 days).

Results of proline accumulation in the bacterial cells growing under 15% PEG indicated that after 5 days of culture autochthonous bacteria show the greatest values and the reference *B. megaterium* the lowest. Nevertheless, the maximum proline production was determined in *Bacillus* sp. in a later growth periods (after 6 and 7 days of growth) (Fig. 4).

In the same way than proline, IAA production (under 15% PEG) by the reference *B. megaterium* had the lowest amount at whatever culture time. The greatest IAA production was reached in *B. thuringiensis* culture irrespective of time of determination (Fig. 4).

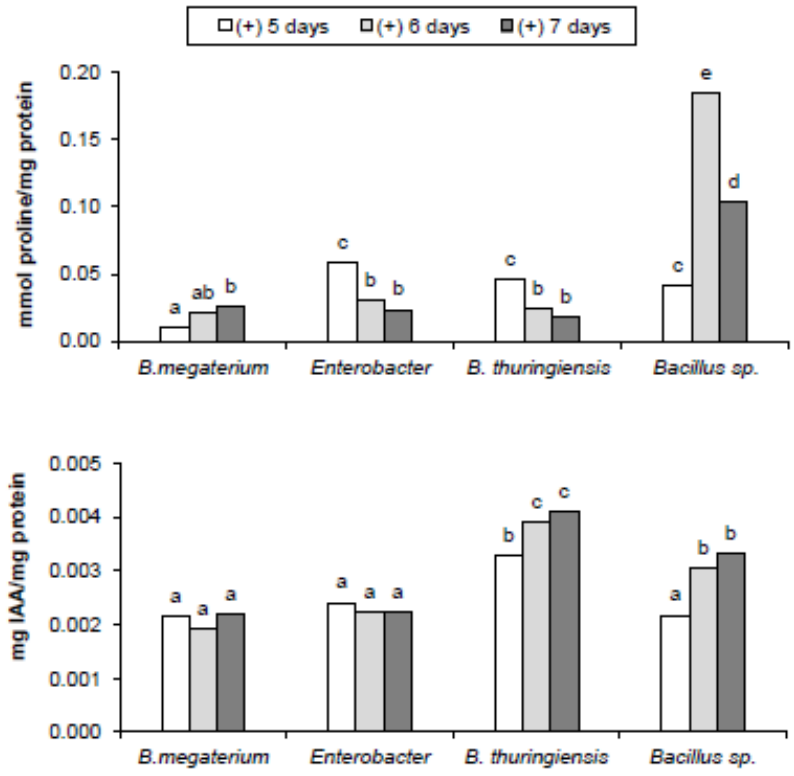


Fig. 4 Cell proline accumulation and indoleacetic acid (IAA) production by native and reference bacteria growing in axenic nutrient medium supplement with PEG (15%) at different time intervals (from 5 to 7 days).

4. Discussion

In this study, we evaluated the effectiveness on plant growth, antioxidant defence and nutrition of three autochthonous drought-adapted rhizosphere bacteria an one allochthonous (also drought-tolerant) of reference under drought conditions in a natural semiarid soil. No previous information reports the results on bacteria inoculation on shrub development in a natural soil under stressful conditions and the results show that the inoculated drought-resistant bacteria were able to enhance growth and to improve plant performance under such stressed drought conditions.

The relationship between plant nutrition and drought stress is important due to nutritional unbalances caused by drought. Results show that particularly *B. thuringiensis* increased K^+, Ca^{++} and Mg^{++} content in shoot of *L. dentata* plants. As it is well-known, K^+ content is an inorganic important osmolyte during drought. K^+ as inorganic osmolyte is important in water homeostasis under water deficit and it is able to regulate the stomatal opening, osmotic balance, maintenance of turgor pressure and reduction of transpiration under

drought stress [29]. Ca^{++} is also an important element controlling several physiological processes under water stress conditions such as transpiration, cell wall synthesis and cell division. Moreover, Ca^{++} is able to stabilize the membrane systems acting as an important cell protectant and Mg^{++} modulates the ion balance in cell, chloroplast, vacuolar membranes and stomatal opening highly related to drought stress [30]. *B. thuringiensis* induced increase in all of these nutrients which indicate that the photosynthetic functioning is affected in a lower extent by drought in inoculated plants. In addition, micronutrients as Zn^{++}, Mn^{++} and Cu^{++} also increased in *B. thuringiensis*-inoculated *L. dentata* plants. It is known that drought stress may affect not only the availability of micronutrients particularly of those slow diffusing but also the competitive uptake and transport is affected. All these changes were considered adaptative responses to the water deficiency. The lack of change or depressing effect in Fe^{++} content after the inoculation may be due to the lack of disturbance of this element by the drought.

In these stressed drought soils, plants are more dependent on microbial activity which is able to increase nutrients and water uptake [4]. The persistence and survival of bacterial community in the rhizosphere soil is very important in stressed environment for the establishment of plants in such environments [31]. But the endophytic condition of these bacteria, as here was tested, in an important mechanisms of inocula survival along time.

The axenic culture studies confirmed that the reference allochthonous *B. megaterium* exhibited the lowest tolerance to water deficit caused by osmotic stress (PEG) while autochthonous strains, particularly *Enterobacter,* resulted the most tolerant to the highest stress (30 and 40% of PEG) in the growing medium. Regarding the proline production by these bacteria as compatible solute able to help cells in the osmoregulation processes and to facilitate water uptake in response to the stress [32], we determine that the reference *B. megaterium* also resulted the lowest proline producers under stress conditions. Proline induces the adjustment of cell osmotic potential and this is indicative of osmotic adaptation by the bacterial cells. In fact the cells of *Bacillus* sp. required a greater proline accumulation than the others bacteria assayed as strategy to cope with drought (applied as PEG). Proline may be used for compensating the bacterial lack of drought tolerance. Similarly, the IAA production by these bacteria under stress conditions evidences their particular ability to promote plant growth under such environmental stress [33-36]. The reference *B. megaterium* also showed the lowest capacity for IAA production under stress conditions. IAA prevents the sensitivity to ethylene suppressing ethylene-initiated abscission signaling [37]. Microorganisms, depending on the environmental damage, can increase the activity preceding the final loss of function a certain threshold values.

In general, in the past, plant growth-promoting rhizobacteria (PGPR) have been used mainly for plant growth promotion by producing plant growth regulators. The ability of autochthonous bacteria to produce auxin-indole derivatives (as here was measured, under

osmotic stress, in the axenic culture medium) can cause part of the stimulating effects tested under these stress conditions. But recent studies show additional beneficial effects on different plant species through the bacterial ability to improve tolerance toward abiotic stresses [1, 38]. Several stress markers analyzed by molecular and biochemical methodologies studied the role of priming on different stress tolerance mechanisms by PGPR [5, 39]. Studies [4] show that plants colonized by the *B. megaterium* strain here used increased water content in *Trifolium repens* under water stress. This effect is particularly important in drought environments for preventing damage and enhancing plant survival under arid conditions. Nevertheless, it seems that various mechanisms were functioning in the stimulation of plant drought tolerance by the inoculated bacteria. In this study, K uptake was increased for the bacterial inocula more in *L. dentata* than in *S. officinalis* being *B. thuringiensis* the most active bacterial strain which resulted very efficient in enhancing K particularly in *L. dentata* (63% over control). Here, in *L. dentata* the K content correlated positively with the enhancement of plant biomass and a decrease of stomatal conductance as affected by the bacterial inoculation. Zhang et al. [40] reported that the salt tolerance in *Arabidopsis thaliana* was mediated through regulation of the HKT1 potassium transporter when inoculated with a *Bacillus* strain. The bacterial activity increasing K in *L. dentata* can be recognized as a very important mechanism to support drought conditions. Concomitantly, stomatal conductance was highly decreased in *L. dentata* inoculated with *B. thuringiensis*. This reduced evapotranspiration by the bacterial inoculation avoided water deficits.

One important mechanism related to stress tolerance is to alter oxidative stress that is necessary for plant survival under drought stress. Few data are available about the mechanism involved in bacterial-mediated plant antioxidant protection and the relevance of such processes in plants surviving and adaptation to drought under arid conditions. Plants have not immune system but they have alternative defence strategies as tools to overcome stress constraints, adapt to the changing environments, and survive. The accumulation of ROS in plant cells under stress are removed by enzymatic systems and the increase in antioxidant enzymatic activities is correlated with the stress severity [41,42]. Here, in *L. dentata,* APX activity was highly decreased (by 85%) when inoculated with *B. thuringiensis* and it is considered the key antioxidant enzyme in the ascorbate-glutathione redox cycle and APX plays an important role in scavenging ROS [43]. In parallel, in *B. thuringiensis*-inoculated *L. dentata*, the GR activity also was highly depressed (by 57%) and it has a central role in maintaining the reduced glutathione pool during the drought stress [44].

Antioxidant activities, particularly APX and GR, decreased in *L. dentata* colonized by the most effective bacteria (*B. thuringiensis*) indicated an important relationship among the level of antioxidant responses and this plant's adaptation to the drought stress but this effect varied

according the plant species involved. The reduction of these antioxidant production in bacterial-inoculated plants means an energy save in favour of vital processes [45]. This is one procedure to decrease the detrimental effects caused by drought. As well, the decrease observed in such antioxidant activities in inoculated plant responses to drought represents the better adaptation to the stress conditions, showing that lower antioxidant activities indicate a reduction of ROS level in stressed plants [5].

Regarding values of these antioxidant activities in *S. officinalis* inoculated by this bacteria (*B. thuringiensis*), different results than in *L. dentata* were found. In fact, both plants differ in antioxidant activities in response to stress. In general, *S. officinalis* shows lower intrinsic GR and APX activities than *L. dentata* which supports the hypothesis about the ability of this plant to have reduced these antioxidants levels under water stress conditions. The low CAT activity of *L. dentata* in response to drought may be caused by the use of GR and APX that have a much higher affinity for H_2O_2 than CAT [46]. *S. officinalis* has lower APX and GR but occasionally higher CAT than *L. dentata* and such antioxidant differences reflected intrinsic osmotic stress tolerance under drought. Such plant diversity in stress tolerance implies that inoculated bacteria may play multifaceted role to sustain drought avoidance in these plants.

In contrast, both *L. dentata* and *S. officinalis* maintained similar SOD activity and without any change in inoculated plants. This is an indication about the nonsignificant role of this activity in the defense against the oxidative stress induced by drought. Nevertheless, changes in plant CAT, APX and GR activities as result of inocula applied would be useful markers for the bacterial effect on strategies of drought tolerance in these plants.

Results show that stress mechanisms are different between these plant species. Thus, according to these results, the plant responses to bacterial inoculation on drought tolerance was different probably due to the relative effect of the bacterial colonization changing nutrition and physiology in each host plant.

The variation found between the protective enzymatic systems as affected by bacterial inocula in these two plants suggest that the bacterial effectiveness in drought tolerance act through particular and more or less specific mechanisms depending on the host plant. There is limited information on the varied growth-promoting effect of particular bacteria on host plant under different environmental natural conditions. Thus, it is important to identify the relevant factors involved in the plant responses under drought stress conditions to ascertain the bacterial effectiveness in arid environments. Previous studies reported that microbial groups as mycorrhizal fungi and/or PGPR also change antioxidant activities [47-48].

In *L. dentata*, whatever inoculated bacteria increased K content, in particular the most efficient *B. thuringiensis*, and in contrast, in *S. officinalis* any of them increase this nutrient. The

osmotic stress tolerance can be modulated by the accumulation of this cation. Potassium is considered the most important inorganic osmolyte.

The bacteria applied also stimulate root growth being such effects strongest in *S. officinalis* that particularly has the greatest root development. An important mechanisms related to the enhancement of plant tolerance to drought may be the change in shoot/root ratio in inoculated plants and it could improve the ability of these plants for increasing their water content. Plants as *S. officinalis* having a well-developed root system, particularly when inoculates with particular bacteria have a highest possibility for taking up water from the medium. Inoculated bacteria were able to produce IAA under drought conditions (as in axenic conditions was observed) and this phytohormone can be responsible for the root enhancement in inoculated plants. IAA production may also improved water use efficiency regulating plants physiological status as here was evidenced. Thus, the bacterial inocula may also affect the adjustment of water partitioning into apoplastic or symplastic space improving the drought tolerance [49].

S. officinalis shows a higher adaptation and/or tolerance and suffer less than *L. dentata* under the stress and results suggest that inoculated *L. dentata* plants have an increased possibility of water acquisition under water limitation.

S. officinalis shows the highest amount of proline and the lowest GR and APX activities. It seems that in this plant proline correlated with a negative regulation of GR and APX activities. In contrast, low proline in *L. dentata* and high GR and APX activities suggest a more direct role of these enzymes in the *L. dentata* protection against oxidative injury. Cells with a greater proline accumulation have a lower lipid peroxidation by drought stress. The efficient and active role of proline in depressing ROS damage has been suggested [50].

Curiously, these nutritional, physiological and biochemical differences between *S. officinalis* and *L. dentata* significantly affected their particular response to the bacterial inocula applied. Differences in whatever parameter here evaluated reflected the diversity and particular stress tolerance of these plants. These and previous results indicate that each plant may play a multifaceted role to maintain health and a multiplicity of factors may be involved in reaching the optimum growth under drought conditions [4, 47, 48, 51].

Inoculated autochthonous bacteria have a varied and strong impact in improving plant stress tolerance mechanisms [1]. Bacteria can help plants in the osmoregulation processes and in improving homeostatic mechanisms upon stress challenge [49]. As results show, a combination of nutritional, metabolic, physiological and morphological changes on the inoculated plants are carried out by the bacteria able to control drought stress in plants. But plant characteristics are important factors affecting the bacterial role in plant adaptation to drought.

According to the results, *B. thuringiensis* produced the highest amount of IAA and proline (at 15% PEG) in axenic culture and this is correlated with the greatest *L. dentata* growth and K nutrition and the lowest stomatal conductance and antioxidant activities. In fact, these measurements resulted an useful marker of bacterial effectiveness in this plant under water stress conditions. As well it is important, from a practical point of view, to know that these bacteria were able to survive and to multiply to reach a sufficient population to express himself activities under stress conditions. This suggest that they can maintain a long time their biochemical traits related to positive effects in inoculated plants under water limiting conditions. The water limitation and osmotic stress negatively affect plant growth but the bacterial inoculation was able to attenuated these detrimental effects.

The use of bacteria to control drought stress in plants is an important and sustainable strategy. But the related processes seem to be regulated differently according to the natural resistance and intrinsic stress tolerance of the plants. The selection of microorganisms involved is important to reach the maximum plant benefit. However, further researches are required to establish the main processes by which bacteria improve plant performance.

Acknowledgements

E. Armada was financed by Ministerio de Ciencia e Innovación. This work was carried out in the framework of the project reference AGL2009-12530-C02-02. We thank to the Instrumentation Service (EEZ) by the plant analysis.

References

1. Dimkpa C, Weinand T, Asch F (2009) Plant-rhizobacteria interactions alleviate abiotic stress conditions, Plant Cell Environ. 32:1682-1694
2. Haferburg G, Kothe E (2007) Microbes and metals: interactions in the environment, J. Basic Microbiol. 47:453-467
3. Marulanda A, Barea JM, Azcón R (2006) An indigenous drought-tolerant strain of *Glomus intraradices* associated with a native bacterium improves water transport and root development in *Retama sphaerocarpa*, Microb. Ecol. 52:670-678
4. Marulanda A, Barea JM, Azcón R (2009) Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments. Mechanisms related to bacterial effectiveness, J. Plant Growth Regul. 28:115-124
5. Kasim WA, Osman ME, Omar MN, Abd El-Daim IA, Bejai S, Meijer J (2013) Control of drought stress in wheat using plant-growth-promoting bacteria, J. Plant Growth Regul. 32:122-130
6. Barea JM, Azcón R, Azcón-Aguilar C (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality, Anton. Leeuw. Int. J. G. 81:343-351
7. Medina A, Azcón R (2012) Reclamation strategies of semiarid mediterranean soil: improvement of the efficiency of arbuscular mycorrhizal fungi by inoculation of plant growth promoting microorganisms and organic amendments. In: Hafidi M, Duponnois R (Eds) The Mycorrhizal Symbiosis in Mediterranean Environment: Importance in Ecosystem Stability and in Soil Rehabilitation Strategies, Nova Science Publishers New York, pp 87-106

8. Marulanda A, Azcón R, Ruíz-Lozano JM (2003) Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress, Physiol. Plant. 119:526-533
9. Zahir ZA, Arshad M, Frankenberger WT (2004) Plant growth promoting rhizobacteria: Applications and perspectives in agriculture, Adv. Agron. 81:97-168
10. Nowak J (1998) Benefits of in vitro "biotization" of plant tissue cultures with microbial inoculants, In Vitro Cell. Dev. Biol.-Plant 34:122-130
11. Potters G, Horemans N, Jansen MAK (2010) The cellular redox state in plant stress biology - A charging concept, Plant Physiol. Biochem. 48:292-300
12. Benabdellah K, Abbas Y, Abourouh M, Aroca R, Azcon R (2011) Influence of two bacterial isolates from degraded and non-degraded soils and arbuscular mycorrhizae fungi isolated from semi-arid zone on the growth of Trifolium repens under drought conditions: Mechanisms related to bacterial effectiveness, Eur. J. Soil Biol. 47:303-309
13. Ruíz-Sánchez M, Aroca R, Munoz Y, Polon R, Ruíz-Lozano JM (2010) The arbuscular mycorrhizal symbiosis enhances the photosynthetic efficiency and the antioxidative response of rice plants subjected to drought stress, J. Plant Physiol. 167:862-869
14. Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic-stress, Microbiol Rev 53:121-147
15. Glick BR (1995) The enhancement of plant-growth by free-living bacteria, Can. J. Microbiol. 41:109-117
16. Phillips JM, Hayman DS (1970) Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection, Trans. Br. Mycol. Soc. 55:159-161
17. Giovannetti M, Mosse B (1980) Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots, New Phytol. 84:489-500
18. Wright SF, Upadhyaya A (1996) Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi, Soil Science 161:575-586
19. Aroca R, Irigoyen JJ, Sánchez-Díaz M (2003) Drought enhances maize chilling tolerance. II. Photosynthetic traits and protective mechanisms against oxidative stress, Physiol. Plant. 117:540-549
20. Burd GI, Dixon DG, Glick BR (2000) Plant growth promoting bacteria that decreased heavy metal toxicity in plants, Can. J. Microbiol. 46:237-245
21. Aebi H (1984) Catalase *in vitro*, Methods Enzymol. 105:121-126
22. Amako K, Chen GX, Asada K (1994) Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants, Plant Cell Physiol 35:497-504
23. Carlberg I, Mannervik B (1985) Glutathione reductase, Methods Enzymol. 113:484-489
24. Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding, Anal. Biochem. 72:248-254
25. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37:911-917
26. Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies, Plant Soil 39:205-207
27. Gordon SA, Paleg LG (1957) Observations on the quantitative determination of indoleacetic acid, Physiol. Plant. 10:39-47
28. Duncan DB (1955) Multiple range and multiple F tests, Biometrics 11:1-42
29. Loutfy N, El-Tayeb MA, Hassanen AM, Moustafa MFM, Sakuma Y, Inouhe M (2012) Changes in the water status and osmotic solute contents in response to drought and salicylic acid treatments in four different cultivars of wheat (*Triticum aestivum*), J. Plant Res. 125:173-184
30. Shaul-Keinan O, Gadkar V, Ginzberg I, Grunzweig JM, Chet I, Elad Y, Wininger S, Belausov E, Eshed Y, Arzmon N, Ben-Tal Y, Kapulnik Y (2002) Hormone

concentrations in tobacco roots change during arbuscular mycorrhizal colonization with *Glomus intraradices*, New Phytol. 154:501-507
31. Rodríguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim Y-O, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis, Isme Journal 2:404-416
32. Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance, Environ. Exp. Bot. 59:206-216
33. Egamberdieva D, Kamilova F, Validov S, Gafurova L, Kucharova Z, Lugtenberg B (2008) High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan, Environ. Microbiol. 10:1-9
34. Mayak S, Tirosh T, Glick BR (2004) Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers, Plant Sci. 166:525-530
35. Belimov AA, Dodd IC, Hontzeas N, Theobald JC, Safronova VI, Davies WJ (2009) Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase yield of plants grown in drying soil via both local and systemic hormone signalling, New Phytol. 181:413-423
36. Yuwono T, Handayani D, Soedarsono J (2005) The role of osmotolerant rhizobacteria in rice growth under different drought conditions, Aust. J. Agric. Res. 56:715-721
37. Sakamoto M, Munemura I, Tomita R, Kobayashi K (2008) Involvement of hydrogen peroxide in leaf abscission signaling, revealed by analysis with an in vitro abscission system in Capsicum plants, Plant J. 56:13-27
38. Yang J, Kloepper JW, Ryu C-M (2009) Rhizosphere bacteria help plants tolerate abiotic stress, Trends Plant Sci. 14:1-4
39. Creus CM, Sueldo RJ, Barassi CA (2004) Water relations and yield in *Azospirillum*-inoculated wheat exposed to drought in the field, Canadian Journal of Botany-Revue Canadienne De Botanique 82:273-281
40. Zhang Y, Wang Z, Zhang L, Cao Y, Huang D, Tang K (2006) Molecular cloning and stress-dependent regulation of potassium channel gene in Chinese cabbage (*Brassica rapa* ssp Pekinensis), J. Plant Physiol. 163:968-978
41. Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V, Mittler R (2008) Ascorbate peroxidase 1 plays a key role in the response of Arabidopsis thaliana to stress combination, J. Biol. Chem. 283:34197-34203
42. Gong HJ, Zhu XY, Chen KM, Wang SM, Zhang CL (2005) Silicon alleviates oxidative damage of wheat plants in pots under drought, Plant Sci. 169:313-321
43. Orvar BL, Ellis BE (1997) Transgenic tobacco plants expressing antisense RNA for cytosolic ascorbate peroxidase show increased susceptibility to ozone injury, Plant J. 11:1297-1305
44. Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA (2009) Plant drought stress: effects, mechanisms and management, Agron. Sustain. Dev. 29:185-212
45. Miller KJ, Wood JM (1996) Osmoadaptation by rhizosphere bacteria, Annu. Rev. Microbiol. 50:101-136
46. Jiménez A, Hernández JA, del Rio LA, Sevilla F (1997) Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves, Plant Physiol. 114:275-284
47. Kohler J, Hernández JA, Caravaca F, Roldán A (2009) Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress, Environ. Exp. Bot. 65:245-252
48. Kohler J, Hernández JA, Caravaca F, Roldán A (2008) Plant-growth-promoting rhizobacteria and arbuscular mycorrhizal fungi modify alleviation biochemical mechanisms in water-stressed plants, Funct. Plant Biol. 35:141-151
49. Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses, Plant Cell Environ. 33:453-467

50. Vendruscolo ECG, Schuster I, Pileggi M, Scapim CA, Correa Molinari HB, Marur CJ, Esteves Vieira LG (2007) Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat, J. Plant Physiol. 164:1367-1376
51. Azcón R, Medina A, Aroca R, Ruíz-Lozano R (2013) Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast interactions. In: de Bruijn FJ (Ed) Molecular Microbial Ecology of the Rhizosphere John Wiley & Sons, Hoboken, New Jersey, pp 991-1002

CHAPTER 2.3

Microbial community analysis by PLFA and Pyrosequencing in autochthonous shrubs species in drought stress and effect of native bacterial of Mediterranean soil

Elisabeth Armada, Almudena Medina, Eiko E Kuramae, Rosario Azcón

1. Introduction

Microbial communities play important roles in soil because of the many functions they perform in nutrient cycling, plant symbioses, decomposition, and other ecosystem processes (Nannipieri *et al.*, 2003). Several studies have shown that plant species have a major selective influence on microbial communities in their rhizospheres (Garland, 1996; Smalla *et al.*, 2001) while soil microbes are important regulators of plant productivity, both through direct effects and through regulation of nutrient availability (Van Der Heijden *et al.*, 2008). Soil microorganisms synthesize and secrete extracellular enzymes, which constitute an important part of the soil matrix (Sinsabaugh *et al.*, 1993). Microbial enzymes play an essential role in soil nutrient cycles and, consequently, factors influencing soil microbial activity will affect the production of the enzymes which control nutrient availability and soil fertility. Soil enzymatic activities have been suggested as potential sensitive indicators of change in soil quality (Bastida *et al.*, 2008; Hu *et al.*, 2011). It has been reported that enzyme activities decreased in Mediterranean ecosystems due to severe drought conditions (Caravaca *et al.*, 2002), which might have a negative effect on nutrient availability.

Soil quality is strongly influenced by microbe-mediated processes, and function can be related to diversity, it is likely that microbial community structure have the potential to serve as an early indication of soil degradation or soil improvement (Jackson *et al.*, 2003; Aboim *et al.*, 2008; Peixoto *et al.*, 2010). Techniques based on molecular biology have given us a way to characterize the structure of the microbial community, and therefore monitor their dynamics. Biomarkers fatty acids are used extensibility in studies of soil microbial ecology since they provide qualitative and quantitative information about microbial communities. Analysis of phospholipids fatty acids (PLFA) from microbial membranes derived from lipid fractionating serve as the main method platform (Frostegard *et al.*, 1993; Frostegård and Bååth, 1996; Zelles, 1997). It provides a set of molecular markers for microbial taxa and indicators of microbial stress that can be used to track changes in composition of the soil microbial community, and it also gives a measure of the total viable microbial biomass (Bossio and Scow, 1995; White *et al.*,

1996). Lipid separation also provides a neutral lipid fatty acid (NLFA) fraction with information about eukaryotic energy reserves useful in studies of fungal nutritional status (Bååth, 2003).

Recent advances in sequencing technology, such as next generation sequencing are a promising approach for evaluating microbial diversity and microbial community structure in different environments (Cristea-FernstrÖm *et al.*, 2007; Roesch *et al.*, 2007).

Earlier experiments have demonstrated that the arbuscular mycorrhizal (AM) fungal diversity in soil can affect the diversity and productivity of plants and, therefore, the stability and sustainability of the ecosystems (Van Der Heijden *et al.*, 1998; Van Der Heijden *et al.*, 2006). And the composition and diversity of the plant community influence the structure of the AM fungi community (Burrows and Pfleger, 2002; Johnson *et al.*, 2003). Thus, mycorrhizal symbiosis seems to be a key ecological factor in the functioning of ecosystems in semiarid Mediterranean regions (Requena *et al.*, 1996). Several studies have shown that AM fungi have host preferences or host specificity and that different plant species are colonized by different AM fungal communities (Vandenkoornhuyse *et al.*, 2003; Öpik *et al.*, 2006; Alguacil *et al.*, 2009), although a lack of specificity for some AM fungal species also has been indicated (Öpik *et al.*, 2006). In addition, plants can interact with several other soil microorganisms than AM fungi, including plant growth-promoting rhizobacteria (PGPR) that make the plant more tolerant to stresses (Barea *et al.*, 2002; Vessey, 2003; Barea *et al.*, 2005). There is ecological interest in the diversity of AM fungi and bacteria PGPR present in roots of different plant species, particularly in revegetation programs for ecosystems using autochthonous shrubs (Armada *et al.*, 2014; Mengual *et al.*, 2014). The selection of autochthonous plants species capable to host high AM fungi diversity in their rhizosphere is a very important a step for the soil restoration. The aim of this work was to examine (1) the importance of 3 different native species (*Thymus vulgaris, Santolina chamaecyparissus* and *Lavandula dentata*) in shaping natural rhizosphere soil community (2) the influence of the inoculation of a beneficial native bacteria in the 3 above plant species on the development and survival of AM fungi and (3) the influence of the inoculation of a beneficial native bacteria in the 3 above plant species on the rhizosphere bacterial community and function (enzymatic activities).

2. Materials and Methods

2.1. Soil bacteria isolation and molecular identification used in the microcosm experiment

The bacterial strain used in this study was isolated from the same natural soil used in the microcosm experiment (see description below). The bacterium was isolated from a mixture of

rhizosphere soils from several autochthonous shrub species. A homogenate of 1 g soil in 9 mL sterile water was diluted (10^{-2} to 10^{-4}), plated on three different media [Yeast Mannitol Agar, Potato Dextrose Agar, Luria-Bertani (LB) Agar (Bertani, 1951)] and then incubated at 28 ºC for 48 h, to isolate bacteria from different taxonomic groups. The selected bacterium was the most abundant bacterial type in such arid soil.

The identification of the selected bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were collected, diluted, lysed, and genomic DNA extracted. The DNAs were used as a template in the PCR reactions. All reactions were conducted in 25 µL volume containing PCR buffer 10X, 50 mM $MgCl_2$, 10 µL each primers: 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), 5 U/µl of *Taq* polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with following conditions: 5 min at 95 ºC, followed by 30 cycles of 45s at 95 ºC, 45s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. The products of PCR were analyzed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST program. Similarity searches at NCBI using BLAST program, unambiguously identified the bacterium as *Bacillus thuringiensis* (Acession NR 043403.1, similarity >98%).

2.2. Isolation and identification of the AM fungi present in the soil used in the microcosm experiment

The identification of the AM fungi species present in the natural soil used in the microcosm experiment (see description below) was realized as follows: first AM fungal spores were isolated from the soil samples by a wet sieving process (Sieverding, 1991). The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG) (Koske and Tessier 1983); a mixture of PVLG and Melzer's reagent (Brundrett, 1994); a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain, 1990). For identification of the AM fungi species, spores were then examined using a compound microscope at up to 400-fold magnification as described for glomeromycotean classification by Oehl et al. (2011). The more predominant AM fungi species identified in the native consortium used in this study area were: *Septoglomus constrictum*, *Diversispora aunantia*, *Archaespora trappei*, *Glomus versiforme*, and *Paraglomus ocultum*, which were catalogued and included in the collection of EEZ (codes EEZ 198 to EEZ 202, respectively).

2.3. Microcosm experimental design and characteristics of soil

The microcosm experimental design was based on two factors: (1) three different autochthonous shrub species: (*Thymus vulgaris* (T); *Santolina chamaecyparissus* (S); *Lavandula dentata* (L)) and (2) inoculation or not of the autochthonous bacteria strain *Bacillus thuringiensis:* non-inoculated (-); *Bacillus thuringiensis* (Bt). Each treatment was replicated five times for total of 30 pots.

The soil used in this experiment is natural soil located in the natural park "Vicente Blanes" in Molina de Segura, Murcia, Spain, (coordinates: 38° 12' N, 1º 13' W; 393 m altitude). The soil in the experimental area is a Typic Torriorthent (SSS, 2006) very little developed with a silty-clay texture that facilitates the degradation of soil structure, and low organic matter content. The main soil characteristics are: organic C 0.94%, total N 0.22%, P $1.36 \cdot 10^{-3}$ g kg^{-1} (Olsen test), pH 8.9 and an electric conductivity of 1.55 dS m^{-1}. The substrate used in this assay consisted in the target soil, screened (5mm), and mixed with sterile sand [5/2 (v/v)]. Substrate was put into pots with a capacity of 0.5 kg. One milliliter of pure culture of *B. thuringiensis* (10^8 cfu mL^{-1}), grown in broth LB medium (Bertani, 1951) for 48 h at 28 ºC was applied to the appropriate pots at sowing time just below the plant seedlings. The bacterial inoculum was applied again 15 days later.

These three plants species were grown for one year in pots containing a mixture of natural soil and quartz sand (5/2 (v/v)) under greenhouse conditions (temperature ranging from 19 to 25 ºC, 16/8 light/dark photoperiod and a relative humidity of 50-70%). The photosynthetic photon flux density (PPFD) was 400-700 µmol m^{-2} s^{-1}, as measured with a light-meter (LICOR, model LI-188B). Plants were grown along the experiment under drought conditions by keeping soil water capacity to 50% each day after water application but water level decreased along day to nearly 30% water capacity to the next water application.

2.4. Plant biomass and nutrient analysis

One year after planting, plants were harvested (five replicates per treatment, n=5), shoots were excised from the roots, and both shoots and roots were weighted to record fresh weights. After that, they were dried for 48 h at 75 ºC to obtain dry weights.

Shoot content (mg $plant^{-1}$) of P, K, Ca and Mg as well as of Zn, Fe, Mn and Cu (µg $plant^{-1}$) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) at Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

2.5. Mycorrhizal counting

Roots were carefully washed and stained. The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples (Phillips and Hayman, 1970), using the gridline intersect method of Giovannetti and Mosse (1980).

2.6. Enzymatic activity in rhizosphere soil

Dehydrogenase activity was determined following Skujins' method (Skujins, 1976), as modified by García et al. (1997). For this, 1 g of soil at 60% of its field capacity was exposed to 0.2 mL of 0.4% INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h, at 22 ºC in darkness. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 mL of methanol, by shaking vigorously for 1 min and filtering through a Whatman N° 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

β-glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG), 0.05 M (Masciandaro et al., 1994) as substrate. This assay is also based on the release and detection of PNP. Two milliliters of 0.1 M maleate buffer (pH 6.5) and 0.5 mL of substrate were added to 0.5 g of sample and incubated at 37 ºC for 90 min. The reaction was stopped with tris-hydroxymethyl aminomethane (THAM) according to Tabatabai (1982). The amount of PNP was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969).

Urease activity was determined by the method of Nannipieri et al. (1980), and expressed as μmol $N\text{-}NH_3$ g^{-1} soil $\cdot h^{-1}$.

Alkaline phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP) 0.115 M as substrate. Two milliliters of 0.5 M sodium acetate buffer adjusted to pH 5.5 using acetic acid (Naseby and Lynch, 1997) and 0.5 mL of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 mL of 0.5 M $CaCl_2$ and 2 mL of 0.5 M NaOH were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969). In controls, the substrate was added before the $CaCl_2$ and NaOH addition.

2.7. Microbial lipid extraction and PLFA analysis

The lipid extraction, fractionation, mild alkaline methanolysis and GC analysis were according to Frostegard et al. (1993). PLFA analysis was carried out in freeze-dried frozen samples kept at −80ºC. Lipids were extracted from 3 g lyophilized soil using a one-phase mixture (1:2:0.8 v/v/v) of chloroform/methanol/citrate buffer (0.15 M pH 4.0). After extraction the lipids were separated into neutral lipids, glycolipids and polar lipids (phospholipids) on

silicic acid (Merck Kieselgel 60 63-200µm) columns followed by a mild alkaline methanolysis to form fatty acid methyl esters for GC analysis. The fatty acids were identified from their retention times in relation to that of the internal standard (fatty acid methyl ester 19:0 and 12:0).

The following fatty acids were used as biomarkers for bacterial biomass: i14:0, i15:0, a15:0, i16:0, 16:1w7t, 17:1w7, a17:1w7, i17:0, cy17:0, 18:1w7c and cy19:0 (Mauclaire *et al.*, 2003). PLFA 16:1w5 was used as an indicator of arbuscular mycorrhizal fungi (Olsson *et al.*, 1995; Drigo *et al.*, 2010). C18:2w6.9 was used as a measure of fungal biomass (Bååth, 2003). Methylated fatty acids (10Me16:0) was used as specific biomarkers for *Actinomycetes* (Frostegard *et al.*, 1993; Welc *et al.*, 2010). The ratios of Gram positive to Gram negative bacteria were calculated by taking the sum of the PLFAs i-C14:0, i-C15:0,a-C15:0,i-C16:0, i-C17:0 and a-C17:0 where designated as Gram positive, whereas C16:1w7, C17:0 cy and C18:1w7 as Gram negative bacterial biomarkers (Frostegård and Bååth, 1996; Zelles, 1997).

2.8. Soil DNA extraction, PCR conditions for fungal and bacterial tag-encoded amplicon and amplicons sequencing

DNA was extracted from 0.5 g of soil using the fast DNA Spin Kit for soil (MO BIO Laboratories inc., Carlsbad CA, USA) and quantified in spectrophotometer (Nanodrop Technology, Wilmington, DE, USA). The integrity of the DNA was verified on 1% agarose gel with TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0).

For fungal 18S rRNA partial gene amplification, the primers described by Verbruggen et al., (2012) were used. The 5′ terminus of primers contained an adaptor sequence and a multiplex identifier tag (MID; 12 different 10-bp-long tags), which resulted in the following primer constructs (adaptor in boldface): Forward (FF390.1), 5′-**CTATGCGCCTTGCCAGCCCGCTCAG**-(MID)-CGWTAACGAACGAGACCT-3′; Reverse (FR1), 5′-**CGTATCGCCTCCCTCGCGCCATCAG**-(MID)-AICCATTCAATCGGTAIT-3′. PCRs contained 2.0 µL (10 µM) of each forward and reverse primer, 5.0 µL 10x PCR-buffer, 5.0 µL dNTP's (2 mM), 0.5 µL BSA, 33.10 µL mili-Q and 0.40 µL of FastStar Expand TAQ DNA polymerase (5 U/µL). The PCR conditions were 95 ºC for 5 min followed by 25 cycles of 95 ºC for 30 s; 57 ºC for 1 min and 72 ºC for 1 min; and a final elongation step at 72ºC for 10 min. Products were purified using QIAquick PCR Purification Kit (Qiagen).

For bacteria, the V4 region of the 16S rRNA gene was amplified by PCR using 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3'). The 515F primer included the Roche 454-B pyrosequencing adapter (a 10-bp barcode) unique to each sample, and a GT linker, while 806R included the Roche 454-A sequencing adapter (a 10-

bp barcode), unique to each sample, and a GG linker. PCRs contained 1.0 µL (5 µM) of each forward and reverse primer, 2.5 µL 10x PCR-buffer, 2.5 µL dNTP's (2 mM), 16.80 µL mili-Q and 0.20 µL of FastStar Expand TAQ DNA polymerase (5 U/µL), was used for PCR under the following conditions: 95 ºC for 5 min followed by 30 cycles of 95 ºC for 30 s; 53 ºC for 1 min and 72 ºC for 1 min; and a final elongation step at 72 ºC for 10 min. Products were purified using QIAquick PCR Purification Kit (Qiagen). Amplicons were quantified and equimoler pooled. The samples were sequenced (Macrogen Inc. Company, South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA).

2.9. Statistical analysis

Statistical analysis involved ANOVA and Duncan's (1955) multiple-range test to determine significant differences. Multiple analysis of variance (MANOVA) applied on principal component analysis (PCA) was carried out in SPSS 21 software package for Windows, used to screen for differences in the PLFA composition of the soil microbial community. All analyzes were conducted at $p \leq 0.05$. The Shannon-Weaver H' diversity index was calculated with two components of diversity (species richness and evenness). Non-metric multidimensional scaling (NMDS) were used to examine the relationship between the treatments (three species autochthonous plants and application or not of *B. thuringiensis*) and the microbial community structure of each soils.

3. Results

3.1. Plant growth, nutrition and symbiotic parameters

The inoculation of *B. thuringiensis* increased the shoot biomass of the three plant species tested in this study and the highest yield was achieved in *L. dentata*, with an increase of 66% of shoot and 39% of root compared to the control. *T. vulgaris* had the highest percentage of AM root colonization among all the non-inoculated plant species. The inoculation of *B. thuringiensis* in *S. chamaecyparissus* significantly increased the percentage AM root colonization in by 92% and total AM fungi colonization in by 145% with respect to the non-inoculated controls (Table 1).

S. chamaecyparissus presented the highest level of P and *L. dentata* the highest shoot content of K, Ca and Mg (Table 2). The inoculation of the native *B. thuringiensis* (Bt) bacteria increased the total P, K, Ca, Mg content in the shoots of the three autochthonous plant species. The highest enhancement was achieved in *T. vulgaris*, with an increase of 51% in P and of 47%

in K shoot content and in *L. dentata*, with an increase of 63% in K, of 27% in Ca and of 36% in Mg shoot content compared to the non-inoculated controls (Table 2).

B. thuringiensis also increased the shoot content of the micronutrients Zn, Fe and Cu in *T. vulgaris* and Zn, Mn and Cu in *S. chamaecyparissus* compared to the non-inoculated controls (Table 2).

Table 1. Plant growth parameters and AMF root colonization of three autochthonous plants species [*Thymus vulgaris* (T)*; Santolina chamaecyparissus* (S); *Lavandula dentata* (L)] grown in natural arid Mediterranean soil under drought stress conditions as affected by the inoculation of the autochthonous bacteria strain *Bacillus thuringiensis* (Bt).

	Shoot dry weight (mg)	Root dry weight (mg)	AMF (%)	Total AMF colonization
T(-)	667 ±71.5b	425 ±46.0b	54 ±3.0b	231 ±37.6c
TBt	779 ±39.9c	336 ±25.2ab	48 ±3.5b	162 ±24.2bc
S(-)	521 ±33.5a	242 ±45.3a	24 ±4.1a	64 ±22.4a
SBt	687 ±35.5bc	339 ±21.0ab	46 ±4.6b	157 ±25.7bc
L(-)	655 ±33.0b	364 ±60.1b	27 ±2.1a	97 ±16.2ab
LBt	1086 ±4.1d	507 ±22.5c	30 ±1.7a	153 ±1.8bc

Standard errors are given. Within each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n= 5).

Table 2. Total content of the macronutrients (P, K, Ca, Mg) and micronutrients (Zn, Fe, Mn and Cu) in the shoot of three autochthonous plants species [*Thymus vulgaris* (T)*; Santolina chamaecyparissus* (S); *Lavandula dentata* (L)] grown in natural arid Mediterranean soil under drought stress conditions as affected by the inoculation of the autochthonous bacteria strain *Bacillus thuringiensis* (Bt).

	P (mg plant^{-1})	K (mg plant^{-1})	Ca (mg plant^{-1})	Mg (mg plant^{-1})	Zn (µg plant^{-1})	Fe (µg plant^{-1})	Mn (µg plant^{-1})	Cu (µg plant^{-1})
T(-)	0.57 ±0.01a	7.07 ±0.25a	6.01 ±1.85a	1.70 ±0.62bc	33.28 ±1.69a	42.79 ±3.67a	40.35 ±14.51b	4.21 ±0.27a
TBt	0.86 ±0.05b	10.42 ±0.07b	7.94 ±0.28a	2.21 ±0.24c	54.06 ±5.90bc	115.22 ±17.83b	46.49 ±0.74b	6.38 ±1.04bc
S(-)	0.87 ±0.03b	10.10 ±0.45b	8.45 ±1.00a	1.01 ±0.06a	60.93 ±3.78c	87.16 ±20.55ab	100.19 ±3.02c	11.05 ±0.59d
SBt	1.01 ±0.04c	12.93 ±0.70c	11.95 ±0.67b	1.43 ±0.10ab	89.72 ±7.46d	78.43 ±19.76ab	121.93 ±2.57d	13.17 ±0.27e
L(-)	0.62 ±0.04a	13.51 ±0.45c	13.30 ±1.68b	2.14 ±0.13c	38.50 ±4.69ab	104.25 ±16.89b	13.41 ±1.32a	5.19 ±0.48ab
LBt	0.64 ±0.00a	21.97 ±1.48d	16.83 ±0.51c	2.91 ±0.04d	47.39 ±2.42abc	100.21 ±15.00b	20.60 ±0.69a	7.23 ±0.69c

Standard errors are given. Within each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n= 5).

3.2. Enzymatic activities in the rhizosphere of the three plant species

β-glucosidase activity was highest in *L. dentata* rhizosphere and the activity of alkaline phosphatase was highest in *S. chamaecyparissus* rhizosphere and dehydrogenase activity was highest in both plant species. The inoculation of the native *B. thuringiensis* had no effect in any of the enzymatic activities measured in any of the three plant species except the urease activity in *S. chamaecyparissus* that increased by 30.5% compared with uninoculated control (Table 3).

Table 3. Soil enzymatic activities in the rhizosphere of three autochthonous plants [*Thymus vulgaris* (T)*; Santolina chamaecyparissus* (S)*; Lavandula dentata* (L)] grown in natural arid Mediterranean soil under drought stress conditions as affected by the inoculation of the autochthonous bacteria strain *Bacillus thuringiensis* (Bt).

	Dehydrogenase (μg INTF g^{-1})	β-glucosidase (μmol PNF g^{-1} soil h^{-1})	Urease (μmol N-NH_3 g^{-1} h^{-1})	Alkaline Phosphatase (μmol PNF g $soil^{-1}$ h^{-1})
T(-)	71.9 ±0.49ab	137.8 ±0.00a	606.5 ±19.96ab	181.2 ±26.90ab
TBt	66.5 ±3.19a	177.8 ±0.02a	577.3 ±21.56ab	141.7 ±28.96a
S(-)	88.6 ±3.36c	201.5 ±0.02a	526.5 ±16.95a	302.4 ±17.71c
SBt	72.9 ±5.11ab	186.9 ±0.04a	687.2 ±71.87b	215.1 ±5.00b
L(-)	87.3 ±6.65bc	367.6 ±56.37b	622.1 ±35.97ab	221.4 ±36.02b
LBt	80.6 ±4.08abc	346.2 ±37.05b	679.6 ±12.09b	224.5 ±12.58b

Standard errors are given. Within each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n= 5).

3.3. Microbial fatty acid composition in rhizosphere soil

The principal component analysis of PLFA data for 16 FAMEs based of microbial community composition (Fig. 1A) explained in total 91.5% of data variation. The PCA1 explained 84.4% of the total variability and was positively related to biomarkers of bacteria (C17:1w6c; C17:1w8c; i17:1w5; i17:1w7; iC15:0; iC16:0; iC17:0), (MeC16; MeC18) of *Actinomycetes*, (C18:1w9c; C18:2w6c) of fungi and (C16:1w5) of arbuscular mycorrhiza, and negatively related to biomarker bacteria (C16:1w7t). The PCA2, explained the 6.9% of the

variability which was positively related to biomarkers of bacteria (iC14:0; i17:1w5), of *Actinomycetes* (MeC17) and of arbuscular mycorrhiza (C16:1w5). The fatty acid C16:1w7t was negatively correlated with the remaining fatty acids.

The PCA1 (Fig. 1B) explained 79.2% of the total variability and was positively related with treatments of *T. vulgaris* and *S. chamaecyparissus* without or with inoculation of *B. thuringiensis* and *L. dentata* [T(-); TBt; S(-); SBt; L(-)] and were clearly separated from Bt inoculation treatment in *L. dentata* (LBt) along this axis. The PCA2 explained 16.0% being more related LBt treatment with this factor. MANOVA analysis of the PCA scores confirmed that soil microbial community was significantly different in the rhizosphere of the three plant species (Wilks' lambda = 17.14, $p \leq 0.05$). Eigenvalues of Table 4 shows a significant effect of plant species in the profile of PLFA in the rhizosphere soil, these significantly differences were showed in the bacterial biomarkers (C17:1w8c; C18:1w9t) and fungal biomarker (C18:1w9c; C18:2w6c). Bacterial inoculation did not influence significantly the profile of fatty acids (Wilks' lambda = 0.39, $p>0.05$).

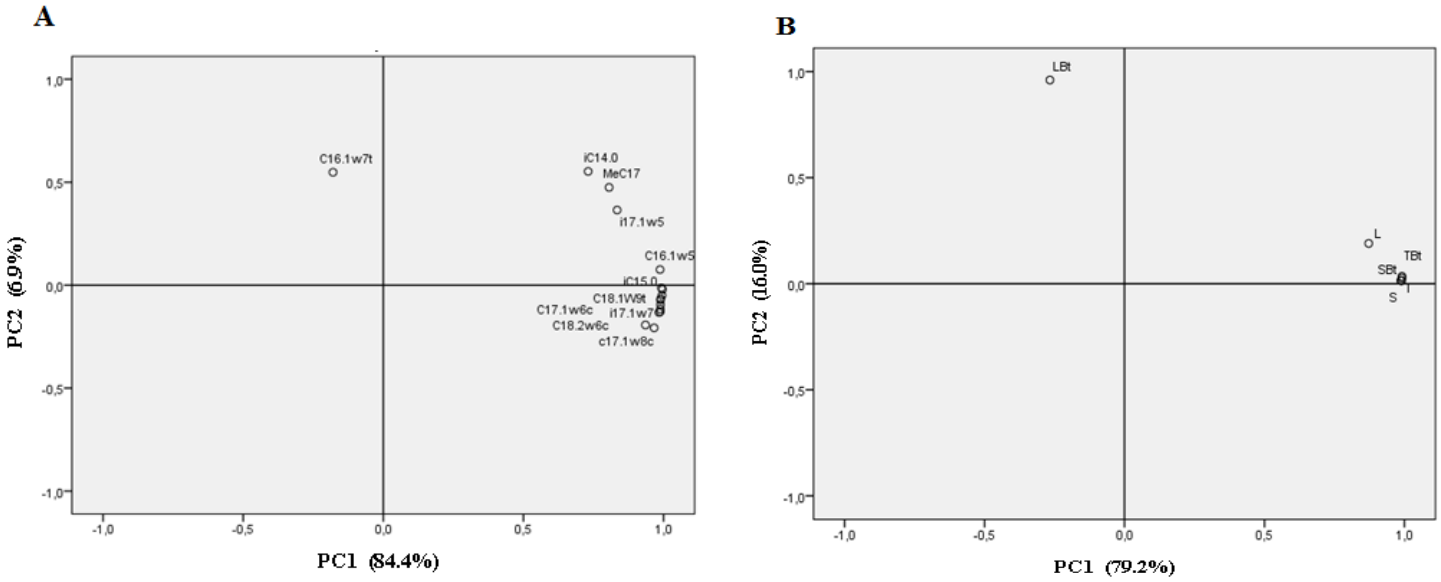


Fig. 1. Principal component analysis (PCA) of: **A**) PLFA data for 16 FAMEs and **B**) the treatments respectively [(T) *Thymus vulgaris*; (S) *Santolina chamaecyparissus*; (L) *Lavandula dentata* and inoculation of *B. thuringiensis* (Bt)] based on the microbial community composition.

Table 4. Eigenvalues of each fatty acid of the autochthonous plants species grown in natural arid Mediterranean soil under drought stress conditions.

Microbial PLFA	F	Sig.
C16:1w7t	1.117	0.357
C17:1w6c	3.054	0.082
C17:1w8c	4.263	0.038*
C18:1W9t	3.841	0.049*
i17:1w5	0.672	0.527
i17:1w7	3.426	0.064
iC14:0	1.117	0.357
iC15:0	3.478	0.062
iC16:0	2.875	0.093
iC17:0	3.170	0.076
MeC16	3.018	0.084
MeC17	0.672	0.527
MeC18	2.945	0.088
C18:1w9c	3.999	0.044*
C18:2w6c	3.995	0.044*
C16:1w5	2.807	0.097

* $p \leq 0.05$

The lipid abundance of the microbial community (Table 5.1A) including bacteria, fungi, *actinomycetes*, AM fungi, Gram-positive (G+) and Gram-negative (G-) bacteria, total PLFA and total NLFA was lower in *L. dentata* rhizosphere.. Bacteria, fungi, mycorrhiza and total PLFA significantly increased in *S. chamaecyparissus* besides *actinomycetes*, G+ and G- bacteria increased both in *T. vulgaris* as *S. chamaecyparissus.* In the ANOVA analysis showed significantly differences ($p \leq 0.05$) in the microbial biomarkers of bacteria, fungi, *actinomycetes*, G+ and G- bacteria and total PLFA of the rhizosphere of autochthonous plants species (Table 5.1B). The inoculation with *B. thuringiensis* on each plant species (Table 5.2) did not significantly affect in rhizosphere soil microbial community structure although observed that *L. dentata* inoculated with *B. thuringiensis* (LBt) presented low content of phospholipids acids biomarkers but the content of neutral lipids (NLFA) significantly increased compared to the non-inoculated control (L(-)) (Table 5.2).

Table 5.1. A) Content of phospholipid acid (μg PLFA g^{-1} sed) and neutral lipids acid biomarkers (μg NLFA gr^{-1} sed) in the rhizosphere of the three autochthonous plants species [*Thymus vulgaris* (T)*; Santolina chamaecyparissus* (S); *Lavandula dentata* (L)] grown in natural arid Mediterranean soil under drought stress conditions, and **B)** ANOVA of each microbial biomarkers.

A)

	Bacterias	Fungi	Actinomycetes	Mycorrhiza	Gram+	Gram-	Total PLFA	Total NLFA
T	0.307 ±0.111ab	0.143 ±0.052ab	0.059 ±0.018b	0.042 ±0.012ab	0.015 ±0.005b	0.020 ±0.006b	0.552 ±0.193ab	14.37 ±2.7a
S	0.581 ±0.249b	0.266 ±0.101b	0.126 ±0.054b	0.069 ±0.025b	0.028 ±0.010b	0.036 ±0.013b	1.042 ±0.428b	9.91 ±5.2a
L	0.015 ±0.004a	0.012 ±0.007a	0.001 ±0.000a	0.003 ±0.000a	0.001 ±0.000a	0.002 ±0.000a	0.032 ±0.011a	10.34 ±2.7a

Standard errors are given. Within each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n= 3).

B)

	Bacterias		Fungi		Actinomycetes		Mycorrhiza		Gram+		Gram-		Total PLFA		Total NLFA	
	F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.
Plants species	3.797	0.046*	4.251	0.034*	4.944	0.022*	2.816	0.092	3.478	0.057*	5.422	0.017*	4.318	0.033*	0.123	0.885

* $p \leq 0.05$

Table 5.2. Content of phospholipid acid (μg PLFA g^{-1} sed) and neutral lipids acid biomarkers (μg NLFA gr^{-1} sed) in the rhizosphere of the three autochthonous plants species [*Thymus vulgaris* (T)*; Santolina chamaecyparissus* (S); *Lavandula dentata* (L)] grown in natural arid Mediterranean soil under drought stress conditions as affected by the inoculation of the autochthonous bacteria strain *Bacillus thuringiensis* (Bt).

	Bacterias	Fungi	Actinomycetes	Mycorrhiza	Gram+	Gram-	Total PLFA	Total NLFA
T(-)	0.307 ±0.111a	0.143 ±0.052a	0.059 ±0.018ab	0.042 ±0.012a	0.015 ±0.005ab	0.020 ±0.006ab	0.552 ±0.193a	14.37 ±2.7ab
TBt	0.601 ±0.478a	0.237 ±0.187a	0.207 ±0.111b	0.098 ±0.078a	0.046 ±0.020b	0.041 ±0.008b	1.144 ±0.840a	18.48 ±0.8ab
S(-)	0.581 ±0.249a	0.266 ±0.101a	0.126 ±0.054ab	0.069 ±0.025a	0.028 ±0.010ab	0.036 ±0.013b	1.042 ±0.428a	9.91 ±5.2a
SBt	0.754 ±0.097a	0.323 ±0.054a	0.165 ±0.021ab	0.110 ±0.019a	0.026 ±0.013ab	0.039 ±0.018b	1.353 ±0.185a	19.42 ±4.7ab
L(-)	0.015 ±0.004a	0.012 ±0.007a	0.001 ±0.000a	0.003 ±0.000a	0.001 ±0.000a	0.002 ±0.000a	0.032 ±0.011a	10.34 ±2.7a
LBt	0.093 ±0.003a	0.046 ±0.017a	0.020 ±0.000a	0.022 ±0.000a	0.006 ±0.000a	0.012 ±0.000ab	0.181 ±0.017a	22.77 ±2.5b

Standard errors are given. Within each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n= 3).

3.4. Correlations of microbial variables, soil enzymatic activity and shoot nutrient acquisition.

Actinomycetes, bacterias, fungi, mycorrhizas, G+ and G- bacterias, and total PLFA were negatively correlated with β-glucosidase activity (Table 6), and G+ and G- bacteria were negatively correlated with dehydrogenase activity, unlike total NLFA was positively correlated with urease. The P- and Mn-biomass nutrient contents were positively correlated with *actinomycetes*, bacteria, fungi, mycorrhiza and total PLFA. However, the Mg content was negatively correlated with bacteria, fungi, mycorrhiza and total PLFA, and the Ca content was negatively correlated with *actinomycetes*. G- bacteria was positively correlated with Mn and Cu, and negatively correlated with Mg.

Table 6. Pearson correlation coefficients between soil enzyme, biomass nutrient contents and microbial variables.

		Actinomycetes	Bacterias	Fungi	Mycorrhiza	G+	G-	Total PLFA	NLFA
Dehydrogenase		-0.407	-0.327	-0.302	-0.422	-0.548*	-0.520*	-0.345	-0.400
β-glucosidase		-0.564*	-0.551*	-0.559*	-0.496*	-0.549*	-0.621**	-0.558*	0.077
Urease		-0.112	-0.087	-0.068	-0.035	0.072	0.138	-0.083	0.616**
Phosphatase		-0.264	-0.046	0.001	-0.171	-0.271	-0.157	-0.077	-0.316
Biomass content	P	0.479*	0.469*	0.470*	0.413*	0.325	0.415	0.473*	0.127
	K	0.335	-0.335	-0.359	-0.257	-0.336	-0.328	-0.339	0.406
	Ca	-0.423*	-0.394	-0.402	-0.316	-0.394	-0.356	-0.399	0.367
	Mg	-0.376	-0.509*	-0.541*	-0.415*	-0.393	-0.472*	-0.497*	0.335
	Zn	.	.	.	.	.	.	.	.
	Fe	-0.287	-0.387	-0.422	-0.282	0.093	-0.040	-0.378	0.197
	Mn	0.485*	0.578*	0.617**	0.495*	0.381	0.587*	0.576*	-0.041
	Cu	0.258	0.357	0.394	0.284	0.213	0.458*	0.351	0.050

G+: Gram-positive bacteria, G-: Gram-negative bacteria, PLFA: phospholipid fatty acids, NLFA: neutral lipid fatty acids. *$p \leq 0.05$; ** $p \leq 0.01$

3.5. Distribution of fungal and bacterial communities, and diversity indexes

The numbers of total sequences were 10 7667 sequences of fungi and 81 135 sequences of bacteria. The sequences reads were assigned to 900 operational taxonomic units (OTUs) of fungi and 3 756 OTUs of bacteria.

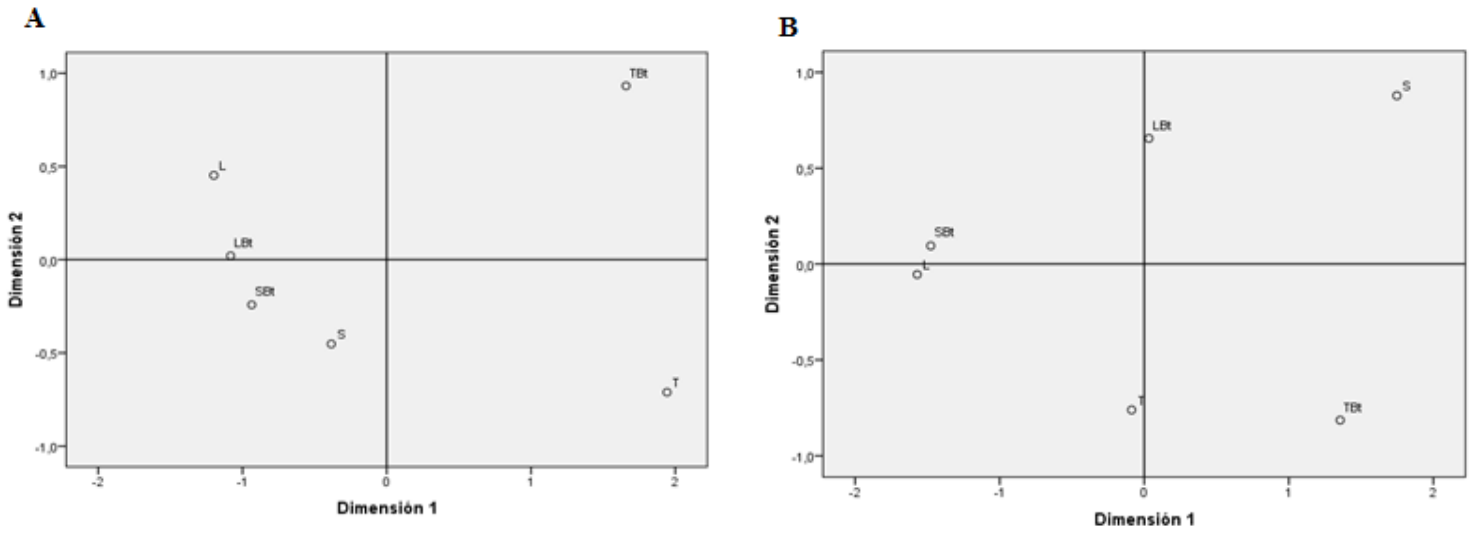
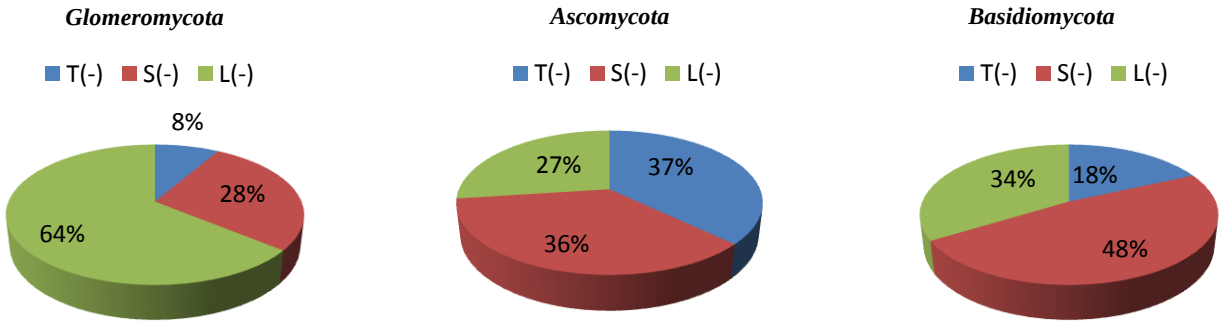


Fig. 2. Non-metric multidimensional scaling (NMDS) analysis based on absolute abundance of fungal OTUs (**A**) and bacterial OTUs (**B**). [(T) *Thymus vulgaris*; (S) *Santolina chamaecyparissus*; (L) *Lavandula dentata* and inoculation of *B. thuringiensis* (Bt)].

Analysis of absolute abundance of fungi in the different environments were used non-metric multidimensional scaling (NMDS) (S = 0.0245; R^2 (RSQ) = 0.99), was presented a proximity of treatments of *S. chamaecyparissus* and *L. dentata* without or with inoculation of *B. thuringiensis* [S(-); SBt; L(-); LBt], however distances in *T. vulgaris* (Fig. 2A). The rhizosphere of *T. vulgaris* had less proportion of OTUs of *Glomeromycota* (8%), and rhizosphere of *L. dentata* had the highest content (64%) (Fig. 3A). *S. chamaecyparissus* rhizosphere had high proportion of *Ascomycota* division (36%) and *Basidiomycota* division (48%), while *T. vulgaris* presented a greater proportion of the *Ascomycota* division and *L. dentata* of the *Basidiomycota* division (Fig. 3A). Inoculation of *B. thuringiensis* increased *Glomeromycota* division in *T. vulgaris* (14% over the T(-)) and *S. chamaecyparissus* (14% over the S(-)). Proportion of *Basidiomycota* in *L. dentata* increased (7% over L(-)) (Fig. 3B). The proportion of the total number of OTUs (Fig. 4) of the different *Glomeromycota* orders which was comparable over the semiarid rhizosphere with different plant species and bacterial inoculation treatments, were higher in the rhizosphere of *S. chamaecyparissus* in particular the *Glomus* order. The

inoculation of *B. thuringiensis* increased the number of OTUs of *Glomus* order in *T. vulgaris* (2% over the T(-)), *S. chamaecyparissus* (5.8% over the S(-)) and *L. dentata* (20% over the L(-)). The proportion of *Paraglomus* and *Diversiporales* were highest in *T. vulgaris* (with or without inoculations of *B.* thuringiensis) and *L. dentata* (non-inoculated). *Archaeosporales* was only found in the rhizospheric of *S. chamaecyparissus* (Fig. 4).

A)



B)

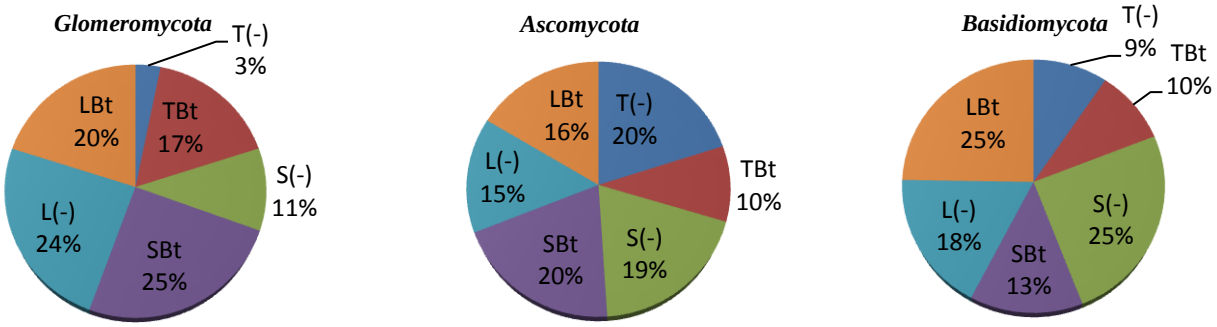


Fig.3. Percentage of OTUs of the different fungi divisions, detected with the FF390/FR1 primers in the soils natural harboring the three plant species non-inoculated **(A)** or inoculated **(B)** with *Bacillus thuringiensis* (Bt).

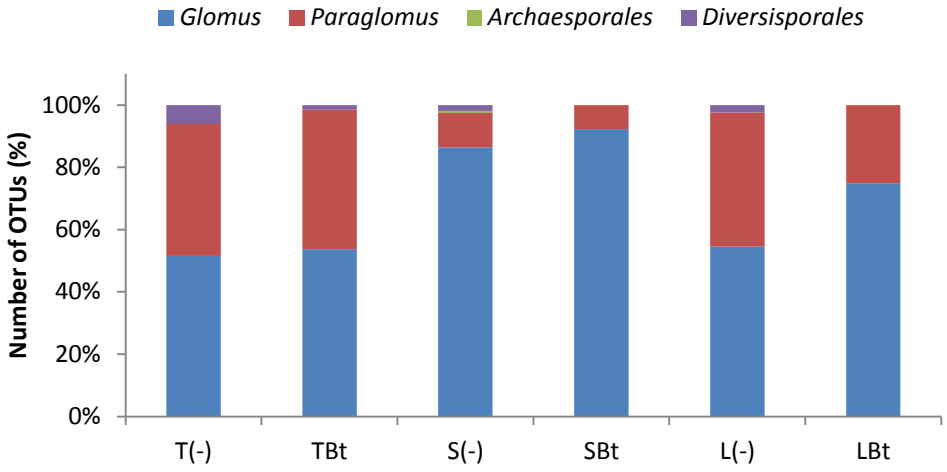


Fig.4. Proportional distribution (% number of OTUs) of the different *Glomeromycota* orders detected with FF390/FR1 primers in the soils natural harboring the three plant species inoculated or not with *Bacillus thuringiensis* (Bt).

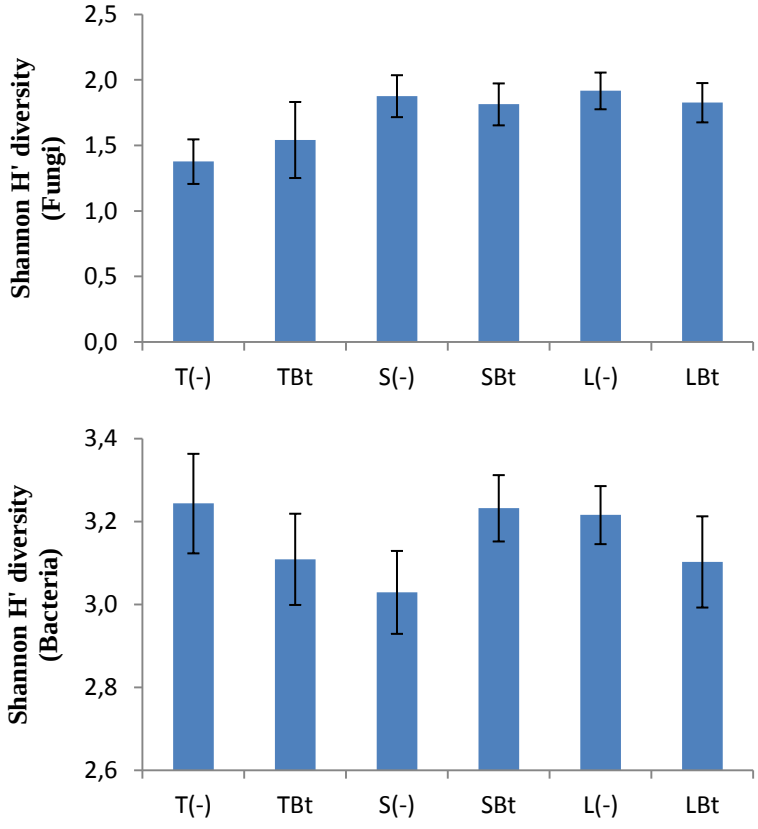


Fig.5. Diversity Index H' (Shannon-Wiener) of fungal **(A)** and bacterial **(B)** community of rhizospheric soil of three plants species, with non-inoculated or inoculated with *B. thuringiensis*. Bars represent average diversity (±SE).

The diversity of fungi in different plant species showed (Fig. 5A) that the treatments with *L. dentata* had highest diversity index (H'=1.92), followed by *S. chamaecyparissus* (H'=1.88), and *T. vulgaris* with the lowest index (H'=1.38). Inoculation of the plants with *B. thuringiensis* increased the diversity of fungi in rhizosphere with treatments of *T. vulgaris* (H'=1.54).

Absolute abundance of bacteria, were used analysis non-metric multidimensional scaling (S =0.039; R^2 (RSQ) =0.99) in the study soils, was presented distance of the S(-) and TBt treatments, unlike the others treatments that was observed a proximity between T(-); LBt and L(-); SBt in the abundances of bacterial communities (Fig. 2B). All treatments had high abundance of phyla *Gemmatimonadetes*, *Actinobacteria* and *Acidobacteria*. *L. dentata* (L(-)) had the greatest numbers of OTUs and highlight the *Gemmatimonadetes* phylum, but the inoculation of Bt decreases the number of OTUs of this phylum and increased the phylum *Actinobacteria* (14.8% over L(-)) (Fig.6A-B). *S. chamaecyparissus* with inoculation of Bt increased the numbers of OTUs of phyla *Acidobacteria*, *Proteobacteria*, *Gemmatimonadetes* and *Planctomycetes* (Fig. 6A).

Besides the percentage of OTUs was mainly very elevated in *alpha-proteobacteria* (T(-) 38%; S(-) 44%; L(-) 31%) but the inoculation of Bt in the plants of *T. vulgaris* and *S. chamaecyparissus* (TBt; SBt) decreased this percentage and increasing in *L. dentata* (8.6% over L(-)). In contrast, *beta-proteobacteria* decreased with Bt inoculated in *L. dentata*. The *delta-proteobacterias* were increased by 8.6% in SBt and *gagma-proteobacterias* were increased by 4% in TBt and LBt treatments (Fig. 6C).

The phylum *Firmicutes* and *Cyanobacteria* were overrepresented in S(-) and T(-), and the phyla *Planctomycetes* and *Verrucomicrobia* phylum were overrepresented in L(-). Inoculation of native bacteria tends decrease the level of *Cyanobacteria* in *S. chamaecyparissus* (Fig. 6B).

Bacterial diversity index (Fig. 5B) was low *S. chamaecyparissus* (H' = 3.03) unlike *T. vulgaris* and *L. dentata* the indexes were high (H' = 3.24; 3.22). *B. thuringiensis* inoculation promotes increase of bacterial diversity in *S. chamaecyparissus* (H' = 3.23).

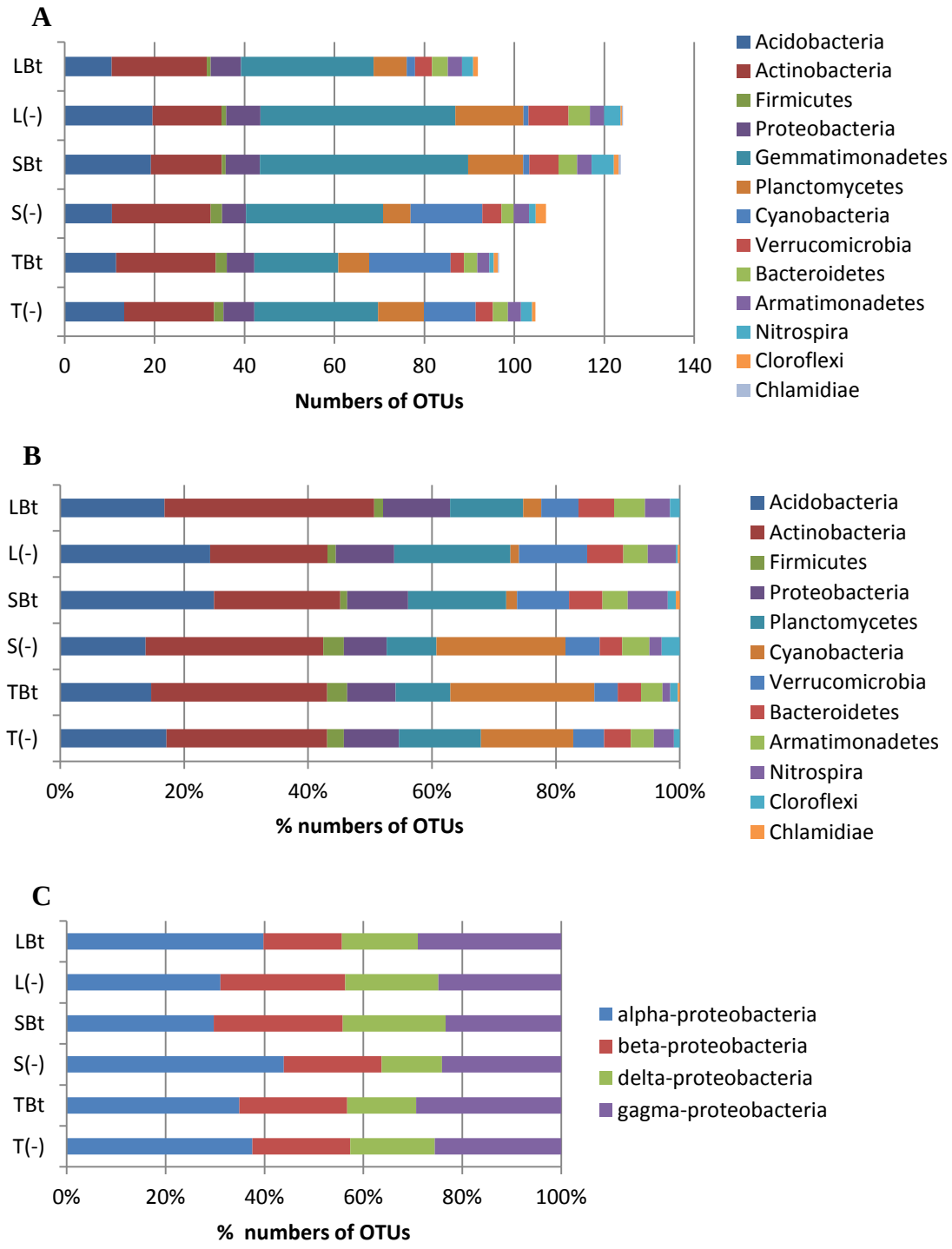


Fig. 6. Absolute abundance of bacterial 16S rDNA genes from soils semi-arids mediterranean with three autochthonous plants species non-inoculated or inoculated with *Bacillus thuringiensis*. **A)** phylum level; **B)** remaining bacterial phylum *Gemmatimonadetes* without. **C)** class of *Proteobacteria*.

4. Discussion

The microbial community of the rhizosphere and the plants were subjected to a high water stress. The plants used in our study belong to different families but they are all autochthonous drought-tolerant shrub species, with deep roots enabling them to cope with nutrient stress in the eroded soil (Francis & Thornes (1990)). They belong to the natural succession of the shrubland community of semiarid Mediterranean ecosystems in the southeast of Spain (Alguacil *et al.*, 2011). The native bacteria inoculation increased the shoot growth in the three plants autochthonous species. It is likely that the bacterial community, in general, employs several physiological modifications in response to changing soil moisture, such as production of exopolysaccharides (Kohler *et al.*, 2009), sporulation (Landesman and Dighton, 2010) and adjustment of internal water potential to match that of the external environment. The bacteria accomplish this by accumulating low-molecular-weight osmoregulatory solutes within their cytoplasm as soil moisture decreases; these are released as soil moisture increases (Landesman and Dighton, 2010). In previous studies (Armada *et al.*, 2014; Armada *et al.*, 2015), *B. thuringiensis* may enhance the plant growth by different mechanisms such as by optimizing the supply of nutrients, as solubilization of inorganic phosphorus or by the synthesis of phytohormones (IAA) and ACC-deaminase. This bacteria can be considered a plant-stress homeostasis-regulating rhizobacteria by the biosynthesis of these phytohormones (Cassán *et al.*, 2014). As well, PHB as carbon storage polymers can support the survival and reproduction of microorganisms under adverse conditions and to improve their tolerance to osmotic stress.

The inoculation of *B. thuringiensis* promoted growth of shoots and roots in *L. dentata*, and increased total AM fungi colonization in *S. chamaecyparissus* and *L. dentata*. This bacterial strain was able to produce indole acetic acid (IAA) under drought conditions and this phytohormone can be responsible for the root enhancement in inoculated plants (Armada *et al.*, 2014). However, *T. vulgaris* inoculated with Bt increased the phosphorus content by 51%. Several strains of bacterial and fungal species have been described and investigated in detail for their phosphate-solubilizing capabilities (Glick, 1995; He *et al.*, 1997; Leggett *et al.*, 2001). The PGPR strains tested have a high nutritional potential, and their mineral content is higher than some organic fertilizer sources (Güneş *et al.*, 2014). Inoculation of Bt in *L. dentata* increased content potassium in leaf of plant by 63% compared with non-inoculated control. Potassium is one the most important inorganic solutes and thereby regulates water uptake capacity by the roots. Determined study found increases in root hydraulic conductivity and potassium, suggesting a close between the processes of water and potassium uptake (Liu *et al.*, 2006).

In addition it should be emphasized the increase in the content of Ca and Mg in inoculated plants. The Ca is important in membrane protection and Mg modulates ionic currents across the chloroplasts and vacuole membranes (regulating stomatal opening and ion balance in cells) under dry conditions (Parida and Jha, 2013). The enhancement of Mg content in inoculated plants suggests that the functioning of photosynthetic apparatus was not affected by drought in these three plants species colonized by *B. thuringiensis* but drought lends to severe damage to membrane integrity in many plants (Silva et al., 2010). This bacterial effect on increasing drought tolerance was related to the decrease of antioxidant enzymatic activity that resulted sensitive indexes of lower cellular oxidative damage involved in the adaptive drought response in *B. thuringiensis*-inoculated *Lavandula* plants (Armada *et al.*, 2014).

The enzymatic activities of soil were especially elevated in two plants species (*S. chamaecyparissus* and *L. dentata*). Dehydrogenase activity was different significantly in *S. chamaecyparissus*, this activity reflected the soil microbial community enhanced, therefore the reactivation of the rhizosphere microbial populations is indication of rehabilitation of degraded soils. The β-glucosidase activity indicates carbohydrates transformation which is important as energy source for microorganisms and this activity was highest in *L. dentata*. The inoculation native Bt decreased dehydrogenase activity but urease activity increased in *S. chamaecyparissus* and mildly in *L. dentata*, it was caused by impoverishment of soil and excess drought this probably raised the demand for N sources by microorganisms, due to the less-efficient use of substrates as a consequence of the stress (Fließbach *et al.*, 1994), this explaining the increase of urease activity under drought. Increased alkaline phosphatase activity in *S. chamaecyparissus* S(-), being plant species that tended to highest content of phosphorus in aboveground biomass (P content increased by 52% and by 40% compared with T(-) and L(-) respectively). The cycles in the soil of such important elements for soil fertility as N, C and P are regulated by hydrolases enzymes such as urease (N cycle), β-glucosidase (C cycle) and phosphatases (P cycle), which are synthesized mainly by soil microorganisms (Ros *et al.*, 2006). Measurement of these soil hydrolases are indication of changes in soil fertility since they are involved in the mineralization of compounds that provide nutrients as N, P and C.

The biomarkers of lipids of each microbial variable or taxa had been correlations with enzymatic activities of soil. The decreases in the activities of soil enzymes involved in the cycles of P, N and C observed in the stressed soils confirms that P and C cycles are altered by severe water stress and that drought will affect, in the long-term, soil nutrient availability, reducing the nutrient supply to plants (Sardans and Peñuelas, 2005) and consequently, altered and changed microbial populations.

According to the results observed, we confirmed that soil microbial community was significantly different in the rhizosphere of the three plant species. And these significantly differences were showed in the bacterial biomarkers (C17:1w8c; C18:1w9t) and fungal biomarker (C18:1w9c; C18:2w6c). However, bacterial inoculation did not influence significantly the profile of fatty acids.

Community-level PLFA profiles are useful in detecting the response of soil microbial communities to various land use or disturbances in other ecosystems (Yao *et al.*, 2000; McKinley *et al.*, 2005). The lipid abundance of the microbial community was lower in rhizosphere of *L. dentata* and significantly increased in *S. chamaecyparissus.* The microbial biomarkers (bacteria, fungi, *actinomycetes,* G+ and G- bacteria and total PLFA) of the rhizosphere of autochthonous plants species showed significantly differences. In contrast, the inoculation with *B. thuringiensis* on each plant species did not significantly affect in soil microbial community structure. Our study demonstrated that *T. vulgaris*, *S. chamaecyparissus* and *L. dentata* had different fungal and bacterial community composition.

Existed similarity of the fungal community between *S. chamaecyparissus* and *L. dentata* species, and differs with *T. vulgaris*. The percentages of number of OTUs of fungal divisions were very lowest in *T. vulgaris*, but *L. dentata* showed highest proportion of *Glomeromycota* division (by 64%) unlike of *S. chamaecyparissus*, these were the divisions of *Ascomycota* and *Basidiomycota*. The inoculation of *B. thuringiensis* increased *Glomeromycota* division in *T. vulgaris* and *S. chamaecyparissus* (by 14% compared with non-inoculated controls) and this promotes a high capacity for nutrient uptake by plants and in the case of *S. chamacyparissus* in higher mycorrhizal colonization. In the respective *Glomeromycota* orders were observed that *S. chamaecyparissus* contained more *Glomus* and the only one that contains the *Archaeosporales* order, unlike of *T. vulgaris* and *L. dentata* were contained high proportion of *Paraglomus* and greater presence of *Diversiporales* in *T. vulgaris*. The inoculation of *B. thuringiensis* increased quite the *Glomus* order in *L. dentata* and this we could explain such significant increased of the content of neutral lipids (NLFA) that shows this plant species inoculated compared with non-inoculated control.

All this confirms the diversity of the rhizosphere soils of different plant species, highlighting that *L. dentata* was plant species with the highest fungal diversity, followed by *S. chamaecyparissus*. According to some authors could relate to arbuscular mycorrhizal fungi (AMF) patterns change root exudation and therefore modifies the physiology of the plant and alter the composition of root exudates (Johansson *et al.*, 2004; Hage-Ahmed *et al.*, 2013). But inoculation of *B. thuringiensis* promoted increased index of fungal diversity in the rhizospheric

soil of *T. vulgaris*, and maintained the fungal diversity in the remaining plants inoculated with *B. thuringiensis*, this inoculation of native bacteria provides high nutritional content of the plants.

In the study of bacterial community was presented distancing of the S(-) and TBt treatments, unlike the others treatments that was observed a proximity. The most remarkable bacterial phylums in different rhizospheric soils were *Gemmatimonadetes*, *Acidobacterias* and *Proteobacterias* (gram- bacteria) and *Actinobacterias* (gram+ bacteria). The rhizosphere of *L. dentata* plant host showed a wide a bacterial diversity, especially gram negative bacteria, the numbers of OTUs of the *Proteobacteria* phylum were highest than the remaining two plants species. The increased activity β-glucosidase in *L. dentata* could be by the existing microbial community, either bacterial or fungal. Indicators that are mainly associated with Gram-negative bacteria increase with organic matter content and high substrate availability (Zelles *et al.*, 1992; Bossio *et al.*, 1998). Gram-negative bacteria are as a group tend to grow more rapidly than do Gram-positive bacteria in high nutrient content environments (Kelly *et al.*, 2007) especially the majority were *alpha-proteobacteria* class. Being the rhizosphere of *L. dentata* the most diverse (with major numbers of OTUs) of the three plant species used in the study. Inoculation of *B. thuringiensis* caused a decrease of bacterial diversity index. However, the inoculation Bt increased diversity of bacterial community in the rhizosphere of *S. chamaecyparissus* besides of fungal community, this provides an increased of urease activity.

Inoculated native bacteria have a varied and strong impact in improving plant stress tolerance mechanisms (Dimpka 2009). *B. thuringiensis* can help plants in the osmoregulation processes and in improving homeostatic mechanisms upon stress challenge. This suggest that they can maintain a long time their biochemical traits related to positive effects in inoculated plants under water limiting conditions. The water limitation and osmotic stress negatively affect plant growth but the Bt inoculation was able to attenuated these detrimental effects.

In conclusion, the results of our study which was located in semiarid areas subject to a high degree of water deficit provides an overview of the composition of the microbial community. Changes in water status could impact the physiology and structure of the soil microbial community (Fang *et al.*, 2001), dissimilar types of microorganisms being affected differently by changing water potential (Griffiths *et al.*, 2003; Williams and Rice, 2007). The soil microbial biomass size and activity help the soil to retain moisture, making it more resistant to drying out, these changes in the microbial community structure, were detected by PLFA analysis also with us pyrosequencing technique facilitated our knowledge of microbial diversity of these nutritionally deficient soils and subjected to extreme environmental conditions. We can

say that the autochthonous shrubs species contribute significantly to the development and enrichment of fungal and bacterial communities of such semiarid areas and consequently, an enhanced functionality and diversity soil. As affect inoculating native bacterial species (*B. thuringiensis*) with PGPR capacity to promote microbial diversity and thus be primarily a potential method for promoting plant growth and nutrient availability, besides of help plants to grow under water deficiency.

Acknowledgments

E. Armada was financed by Ministry of Science and Innovation. This work was carried out in the framework of the project reference AGL2009-12530-C02-02 with a grant of short stay (ref. BES-2010-042736) at NIOO-KNAW in Wageningen, Netherlands. We thank Domingo Álvarez for the morphological identification of autochthonous mycorrhizal fungus.

References

Aboim, M.C.R., Coutinho, H.L.C., Peixoto, R.S., Barbosa, J.C., Rosado, A.S., 2008. Soil bacterial community structure and soil quality in a slash-and-burn cultivation system in Southeastern Brazil. Applied Soil Ecology 38, 100-108.

Alguacil, M.M., Roldán, A., Torres, M.P., 2009. Complexity of semiarid gypsophilous shrub communities mediates the AMF biodiversity at the plant species level. Microbial Ecology 57, 718-727.

Alguacil, M.M., Torres, M.P., Torrecillas, E., Díaz, G., Roldán, A., 2011. Plant type differently promote the arbuscular mycorrhizal fungi biodiversity in the rhizosphere after revegetation of a degraded, semiarid land. Soil Biology and Biochemistry 43, 167-173.

Armada, E., Azcón, R., López-Castillo, O.M., Calvo-Polanco, M., Ruiz-Lozano, J.M., 2015. Autochthonous arbuscular mycorrhizal fungi and Bacillus thuringiensis from a degraded Mediterranean area can be used to improve physiological traits and performance of a plant of agronomic interest under drought conditions. Plant Physiology and Biochemistry 90, 64-74.

Armada, E., Roldán, A., Azcón, R., 2014. Differential activity of autochthonous bacteria in controlling drought stress in native *Lavandula* and *Salvia* plants species under drought conditions in natural arid soil. Microbial Ecology 67, 410-420.

Bååth, E., 2003. The use of neutral lipid fatty acids to indicate the physiological conditions of soil fungi. Microbial Ecology 45, 373-383.

Barea, J.M., Azcón, R., Azcón-Aguilar, C., 2002. Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 81, 343-351.

Barea, J.M., Pozo, M.J., Azcón, R., Azcón-Aguilar, C., 2005. Microbial co-operation in the rhizosphere. Journal of Experimental Botany 56, 1761-1778.

Bastida, F., Kandeler, E., Moreno, J.L., Ros, M., García, C., Hernández, T., 2008. Application of fresh and composted organic wastes modifies structure, size and activity of soil microbial community under semiarid climate. Applied Soil Ecology 40, 318-329.

Bertani, G., 1951. STUDIES ON LYSOGENESIS I. : The Mode of Phage Liberation by Lysogenic *Escherichia coli*. Journal of Bacteriology 62, 293-300.

Bossio, D.A., Scow, K.M., 1995. Impact of carbon and flooding on the metabolic diversity of microbial communities in soils. Applied and Environmental Microbiology 61, 4043-4050.

Bossio, D.A., Scow, K.M., Gunapala, N., Graham, K.J., 1998. Determinants of soil microbial communities: Effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microbial Ecology 36, 1-12.

Brundrett, M., 1994. Practical Methods in Mycorrhizal Research.

Burrows, R.L., Pfleger, F.L., 2002. Host responses to AMF from plots differing in plant diversity. Plant and Soil 240, 169-179.

Caravaca, F., Masciandaro, G., Ceccanti, B., 2002. Land use in relation to soil chemical and biochemical properties in a semiarid Mediterranean environment. Soil and Tillage Research 68, 23-30.

Cassán, F., Vanderleyden, J., Spaepen, S., 2014. Physiological and Agronomical Aspects of Phytohormone Production by Model Plant-Growth-Promoting Rhizobacteria (PGPR) Belonging to the Genus Azospirillum. Journal of Plant Growth Regulation 33, 440-459.

Cristea-FernstrÖm, M., Olofsson, M., Chryssanthou, E., Jonasson, J., Petrini, B., 2007. Pyrosequencing of a short hypervariable 16S rDNA fragment for the identification of nontuberculous mycobacteria - A comparison with conventional 16S rDNA sequencing and phenotyping. APMIS 115, 1252-1259.

Drigo, B., Pijl, A.S., Duyts, H., Kielak, A.M., Gamper, H.A., Houtekamer, M.J., Boschker, H.T.S., Bodelier, P.L.E., Whiteley, A.S., Van Veen, J.A., Kowalchuk, G.A., 2010. Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO2. Proceedings of the National Academy of Sciences of the United States of America 107, 10938-10942.

Duncan, D.B., 1955. Multiple Range and Multiple F Tests. Biometrics 11, 1-42.

Fang, C., Radosevich, M., Fuhrmann, J.J., 2001. Characterization of rhizosphere microbial community structure in five similar grass species using FAME and BIOLOGY analyses. Soil Biology and Biochemistry 33, 679-682.

Fließbach, A., Martens, R., Reber, H.H., 1994. Soil microbial biomass and microbial activity in soils treated with heavy metal contaminated sewage sludge. Soil Biology and Biochemistry 26, 1201-1205.

Francis, D.F., & Thornes J.B. (Ed), 1990. Matorral: erosion and reclamation. In: Albaladejo, J.,Stocking, M.A., Díaz, E. (Eds.), Soil Degradation and Rehabilitation in Mediterranean Environmental Conditions. Consejo Superior de Investigaciones Científicas, Murcia, Spain. pp. 87-115

Frostegård, A., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59-65.

Frostegård, Å., Bååth, E., Tunlio, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology and Biochemistry 25, 723-730.

Frostegard, A., Tunlid, A., Baath, E., 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Applied and Environmental Microbiology 59, 3605-3617.

García, C., Hernández, M.T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plant Nutr. 28, 123-134.

Garland, J.L., 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biology and Biochemistry 28, 213-221.

Giovannetti, M., Mosse, B., 1980. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84, 489-500.

Glick, B.R., 1995. The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology 41, 109-117.

Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey, M.J., 2003. Physiological and Community Responses of Established Grassland Bacterial Populations to Water Stress. Applied and Environmental Microbiology 69, 6961-6968.

Güneş, A., Turan, M., Güllüce, M., Şahin, F., 2014. Nutritional content analysis of plant growth-promoting rhizobacteria species. European Journal of Soil Biology 60, 88-97.

Hage-Ahmed, K., Moyses, A., Voglgruber, A., Hadacek, F., Steinkellner, S., 2013. Alterations in Root Exudation of Intercropped Tomato Mediated by the Arbuscular Mycorrhizal Fungus Glomus mosseae and the Soilborne Pathogen Fusarium oxysporum f.sp. lycopersici. Journal of Phytopathology 161, 763-773.

He, Z.L., Baligar, V.C., Martens, D.C., Ritchey, K.D., 1997. Effect of phosphate rock, lime and cellulose on soil microbial biomass in acidic forest soil and its significance in carbon cycling. Biology and Fertility of Soils 24, 329-334.

Hu, J., Lin, X., Wang, J., Dai, J., Chen, R., Zhang, J., Wong, M., 2011. Microbial functional diversity, metabolic quotient, and invertase activity of a sandy loam soil as affected by long-term application of organic amendment and mineral fertilizer. J Soils Sediments 11, 271-280.

Jackson, L.E., Calderon, F.J., Steenwerth, K.L., Scow, K.M., Rolston, D.E., 2003. Responses of soil microbial processes and community structure to tillage events and implications for soil quality. Geoderma 114, 305-317.

Johansson, J.F., Paul, L.R., Finlay, R.D., 2004. Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiology Ecology 48, 1-13.

Johnson, D., Booth, R.E., Whiteley, A.S., Bailey, M.J., Read, D.J., Grime, J.P., Leake, J.R., 2003. Plant community composition affects the biomass, activity and diversity of microorganisms in limestone grassland soil. European Journal of Soil Science 54, 671-677.

Kelly, J.J., Favila, E., Hundal, L.S., Marlin, J.C., 2007. Assessment of soil microbial communities in surface applied mixtures of Illinois River sediments and biosolids. Applied Soil Ecology 36, 176-183.

Kohler, J., Caravaca, F., Roldán, A., 2009. Effect of drought on the stability of rhizosphere soil aggregates of Lactuca sativa grown in a degraded soil inoculated with PGPR and AM fungi. Applied Soil Ecology 42, 160-165.

Landesman, W.J., Dighton, J., 2010. Response of soil microbial communities and the production of plant-available nitrogen to a two-year rainfall manipulation in the New Jersey Pinelands. Soil Biology and Biochemistry 42, 1751-1758.

Leggett, M., Gleddie, S., Holloway, G., 2001. Phosphate-Solubilizing Microorganisms and Their Use. In: Ae, N., Arihara, J., Okada, K., Srinivasan, A. (Eds.), Plant Nutrient Acquisition. Springer Japan, pp. 299-318.

Liu, H.Y., Sun, W.N., Su, W.A., Tang, Z.C., 2006. Co-regulation of water channels and potassium channels in rice. Physiologia Plantarum 128, 58-69.

Masciandaro, G., Ceccanti, B., García, C., 1994. Anaerobic digestion of straw and piggery wastewater: II. Optimization of the process. Agrochimica 3, 195-203.

Mauclaire, L., Pelz, O., Thullner, M., Abraham, W.R., Zeyer, J., 2003. Assimilation of toluene carbon along a bacteria-protist food chain determined by 13C-enrichment of biomarker fatty acids. Journal of Microbiological Methods 55, 635-649.

McKinley, V.L., Peacock, A.D., White, D.C., 2005. Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. Soil Biology and Biochemistry 37, 1946-1958.

Mengual, C., Schoebitz, M., Azcón, R., Roldán, A., 2014. Microbial inoculants and organic amendment improves plant establishment and soil rehabilitation under semiarid conditions. Journal of Environmental Management 134, 1-7.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions. European Journal of Soil Science 54, 655-670.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, proteases, organic-carbon, and nitrogen from soil. Soil Sci. Soc. Am. J. 44, 1011-1016.

Naseby, D.C., Lynch, J.M., 1997. Rhizosphere soil enzymes as indicators of perturbations caused by enzyme substrate addition and inoculation of a genetically modified strain of *Pseudomonas fluorescens* on wheat seed. Soil Biol. Biochem. 29, 1353-1362.

Oehl, F., 2011. Advances in Glomeromycota taxonomy and classification. IMA fungus 2, 191-199.

Olsson, P.A., Baath, E., Jakobsen, I., Soderstrom, B., 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99, 623-629.

Öpik, M., Moora, M., Liira, J., Zobel, M., 2006. Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. Journal of Ecology 94, 778-790.

Parida, A.K., Jha, B., 2013. Physiological and biochemical responses reveal the drought tolerance efficacy of the halophyte *Salicornia brachiata*. J. Plant Growth Regul. 32, 342-352.

Peixoto, R.S., Chaer, G.M., Franco, N., Junior, F.B.R., Mendes, I.C., Rosado, A.S., 2010. A decade of land use contributes to changes in the chemistry, biochemistry and bacterial community structures of soils in the Cerrado. Antonie Van Leeuwenhoek 98, 403-413.

Phillips, J.M., Hayman, D.S., 1970. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 159-161.

Requena, N., Jeffries, P., Barea, J.M., 1996. Assessment of natural mycorrhizal potential in a desertified semiarid ecosystem. Applied and Environmental Microbiology 62, 842-847.

Roesch, L.F.W., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K.M., Kent, A.D., Daroub, S.H., Camargo, F.A.O., Farmerie, W.G., Triplett, E.W., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. ISME Journal 1, 283-290.

Ros, M., Pascual, J.A., Garcia, C., Hernandez, M.T., Insam, H., 2006. Hydrolase activities, microbial biomass and bacterial community in a soil after long-term amendment with different composts. Soil Biology and Biochemistry 38, 3443-3452.

Sardans, J., Peñuelas, J., 2005. Drought decreases soil enzyme activity in a Mediterranean Quercus ilex L. forest. Soil Biology and Biochemistry 37, 455-461.

Sieverding, E., 1991. Vesicular-Arbuscular Mycorrhiza Management in Tropical Agrosystems.

Silva, E.N., Ferreira-Silva, S.L., Fontenele, A.d.V., Ribeiro, R.V., Viégas, R.A., Silveira, J.A.G., 2010. Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in *Jatropha curcas* plants. J. Plant Physiol. 167, 1157-1164.

Sinsabaugh, R.L., Antibus, R.K., Linkins, A.E., McClaugherty, C.A., Rayburn, L., Repert, D., Weiland, T., 1993. Wood Decomposition: Nitrogen and Phosphorus Dynamics in Relation to Extracellular Enzyme Activity. Ecology 74, 1586-1593.

Skujins, J., 1976. Extracellular enzymes in soil. CRC critical reviews in microbiology 4, 383-421.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G., 2001. Bulk and Rhizosphere Soil Bacterial Communities Studied by Denaturing Gradient Gel Electrophoresis: Plant-Dependent Enrichment and Seasonal Shifts Revealed. Applied and Environmental Microbiology 67, 4742-4751.

Spain, J.L., 1990. Arguments for diagnoses based on unaltered wall structures. Mycotaxon 38, 71-76.

SSS, 2006. Soil Survey Staff (SSS). "Keys to Soil Taxonomy" 10th ed. USDA. Natural Resources, Conservation Service, Washington DC.

Tabatabai, M.A., 1982. Soil enzymes. In: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2 2nd ed. Agron Monogr 9. ASA and SSSA, Madison, Wisconsi, pp. 501-538.

Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301-307.

Van Der Heijden, M.G.A., Bardgett, R.D., Van Straalen, N.M., 2008. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems (Ecology Letters (2008) 11, 3 (296-310)). Ecology Letters 11, 651.

Van Der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 69-72.

Van Der Heijden, M.G.A., Streitwolf-Engel, R., Riedl, R., Siegrist, S., Neudecker, A., Ineichen, K., Boller, T., Wiemken, A., Sanders, I.R., 2006. The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytologist 172, 739-752.

Vandenkoornhuyse, P., Ridgway, K.P., Watson, I.J., Fitter, A.H., Young, J.P.W., 2003. Co-existing grass species have distinctive arbuscular mycorrhizal communities. Mol. Ecol. 12, 3085-3095.

Verbruggen, E., Kuramae, E.E., Hillekens, R., de Hollander, M., Kiers, E.T., Röling, W.F.M., Kowalchuk, G.A., van der Heijden, M.G.A., 2012. Testing Potential Effects of Maize Expressing the Bacillus thuringiensis Cry1Ab Endotoxin (Bt Maize) on Mycorrhizal Fungal

Communities via DNA- and RNA-Based Pyrosequencing and Molecular Fingerprinting. Applied and Environmental Microbiology 78, 7384-7392.

Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255, 571-586.

Welc, M., Ravnskov, S., Kieliszewska-Rokicka, B., Larsen, J., 2010. Suppression of other soil microorganisms by mycelium of arbuscular mycorrhizal fungi in root-free soil. Soil Biology and Biochemistry 42, 1534-1540.

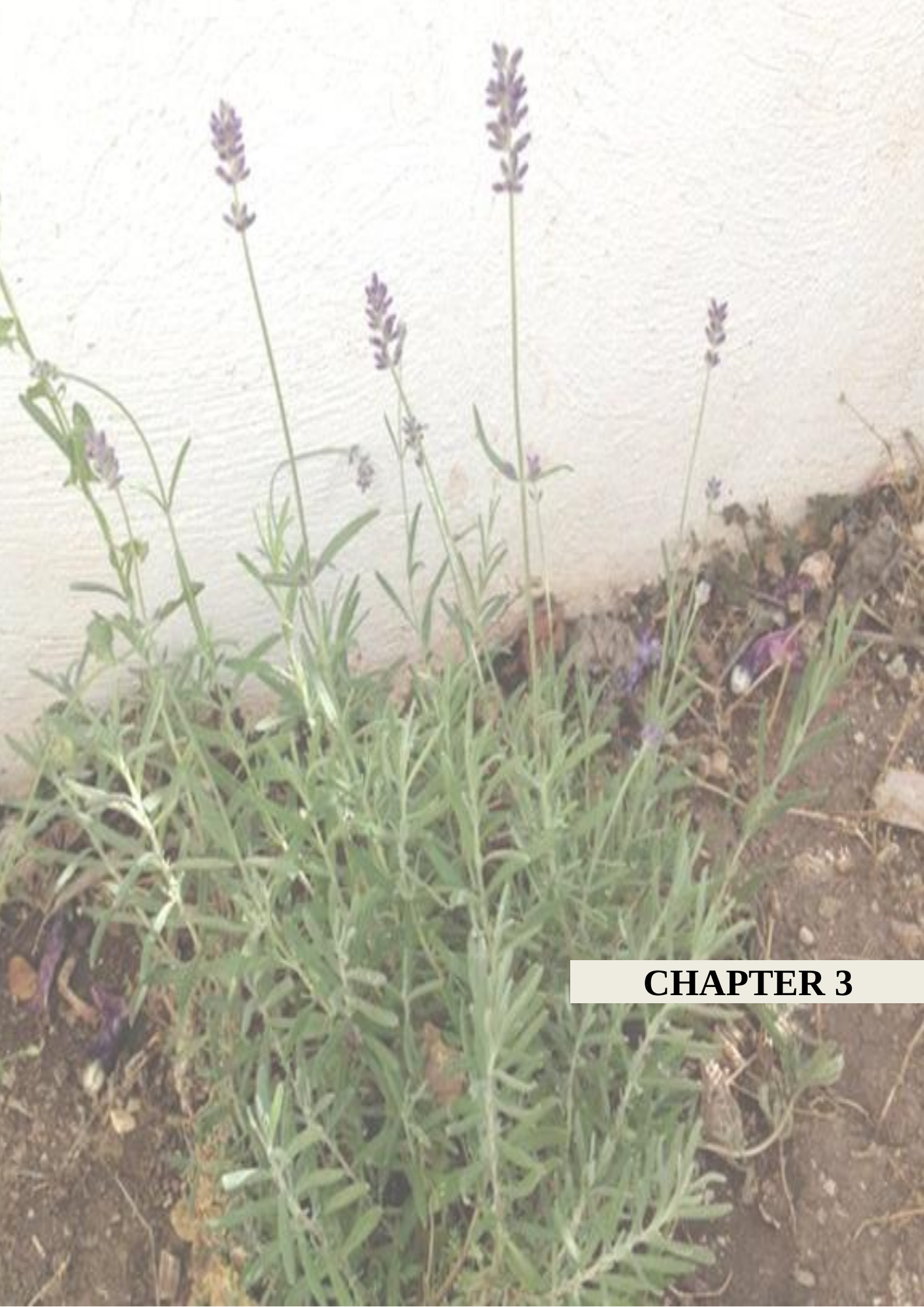
White, D.C., Pinkart, H.C., Ringelberg, D.B., 1996. Biomass measurements: biochemical approaches. In: In: Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. (Eds.), Manual of Environmental Microbiology. American Society for Microbiology Press, Washington, DC, pp. 91 - 101. (Ed.).

Williams, M.A., Rice, C.W., 2007. Seven years of enhanced water availability influences the physiological, structural, and functional attributes of a soil microbial community. Applied Soil Ecology 35, 535-545.

Yao, H., He, Z., Wilson, M.J., Campbell, C.D., 2000. Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. Microbial Ecology 40, 223-237.

Zelles, L., 1997. Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35, 275-294.

Zelles, L., Bai, Q.Y., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology and Biochemistry 24, 317-323.



CHAPTER 3

CHAPTER 3.1

Native plant growth promoting bacteria *Bacillus thuringiensis* and mixed or individual mycorrhizal species improved drought tolerance and oxidative metabolism in *Lavandula dentata* plants

E. Armada, A. Probanza, A. Roldán and R. Azcón

1. Introduction

Plants inhabiting arid or semiarid areas have many abiotic stresses such as water deficiency, limitation in essential macronutrients and low organic matter, the latter due mainly to the plants' limited establishment and production. Native plant species establishment are used as the most effective strategy in arid ecosystems and in semiarid Mediterranean areas for reclaiming these degraded soils [1]. Mycorrhizae may help plants to thrive in semiarid ecosystems [2]. This symbiosis is widespread under natural stress conditions and it occurs in nearly all environments. AM fungi are able to colonize and function in poor degraded ecosystems such as mine soil [3] or under arid/saline conditions [4], but such detrimental environmental factors have a negative effect on the development of AM symbiosis. The arbuscular mycorrhizal (AM) fungi have the ability to colonize the roots of most vascular plants and AM colonized plants cope more effectively with water deficit. The mycorrhizal effect is based on direct and indirect mechanisms, for example, mycorrhizal myceliums have access to soil pores therefore being more efficient than roots for nutrient and water extraction [5]. It is well known that mycorrhizal plants enhanced the uptake of nutrients, especially these with low mobility such as P, Zn, Cu and others. Physiological and biochemical changes related to mycorrhizal plant drought tolerance have been described [6-7]. Thus, the plants' ability to cope with environmental stresses is enhanced by AM fungal colonization and AM fungi have been considered an important functional component of the soil/plant system in disturbed soils. There is a lot of evidence that AM fungi are adapted to edaphic conditions but differences in fungal behaviour, efficiency on plant growth and stress tolerance can be, at least partly, due to the fungus involved. Nevertheless, the whole extent to which the plant benefits from particular AM species is still unknown.

The value to AM-derived nutrients, in terms of its C cost, is therefore likely to vary between particular plant-fungus associations. Carbon demand by each AM fungus is considered

a cost of the symbiotic association and this need to be compensated [8]. Drought highly reduced C assimilation processes by the host plant but plants respond differently to particular AM colonization according to how each fungus affects the process of C assimilation in the host-plant and the C requirements of each fungus. These results highlight the diversity in the way, function and reaction of AM colonization according to partners involved and the environmental conditions. The existence of species' specific interactions between the host-plant and the fungal species underlines the importance of screening of fungal species to maximize the benefits of the symbiosis [9]. Authors reported that the inoculation with a mix of native AM fungi was a more effective treatment for the development of *Retama sphaerocarpa* than an allochthonous fungus, *Glomus claroideum* in a semiarid ecosystem. Other studies have focused on the importance of the origin of the AM fungi to be used as inocula in dry soil when plants were colonized by drought-sensitive or drought-tolerant *Glomus* species [7].

The assimilation of nutrients by AM colonized plants reflects the amount of direct plant uptake plus the indirect contribution from the AM fungus. However, both ways became weaker when water availability decreased, that even AM colonization decreased. Drought may induce changes in the metabolic capacity reducing the infective fungal capacity but these characteristics have not yet been studied.

Adverse environmental conditions can negatively affect the diversity and number of spores in soil and also the infectivity of AM propagules [10]. The negative effect of drought stress can be compensated by rhizosphere bacteria that are able to improve the growth of AM fungi [3, 11].

Plant growth promoting bacteria (PGPB), as component of soil microbiota, have the potential role of improving the establishment of plant species under arid soil conditions [12]. They can colonize root surface and/or intercellular spaces in plant tissues.

Many mechanisms lead to plant growth promotion as phytohormones production, nutrients and water acquisition and others have been described [13]. But unpredictable results of PGPB inoculation can be found mainly caused by the quality and resistance/tolerance of inoculants to the severe stress conditions. Thus, the bacterial ability to produce compounds that play important role in the process of osmotic adjustment decreasing the cell osmotic potential allowing greater water retention during drought were evaluated under axenic conditions using polyethylene glycol (PEG) as an osmotic stress agent.

Regarding plant biochemical parameters affected by water stress, reactive oxygen species (ROS) have been used as an indicator of drought tolerance. Water stress generates ROS production that may cause lipid peroxidation, protein degradation, membrane injury and cell

death [14]. Major ROS scavenging enzymes include antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) to control the cellular redox status under stress conditions such as drought [14-16]. These antioxidant enzymes increase the ability of plants to resist environmental stresses. Nevertheless, the effectiveness of autochthonous microorganisms in plant drought tolerance has been scarcely reported [6-7].

The diversity of AM fungi is low compared to that of host plants, although evidence from molecular methods suggests that the diversity of AM fungi is higher than expected [17]. However, the relatively low AM diversity shows differences according to fungus, habitants and host species involved [18]. It seems attributable to functional differences between AM fungi [19].

For this paper, the plant *Lavandula dentata* was selected as a representative shrub species from semiarid scrublands in the southeast of Spain. This plant is well-adapted to drought conditions and it was a prevailing plant species growing in the arid zone of study.

Combined microbial inoculations resulted more effective to induce resistance to drought conditions and in the protection of plants against a drought stress enhance revegation process. We conducted a pot experiment in a semiarid Mediterranean soil under drought conditions and we assayed if *L. dentata* was more benefited from the inoculation with a whole autochthonous AM fungal consortium or from each one of the single native fungal isolates (selecting the five most abundant and representative ecotypes). In addition, the autochthonous beneficial bacteria *B. thuringiensis* was assayed in interaction with native AM fungi (single or mixture) stimulating plant growth, nutrition and drought tolerance. Thus, here we hypothesised that the combined inoculation involving autochthonous microorganism (single or mixed AM fungi and *Bacillus thuringiensis*) could be beneficious to enhance *L. dentata* growth under water stress conditions. The drought tolerance, PGPB characteristics and endophytic conditions of *B. thuringiensis* here used were also evaluated. The aim is to verify the potential of plant coinoculation to increase drought tolerance and to alleviate the impact of water stress. Selected soil microorganism may help an important role in the establishment of autochthonous plant cover under arid environmental conditions.

2. Material and Methods

Independent experiments were carried out in the present study. Firstly, an autochthonous bacteria, isolated from the semiarid experimental soil from the province of Murcia (Spain), was identified using molecular methods and in an *in vitro* assay, we determined changes on maintenance of growth of the bacterial cells in axenic culture medium under non stress and stress osmotic conditions [by 40% polyethylene glycol (PEG) application] and their abilities to produce proline, lipid peroxidation (MDA) or poly-ß-hydroxybutyrate (PHB) and PGPB characteristics tested as α-ketobutyrate (ACC deaminase), indole acetic acid (IAA) production and phosphate solubilization under such non-stress and stress conditions. Secondary, a microcosm experiment under drought conditions analyzed the effectiveness of five autochthonous AM fungal species single or in consortium inoculated and the impact of autochthonous bacteria in improving plant growth, physiology, nutrition and antioxidant activities as indexes of drought tolerance.

2.1. Isolation and molecular identification of the bacterial strain

The autochthonous bacteria strain used throughout this study were isolated from the same natural soil used in the bioassay (see description below). The bacterium was isolated from a mixture of rhizosphere soils from several autochthonous shrub species.

A homogenate of 1 g of soil in 9 mL of sterilized water was diluted (10^{-2} to 10^{-4}), plated on three different media [Yeast Mannitol Agar, Potato Dextrose Agar, Luria-Bertani (LB) Agar] and then incubated at 28 ºC for 48 h, to isolate bacteria from different taxonomic groups. The selected bacterium was the most abundant bacterial type in such arid soil.

Identification of isolated bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were collected, diluted, lysed, and their DNA used as a template in the PCR reactions. All reactions were conducted in 25 µL volume containing PCR buffer 10X, 50 mM $MgCl_2$, 10 µM each primers: 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), [20] 5 U/µL of *Taq* polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with the following conditions: 5 min at 95 ºC, followed by 30 cycles of 45s at 95 ºC, 45s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. The products of PCR were analyzed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (NCBI) using BLAST program.

2.2. Isolation and identification of the arbuscular mycorrhizal (AM) fungi

The method used in the isolation of spores of the arbuscular mycorrhizal fungi from rhizosphere soil samples, called "method wet sieving and decanting" [21] optimizes the separation of the spores from other mineral and organic soil particles.

A suspension of soil in water was filtered through a chain of different diameter mesh strainers (500, 250 and 50 μm). The contents of each sieve were then collected and they were counted using a stereo-microscope (30-40X). The population of arbuscular mycorrhizal was increased through the establishment of plants 'trap' [22]. This method involves growing plants with a strong dependence on mycorrhizal, in the soil of study. Thus fungal species can complete their life cycles and sporulate mass, resulting in a diverse population of species of AM fungi at different stages of ontogenic.

The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG) [23]; a mixture of PVLG and Melzer's reagent [24] a mixture of lactic acid to water at 1:1; Melzer's reagent; and water [25]. For identification of the AM fungi species, spores were then examined using a compound microscope at up to 400-fold magnification as described for glomeromycotean classification [26].

2.3. Evaluation in axenic culture of *B. thuringiensis* growth, stress tolerance abilities and PGPB characteristics under non-stress and stress (40% of PEG) conditions

2.3.1. B. thuringiensis growth

Bacterial strain were cultivated at 28 ºC in nutrient broth (Luria-Bertani (LB)) medium supplemented with PEG (40%) to generate osmotic stress (equivalent to -3.99 MPa). This level of PEG was selected in preliminary studies as the maximum PEG concentration supportable by bacterial strain. The number of viable cells was estimated after 4 days of growth following the conventional procedure: 1 mL of suspension was plated in nutrient broth medium. The bacterial growth was monitored by measuring optical density at 600 nm [13].

2.3.2. B. thuringiensis stress tolerance abilities

The bacterial isolates were cultivated at 28 ºC at 120 rpm in 100 mL of liquid nutrient (LB) medium supplemented or not with 40% of PEG (-3.99 MPa) in order to induce drought stress.

The accumulation of proline was estimated by spectrophotometric analysis at 530 nm [27]. The bacterial extract reacts with ninhydrin and glacial acetic acid for 1 h at 100 ºC. The reaction stops by introducing the tubes in an ice bath. The reaction mixture is extracted with 2 mL of toluene, shaking vigorously for 20 seconds. A standard curve which was prepared with known concentrations of proline.

Measurement of lipid peroxidation was done by the method based on the reaction of 2-thiobarbituric acid (TBA) with reactive species derived from lipid peroxidation, particularly malondialdehyde (MDA). Detection of 2-thiobarbituric acid reactive substances (TBARS) was carried out by a colorimetric assay described by Buege and Aust [28] with some modifications [29]. 50 mg of cells were resuspended in 500 µL of 50 mM phosphate buffer, pH 6.0, containing 10% trichloroacetic acid (TCA), and 0.3 g glass beads were added. The samples were broken by three cycles of 1 min agitation on a vortex mixer followed by 1 min on ice. After centrifugation, supernatants were mixed with 0.1 mL of 0.1M EDTA and 0.6 mL of 1% (w/v) TBA in 0.05 M NaOH. The reaction mixture was incubated at 100 ºC for 15 min and then cooled on ice for 5 min. The absorbance was measured at 532 nm. Lipid peroxidation was expressed as µmoles of malondialdehyde g^{-1} of dry cell weight.

The poly-β-hydroxybutyrate (PHB) production of the bacterial strain on different osmotic concentrations (0% and 40% PEG) in N_2 deficient medium (pH 7) and incubated at 28 ºC for 72 h at 120 rpm was measured. PHB produced were extracted as described in the method of Ramsay et al. [30]. The amount of PHB in the extracts was determined spectrophotometrically at 235 nm [31-32]. A standard curve was prepared to determine PHB in mg mL^{-1}.

2.3.3. B. thuringiensis PGPB characteristics

The activity of ACC deaminase enzyme in isolates was measured as described by Penrose and Glick [33]. The enzyme activity was assayed according to a modification of the method of Honma and Shimomura [34] which measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase hydrolyses ACC. The quantity of µmol of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 1.0 mmol and 1.0 µmol. Protein concentration of cellular suspension in the toluenized cells was determined by the method of Bradford [35].

The production of indole-3- acetic acid (IAA) by these bacteria was determined using Salper's reagent [36]. Three milliliters of fresh Salper's reagent (1mL 0.5 M $FeCl_3$ in 50 mL 37% $HClO_4$) was added to free-cell supernatant and kept in complete darkness for 30 minutes

at room temperature, and the optical density at 535 nm was measured in each treatment [37]. A standard curve was also prepared for IAA determination.

To determine phosphate solubilization index (PSI), each bacterial culture was assayed on Pikovskaya agar plates [38] containing tricalcium phosphate ($Ca_3(PO_4)_2$) as insoluble phosphate source. Cells were grown overnight in LB medium, next they were washed twice with 0.9% NaCl and resuspended in 0.9% NaCl to produce equal cell densities among. Solutions were inoculated on the agar plates and incubated at 30°C, and observed daily for 7 days for the appearance of transparent "halos" [39]. Experiments were performed in triplicate. Phosphorus solubilization index was measured using the following formula [40].

$$PSI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

2.4. Microbial inoculation in *Lavandula dentata* plants under greenhouse conditions

2.4.1. Experimental design

The experimental work was based on a design with two factors: isolates of arbuscular mycorrhizal fungi species predominant in the study area (see results, five different AM fungi species: *Septoglomus constrictum* EEZ 198; *Diversispora aunantia* EEZ 199; *Archaeospora trappei* EEZ 200; *Glomus versiforme* EEZ 201; *Paraglomus ocultum* EEZ 202 and a mixture or consortium of these AM fungi) and bacterial inoculation treatments [bacteria native isolated of study zone: control (-); *Bacillus thuringiensis* (B.t)].

2.4.2. Test soil and inoculation of microorganisms

The soil used in this experiment is located in the National Park of "Vicente Blanes" in the town of Molina de Segura, Murcia (Spain), (coordinates: 38° 12' N, 1º 13' W; altitude 393 m). The main features of this soil was that it was a soil with low organic matter content and a silty-clay texture which are both causes for very easy ground degradation. The main characteristics of the soil were pH 8.90, P $1.36 \cdot 10^{-3}$ g kg^{-1} (Olsen test), organic carbon 0.94%, total nitrogen 0.22%, electrical conductivity of 1.55 dS m^{-1}. The substrate used in this assay consisted in using the previously mentioned Mediterranean soil (sterilized and sieved by 5mm), and mixed with sterile sand to the ratio of (5:2, (v/v)). Substrate was put into pots with a capacity of 0.5 kg. The plant used in this study was *Lavandula dentata* and was grown under drought conditions for six months in greenhouse.

One milliliter of pure bacterial culture (10^7 cfu mL^{-1}) grown in nutrient broth medium for 48 h at 28 ºC, was applied to the appropriate pots at sowing time just below plant seedlings, and 15 days later the bacterial culture (1 mL, 10^7 cfu mL^{-1}) was applied around the plant on the soil. Five grams of different isolates of arbuscular mycorrhizal fungi (AMF) species and consortium of AMF per pot were applied to each one of the appropriate pots at sowing time just below the seeds. Five replicates of each treatment were used, making a total of 70 pots.

2.4.3. Plant growth conditions

These plants were grown for six months in pots containing a mixture of sterile soil and sterile quart sand (5:2, (v/v)) under green house conditions (temperatures ranging from 15 ºC to 21 ºC; 16/8 light/dark photoperiod, and a relative humidity of 50-70%). A photosynthetic photon flux density of 400-700 μmol m^{-2} s^{-1} was applied as supplementary light. Plants were grown during the experiment under drought conditions by keeping soil water capacity to 50% each day after water application but water levels decreased gradually during the day to nearly 20% water capacity until the next water application.

2.4.4. Plant biomass analyses

After six months of growth, plants were harvested (five replicates of each treatment) shoots and roots were weighed and dried for 48 h at 75 ºC to obtain dry weights. Shoot/root ratio (g) was also calculated.

Shoot content (mg $plant^{-1}$) of C, N, P, K, Mg and Ca as well as of Mn, Cu, Fe, and Zn (μg $plant^{-1}$) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Mineral analysis was carried out by the Analytical Service of the "Centro de Edafología y Biología Aplicada del Segura, CSIC", Murcia, Spain.

2.4.5. Root colonization

2.4.5.1. Mycorrhizal colonization

Fungal colonization was assessed after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Philips and Hayman [41]. The extent of mycorrhizal colonization was calculated according to the gridline intersect method [42] after counting 150 intersections. Mycorrhizal development was evaluated by the method of Trouvelot et al. [43] using MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The parameters measured according to this method were the frequency of AM colonization in the sample (F%), intensity of AM colonization in the whole root system (M%), and absolute arbusculum richness (A%) referred to the calculated whole root system

respectively. The images were performed with a Nikon Eclipse 50i microscope equipped with a Nikon DS-Fi1 camera.

2.4.5.2. *B. thuringiensis* endophytic colonization

Bacterial endophytic colonization was realized only in two treatments (B.t; B.t+MIX) due to lack of root biomass in the remaining treatments and also by our interest in evaluates the bacterial endophytic colonization mainly in these two treatments. Roots containing rhizospheric soil were washed with sterile distilled water, desinfected with 70% ethanol, rinsed, disinfected superficially with 3% sodium hypochlorite, rinsed again to eliminate hypochlorite, and spread on nutritive agar to confirm root surface sterility [44]. One centimeter root section from the two treatments was aseptically excised, and homogenates were serially diluted in 0.1 M $MgSO_4$ to enumerate the bacteria colonizing the root (cfu per cm) [45].

Transmission electron microscopy (TEM) in root of two treatments mentioned above (B.t; B.t+MIX) were fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) dehydrated in a graded series of ethanol and embedded in unicryl resin. Ultrathin sections were examined with a transmission electron microscope JEOL 1011.

2.4.6. Oxidative damage to lipids in shoots

The non-mycorrhizal plants [non-inoculated (-) and inoculated with *B. thuringiensis* (B.t)] showed insufficient shoot biomass for the following determinations.

Lipid peroxides were extracted by grinding 0.5 g of shoot with ice-cold mortar and 5 mL of TCA 5%. Homogenates were centrifuged at 12,290 *g* for 10 min. The chromogen was formed by mixing 0.5 mL of supernatant with 1.5 mL of a reaction mixture containing 20% (w/v) TCA, 0.5% (w/v) TBA, and by incubating the mixture at 95 ºC for 30 min [46]. After cooling at room temperature, absorbance of samples was measured at 532 nm. Lipid peroxidation was estimated as the content of TBARS and expressed as equivalents of MDA according to Halliwell and Gutteridge [47]. The calibration curve was made using MDA in the range of 0.1-100 μmol. A blank for all samples was prepared by replacing the sample with extraction medium.

2.4.7. Antioxidant enzymatic activities in shoot (SOD, CAT, APX and GR)

The antioxidant enzymatic activities of the non-mycorrhizal plants [non-inoculated (-) and inoculated with *B. thuringiensis* (B.t)] were not performed due to small amount of shoot biomass, insufficient to proceed for its determination. The method followed for the extraction of enzymes, shoot tissues was that described by Aroca et al. [48]. Thus, plant material was homogenized in cold mortar with 4 mL 100 mM phosphate buffer (pH 7.2) containing 60 mM

KH_2PO_4, 40 mM K_2HPO_4, 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 1 % (w/v) PVPP. The homogenate was centrifuged at 18,000 *g* for 10 min at 4 ºC, and the supernatant was used for enzyme activity determination. Total SOD activity (EC 1.15.1.1) [49] was measured on the basis of SOD's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25ºC. CAT activity (EC 1.11.1.6) was measured as described by Aebi [50], conducted in 2 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µL of enzyme extract. It was determined the consumption of H_2O_2 and followed bydecrease in absorbance at 240 nm for 1 min (extinction coefficient (ε_{240}) of 39.6 mM^{-1} cm^{-1}). APX activity (EC 1.11.1.11) was measured in a 1 mL reaction volume containing 80 mM potassium phosphate buffer (pH 7.0), 0.5 mM hydrogen peroxide and 0.5 mM sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate [51]. GR activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH [52]. The reaction mixture (1 mL) contained 50 mM Tris buffer, 3 mM $MgCl_2$ (pH 7.5), 1 mM oxidized glutathione, 100 µL enzyme extract and 0.3 mM NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in mmol NADPH oxidized mg^{-1} protein, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH (ε_{340}=6.22 mM^{-1} cm^{-1}). Total soluble protein amount was determined using the Bradford method [35] and bovine serum albumin as standard.

2.5. Statistical analyses

Data from both experiments were analyzed using the SPSS 21 software package for Windows and were subjected to a one-way general linear model ANOVA (analysis of variance) which was used to determine the effect of each treatment. Duncan's multiple-range test [53] was used for post hoc analysis to determine differences between means. Differences were considered significant at $p \leq 0.05$. Percentage values were arcsine-transformed before statistical analysis.

3. Results

3.1. Identification of bacterial strain and of arbuscular mycorrhizal (AM) fungi

Each sequence obtained was compared with the database of 16S rDNA from the NCBI/BLAST. The similarity unambiguously identified the bacterium as *Bacillus thuringiensis* (Acession NR 043403.1, identity 98%).

Fungal characterization has been done using morphological techniques [54]. The more predominant AMF species identified in the study area were: *Septoglomus constrictum*, *Diversispora aunantia*, *Archaespora trappei*, *Glomus versiforme* and *Paraglomus ocultum*, which were cataloged and included in the collection of EEZ (codes 198-202).

3.2. Characterization of bacterial osmotic stress tolerance and PGPR activities

Table 1 shows the *B. thuringiensis* growth, the bacterial stress tolerance and its PGPB characteristics under non-stress and stress (40% PEG) conditions. Osmotic stress decreased more bacterial growth than its PGPB abilities. In fact, the stress highly increased ACC deaminase production, slightly reduced IAA and it does not change phosphate solubilization. The stress tolerance parameters either did not change as PHB production or increased as proline or MDA. Regarding these *in vitro* results, the stress applied in the culture medium did not reduce the bacterial potential to improve plant growth.

Table 1. Bacterial growth (cfu mL^{-1}), drought tolerance abilities [proline, lipid peroxidation (MDA) and poly-β-hydroxybutyrate (PHB) production] and PGPB activities [indole acetic acid (IAA), phosphate solubilization index (PSI) and α-ketobutyrate (ACC) accumulations] by *Bacillus thuringiensis* (B.t) grown for four days under non-stress and osmotic stress conditions produced by a concentration of 40% polyethylene glycol (PEG) in the growing medium.

	[PEG]	cfu mL^{-1}	mmol proline mg^{-1} protein	µmol MDA g^{-1}dry cell weight	mg PHB mL^{-1}	µg IAA mg^{-1} protein	PSI	mmol α-ketobutyrate mg^{-1} protein
B.t	0%	2.18 b	0.12 a	0.7 a	0.33 a	18.2 b	1.56 a	0.20 a
	40%	0.83 a	0.31 b	4.4 b	0.38 ab	13.0 a	1.37 a	0.41 b

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=4).

3.3. Plant biomass production and nutrients uptake

L. dentata inoculated plants have higher root and shoot biomass under the drought condition compared to non-inoculated plants. *L. dentata* showed significant growth difference according to the single mycorrhizal species (or mixture) inoculated and the particular interaction of each AM fungus with *B. thuringiensis* (Fig 1).

The most efficient mycorrhizal fungus in increasing shoot biomass were *A. trappei* and *P. occultum* yielding 0.61 and 0.51 g shoot dry weight respectively while control non-inoculated plants yielded 0.14 g. These fungal inocula promoted increases in plant growth of 336% (A.t) and 264% (P.o). Single Bt increased shoot growth by 21% and *B. thuringiensis* associated with *S. constrictum*, *G. versiforme* or the mixture of native fungi improved the effectiveness of these fungi in enhancing shoot growth by 12.8% (S.c), 27.3% (G.v) and 22.9% the fungal mixture (Fig. 1A). However, the opposite effect was observed when *B. thuringiensis* was associated to *D. aunantia*.

Great differences in root development between inoculated and non-inoculated plants were also observed. The bacteria improved root growth by 50% but this effect was always higher for the mycorrhizal inoculated plants. In some cases, *B. thuringiensis* decreased this value in AM colonized plants. The dual inoculation of *G. versiforme* plus *B. thuringiensis* resulted to be the most effective treatments in increasing the root growth over non-inoculated plants by 412% (Fig. 1B).

AM fungal colonization by *S. constrictum*, *D. aunantia*, *G. versiforme* and mix increased more root than shoot biomass under drought conditions. But when associated to *B. thuringiensis* (*D. aunantia* and mix) increased this ratio (Fig. 1C).

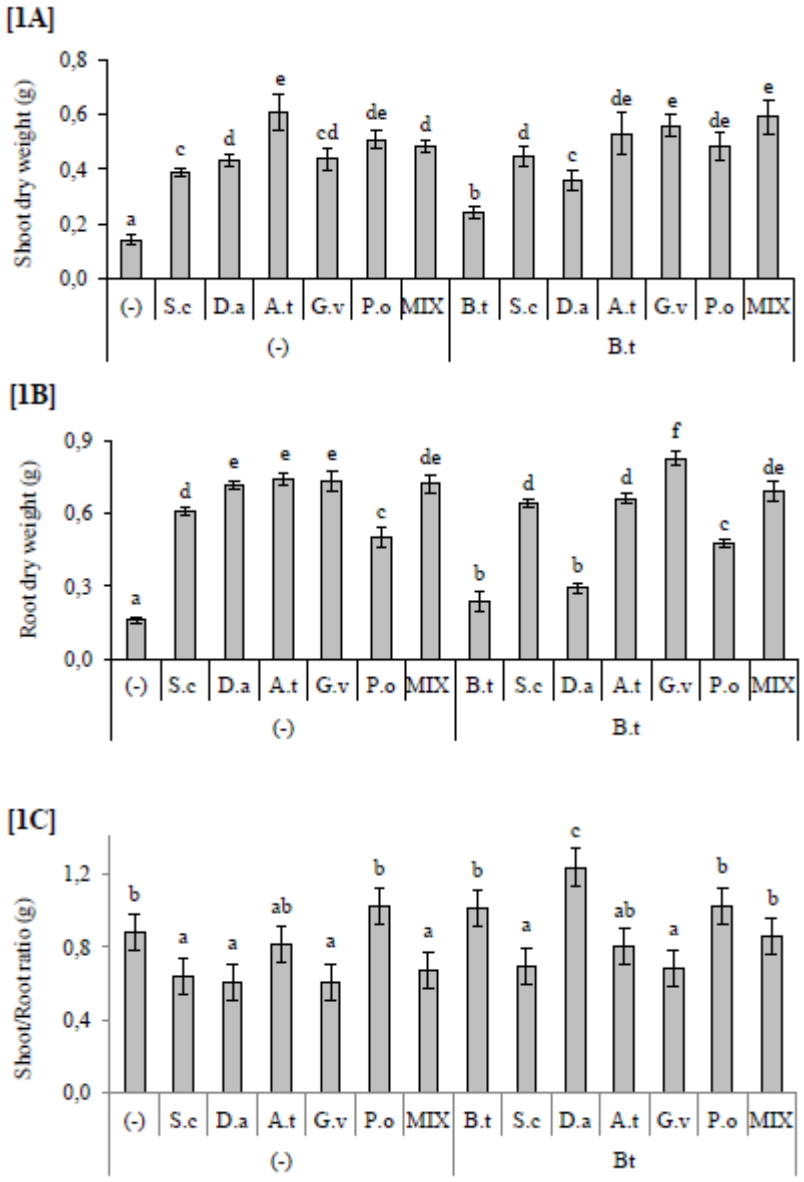


Fig. 1. Shoot dry weight (g) [1A], root dry weight (g) [1B] and shoot/root ratio (g) [1C] in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan's multiple-range test (n=5).

Under these drought conditions, the uptake of whatever nutrient analyzed was enhanced by the mycorrhizal colonization. But this effect was variable according to the colonizing fungal species. The colonization with *A. trappei* enhanced the C, N, P, K, Mg and Ca shoot content in a

higher extent than the rest of the colonizing AM fungal species or the mix (Fig. 2 and 3). *B. thuringiensis* maximized the shoot accumulation of some of these nutrients (C, N and Ca) when associated to *G. versiforme* or (C, N and K) when associated to the fungal mixture. As results show micronutrients such as Mn, Cu, Fe and Zn were differently increased by the microorganisms applied and the highest values were observed in dually inoculated plants.

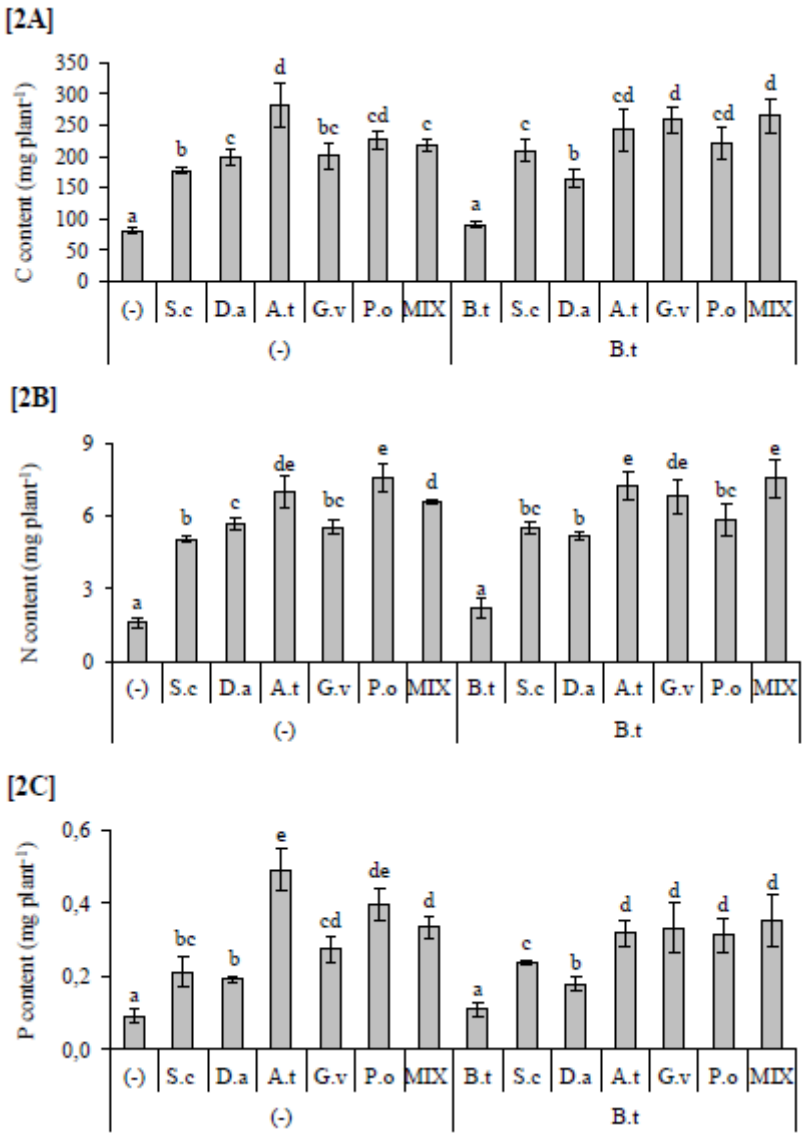


Fig. 2. C [2A], N [2B] and P [2C] content (mg plant^{-1}) in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan's multiple-range test (n=3).

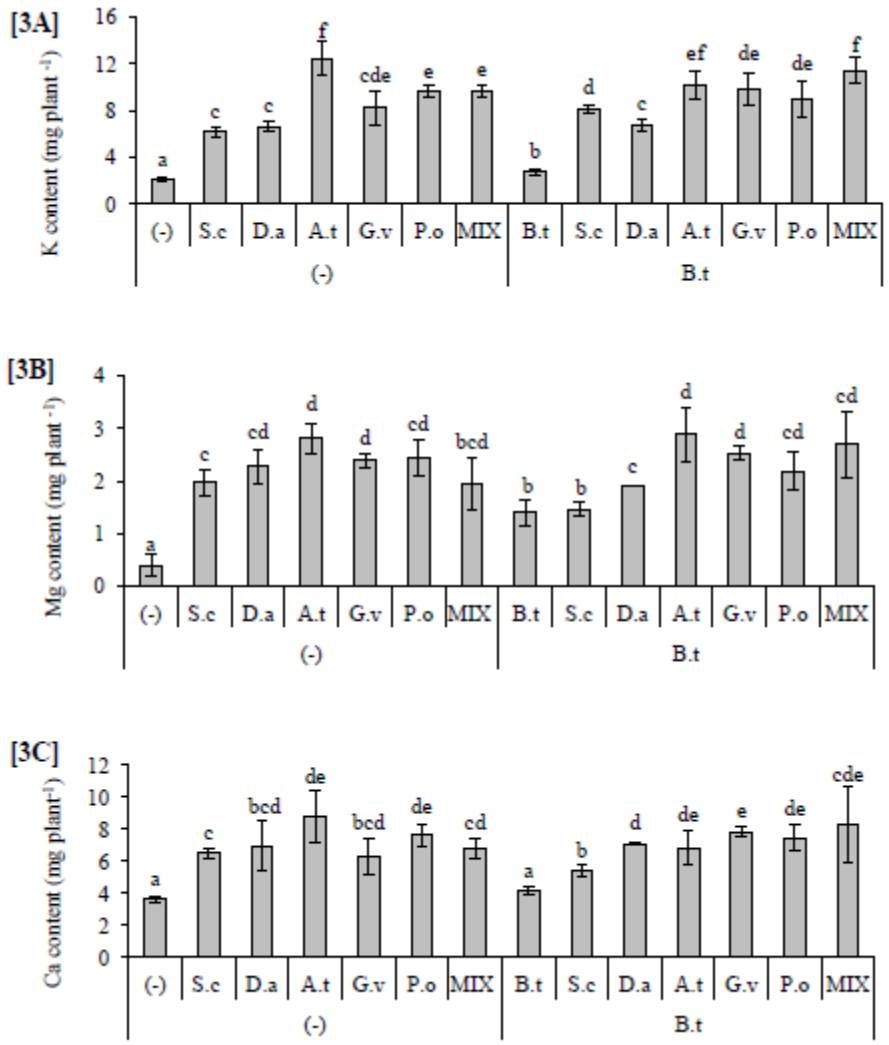


Fig. 3. K [3A], Mg [3B] and Ca [3C] content (mg plant^{-1}) in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan's multiple-range test (n=3).

The positive effect of *B. thuringiensis* in increasing micronutrients content was only observed in interaction with *S. constrictum* (Cu content) or with *D. aunantia* and *G. versiforme* (Fe content) (Fig. 4). Non-mycorrhizal control plants show that micronutrients are present in low concentrations in the soil solution and particularly Zn and Cu have a low mobility which normally causes deficiencies.

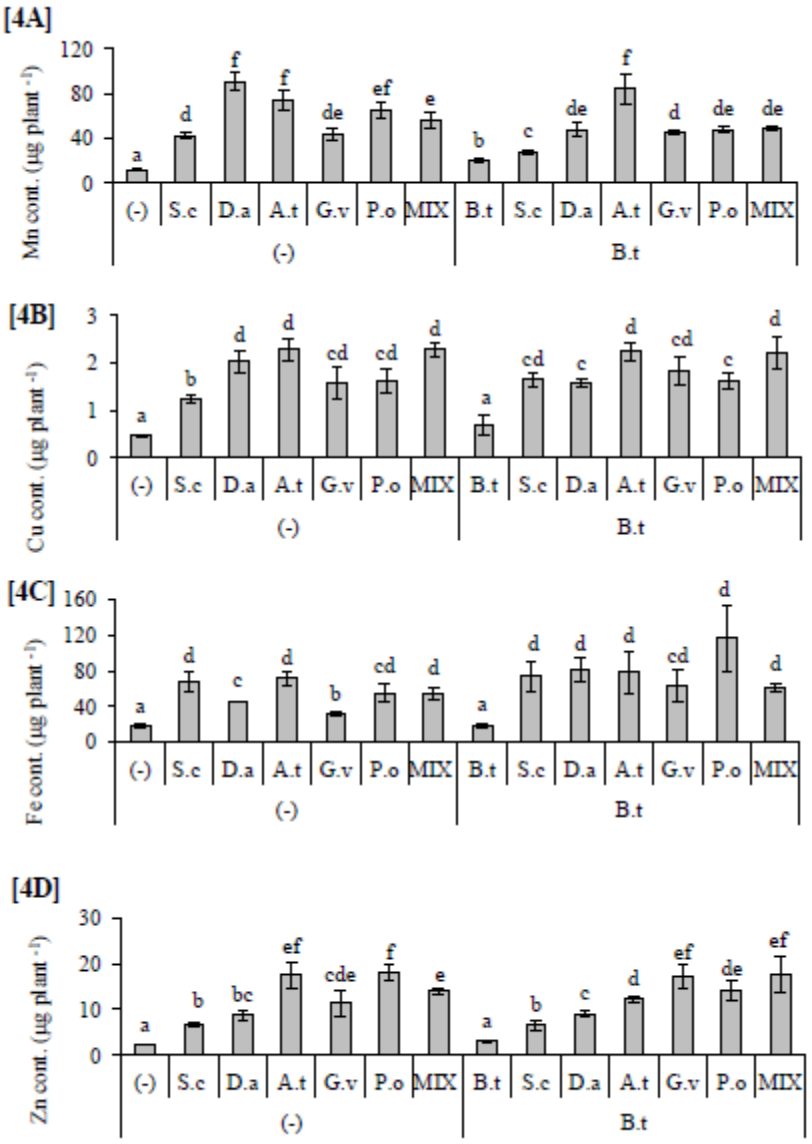


Fig. 4. Mn [4A], Cu [4B], Fe [4C] and Zn [4D] content (µg plant^{-1}) in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan's multiple-range test (n=3).

3.4. Root colonization

The percentage of AM colonized roots (% AMF) (Fig. 5A) was highly variable depending on the fungal inoculum involved and ranged from 13% (*D. aunantia*) to 52% (MIX + B.t). Regarding whatever colonizing parameters (%AMF, %F, %M or %A) the highest values were observed in roots colonized by the Mix that significantly differed from single fungi. The bacterial inoculation tends to increase % AMF by 18% in single *A. trappei* and by 24% in *S. constrictum* colonized plants. But the highest %AMF colonization was obtained in plants inoculated with the fungal mixture irrespective of bacteria (Fig. 5A and 6). Nevertheless, the most important effect of *B. thuringiensis* on the symbiotic development was the improvement of the most important mycorrhizal parameters as %M and particularly %A in plants colonized by most of the fungi.

The arbuscular colonization was limited (less than 10%) in single AM-colonized plants but in plants colonized by the fungal mixture it was much higher (24.6%). The interaction with *B. thuringiensis* enhanced the formation of this important propagule by 545% (*A. trappei*), by 294% (*G. versiforme*) and by 561% (*P. occultum*). Thus the arbuscular development was maximized in plants colonized by dual *B. thuringiensis* and the fungal mixture.

The different species of arbuscular mycorrhizal fungi colonize the roots of a more or less intense way as was also observed on microscopic images (Fig. 6). The photos show that the strains *S. constrictum* (A1), *G. versiforme* (A4), *P. ocultum* (A5) and the mixture of AM fungi (A6) developed the highest level of intracellular hyphae. In root colonized by *A. trappei* (A3) and *P. ocultum* (A5) is observed the formation of extracellular hyphae but the dual inoculation of these fungi with *B. thuringiensis* decreases this extracellular development. In general, the co-inoculated roots increased the intraradical growth and development of most of AMF species (except in *D. aunnatia*) as also values of %M and %A showed. Species such as *G. versiforme* (B4), *P. ocultum* (B5) and mixture of AM fungi (B6) co-inoculated with *B. thuringiensis* increased the presence of arbuscules as Fig. 5 also shows but B.t as well promotes the formation of vesicles in the roots colonized with mixture of AM fungi (B6).

The endophytic colonization of *B. thuringiensis* in roots of *L. dentata* was also determined (Fig. 7.1). It was of $5.6 \cdot 10^6$ cfu cm^{-1}, being the B.t cells mainly located in the intracellular zone (Fig. 7.2A). But B.t inoculation with the mixture of AM fungi reached the cells number to $9.0 \cdot 10^6$ cfu cm^{-1}. As consequence, the presence of fungal mixture increased the population of *B. thuringiensis* by 61% (Fig. 7.1), located in the intracellular and extracellular zones of the plant cortex cells (Fig. 7.2B). Thus, an interactive relationship was detected

between both microorganisms and the competence by roots occupancy by these endophytic organisms is excluded.

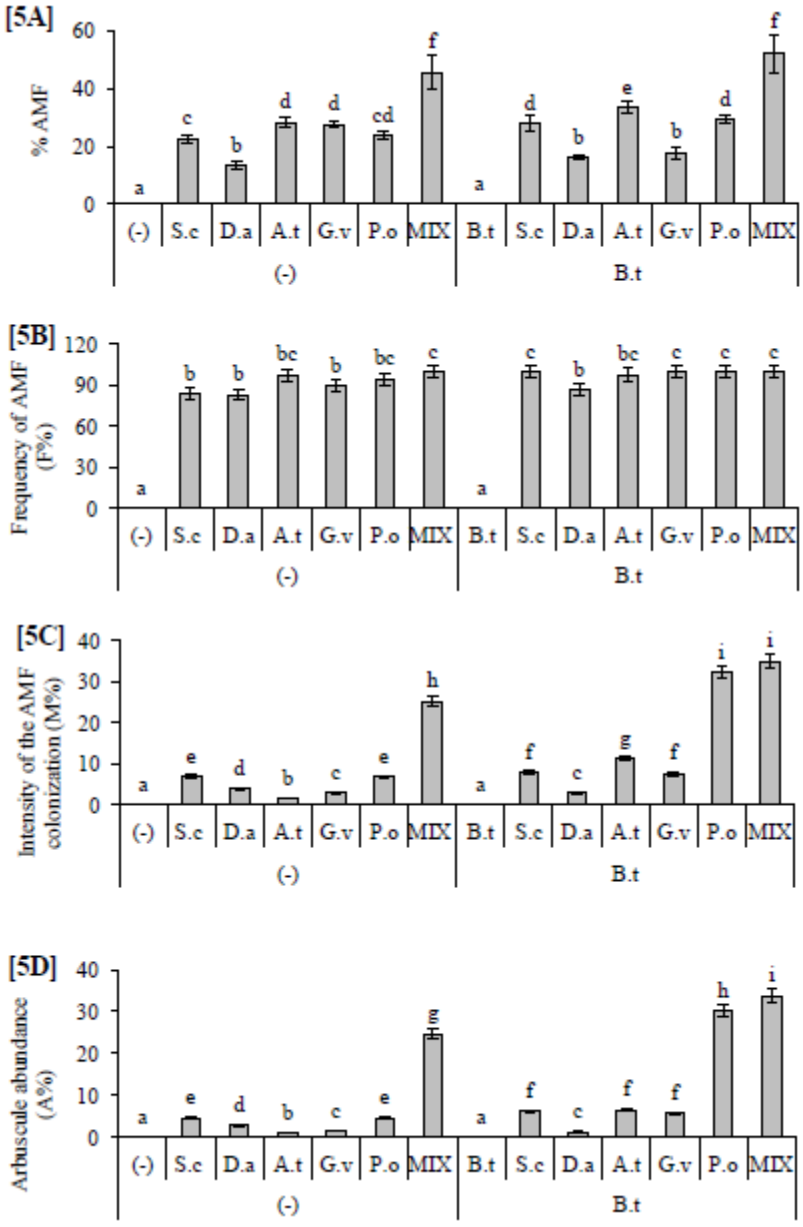


Fig. 5. Percentage of AM colonization [5A], frequency of AMF colonization in the whole root system (F%) [5B], intensity of AMF colonization in the whole root system (M%) [5C] and arbusculum abundance (A%) [5D] in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their interaction with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan's multiple-range test (n=3).

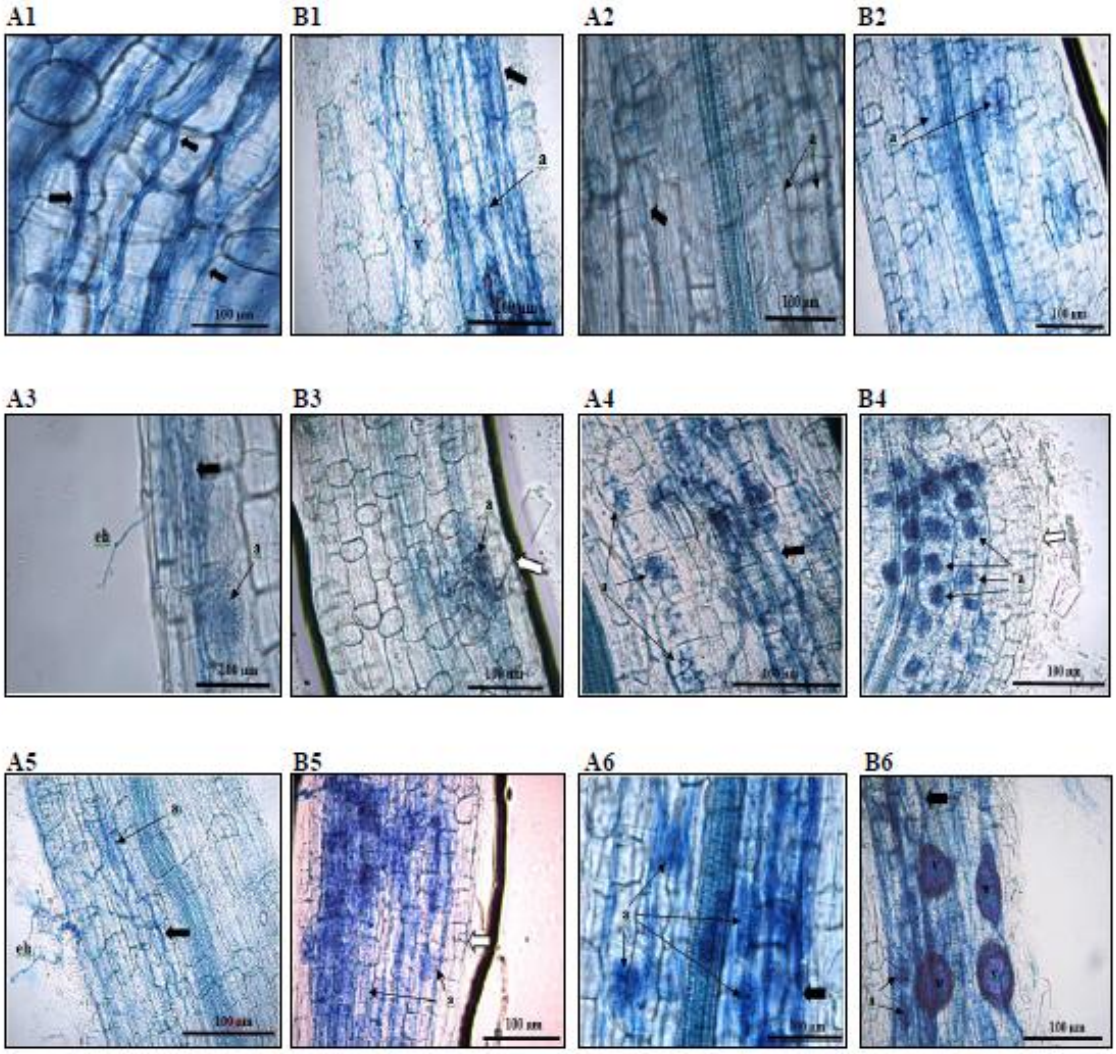


Fig. 6. Single arbuscular mycorrhizal fungal species (A1-5) or mixed (A6) colonizing *Lavandula dentata* roots. (A1: *Septoglomus constrictum* EEZ 198; A2: *Diversispora aunantia* EEZ 199; A3: *Archaeospora trappei* EEZ 200; A4: *Glomus versiforme* EEZ 201; A5: *Paraglomus ocultum* EEZ 202 and A6: a mixture or consortium of these AMF). And the effect of endophytic *B.thuringiensis* (B) on each one of AM fungal species (B1-5) and then mixture (B6). Intracellular hyphae (black arrow); extracellular hyphae (eh) and entry point (white arrow); arbuscule (a); vesicle (v). Bars= 100 µm.

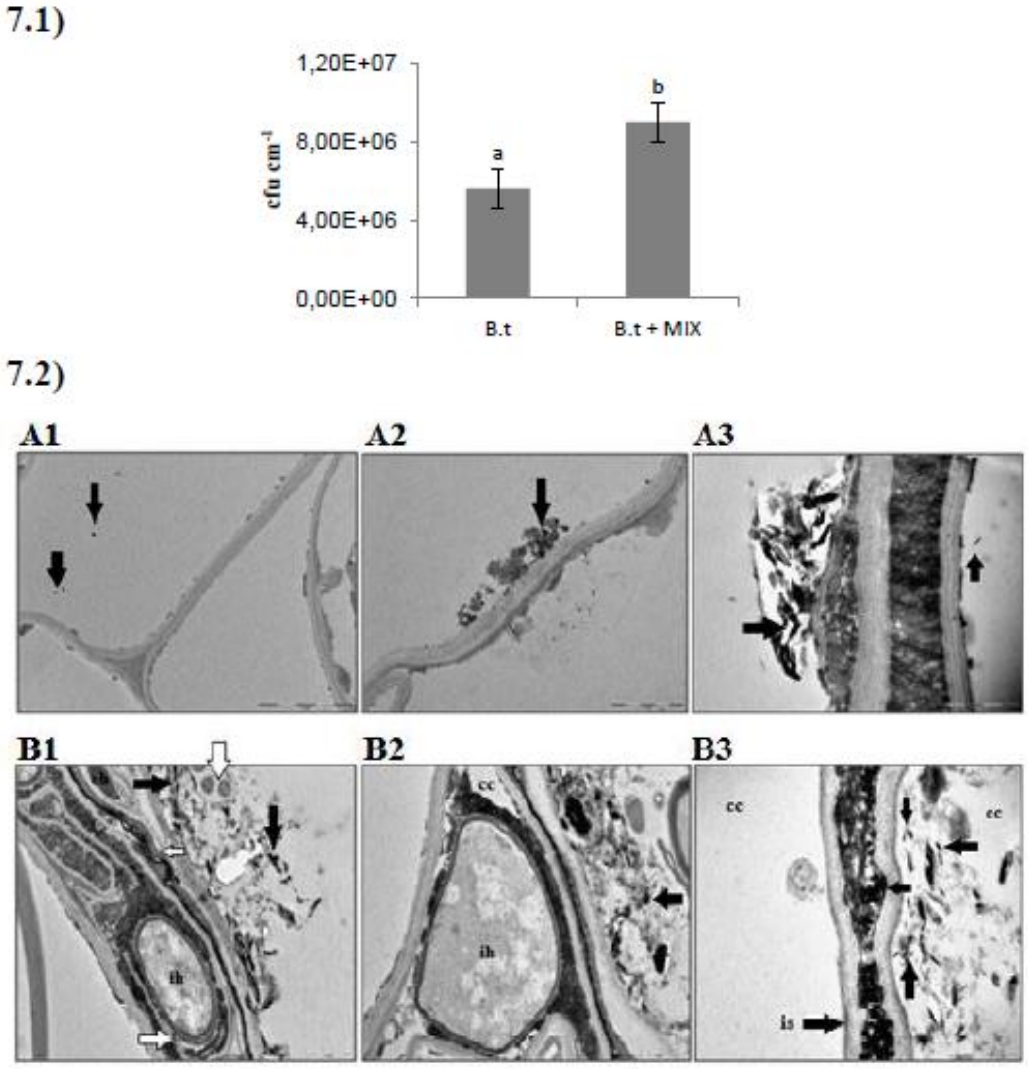


Fig. 7.1. Bacterial endophytic colonization in roots of *Lavandula dentata* inoculated with autochthonous (B.t) dually inoculates with mixture autochthonous arbuscular mycorrhizal fungi (MIX). Different letters indicate significant differences ($p \leq 0.005$) determined by Duncan's multiple-range test (n=5). **Fig. 7.2)** Transmission electron micrographs (TEM) showing intraradical hyphae (ih) penetrating cortical root cell of *Lavandula dentata* colonized by endophytic bacteria (*Bacillus thuringiensis*). **A**) Presence of *B. thuringiensis* in intracellular cell (black arrow). **B**) Bacteria endophytic colonization in intra and extracellular zones of cortex cells harbouring a functional arbuscule with well-separated hyphal structures and details of arbuscular branches (white arrow). The organelle-rich plant cytoplasm surrounding each arbuscule branch, which is enclosed by the periarbuscular membrane (white arrow). Chloroplats (ch); cell cytoplasm (cc); intercellular space (is). Bars= 5 µm (A1); 2 µm (A2; A3; B1; B2); 1 µm (B3).

3.5. Oxidative stress

Drought stress is accompanied by an increase in oxidative stress indicators (MDA). In non-mycorrhizal plants the limited and insufficient shoot of biomass made impossible to carry out these determinations. MDA content was quite similar among mycorrhizal plants. However, the coinoculation with *B. thuringiensis* highly decreased this oxidative stress. This decreasement ranged from 47% in *S. constrictum* colonized plants until 73% in *P. occultum* inoculated *L. dentata* (Fig. 8).

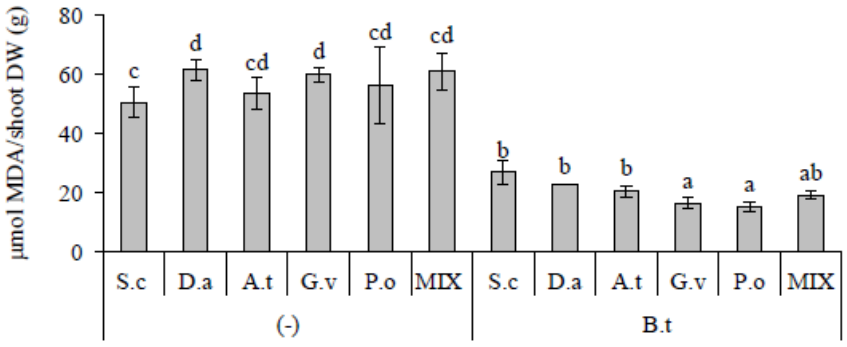


Fig. 8. Oxidative damage to lipids in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan's multiple-range test (n=3).

3.6. Antioxidant enzymes activity

Antioxidant activities were only determined in mycorrhizal plants because of the lack of material in control plants (Fig. 9). *P. occultum* colonized plants showed the lowest SOD, CAT, APX and GR and although differences with others fungi were non-significant in most of the cases. Nevertheless, it is worthy to note that the highest activities were observed in plants inoculated by *D. aunantia*, *A. trappei* or by the fungal mixture with *B. thuringiensis*. These microbial treatments were the most active in increasing SOD, CAT and APX activities in *L. dentata* (Fig 9).

The antioxidant enzymes activities of *L. dentata* varied slightly depending on the activity and the reaction of the bacteria was also different according to the antioxidant activity and the fungus involved.

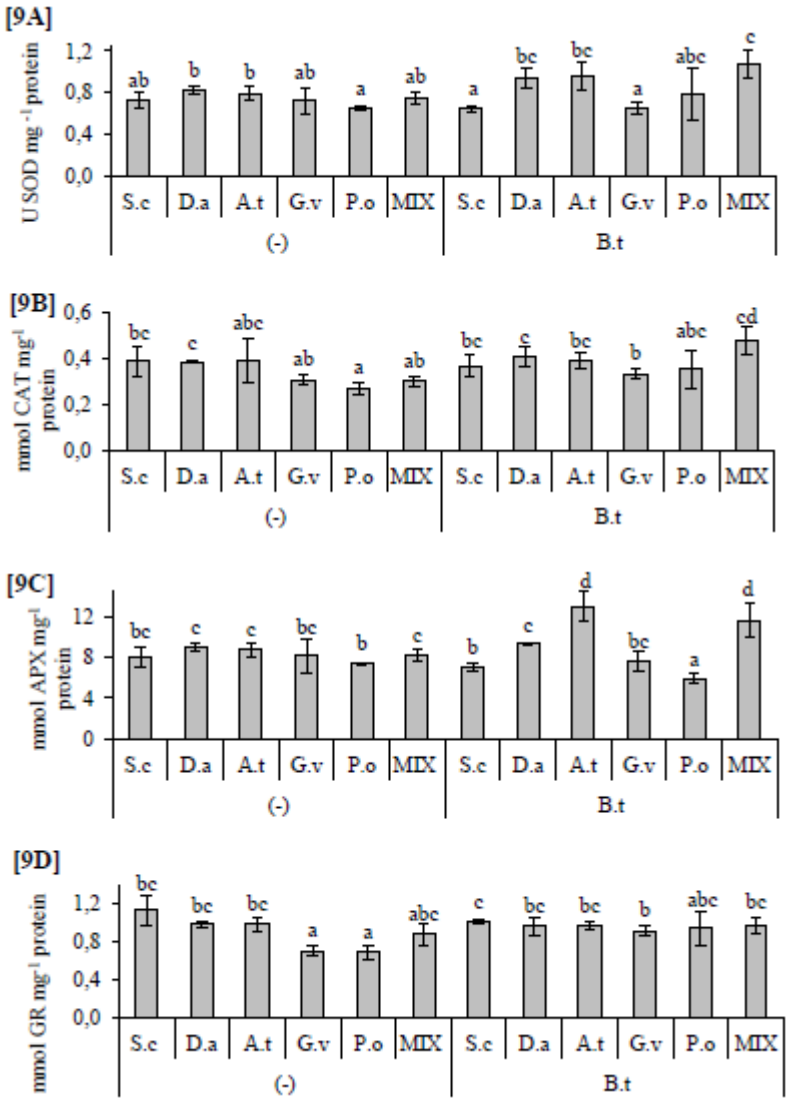


Fig. 9. Antioxidant enzymatic activities (Superoxide dismutase [9A], Catalase [9B], Ascorbate peroxidase [9C] and Glutathione reductase [9D]) in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan's multiple-range test (n=3).

4. Discussion

In this study as in others previous the applications of microbial inoculants have proved to be a useful strategy for the establishment of native shrubs species in degraded environments [13, 55].

L. dentata was selected as a representative shrubs species in Mediterranean zones and inoculated plants improved defense mechanisms to cope with drought in the nutrient deficient semiarid experimental soil here used. The inocula effectiveness were based on to enhance nutrients uptake and physiological/biochemical values [7, 13, 56]. The excellent efficiency of nutrient acquisition in mycorrhizal plants colonized by whatever inoculated fungal strain could explain the differences in some parameters evaluated in these plants. Particularly, arbuscular-mycorrhizal fungi enhanced the supply of nutrients of low mobility such as P and K, contributing to the best availability of these nutrients from soil to associated plants. The most efficient single autochthonous fungus on nutrients uptake was *A. trappei* or the fungal mixture plus *B. thuringiensis* that respectively increased, over non inoculated control, C by 251% or 231%; N by 335% or 371%; P by 444% or 288% and K 493% or 444%. The highest C content in inoculated plants is one of the main plant strategies for drought stress tolerance [57]. These two fungal treatments were also similarly effective in increasing shoot biomass production over control plants.

The highest AM colonization and mycorrhizal activity was found in dually inoculated plants as the arbuscular production shows. In fact, the impact of bacteria in increasing drought tolerance processes seem more associated to the proportion of intraradical structures as arbuscules than to the percentage of root colonized as previously reported [6, 58-59]. The effectiveness of *B. thuringiensis* in enhancing the mycorrhizal functional and metabolic status of whatever autochthonous fungal ecotype here used had a greater relevance on plant biochemical status than on growth and nutrition [60-61]. With regard mycorrhizal intensity (%M) less than 10% was observed in plants inoculated with any of the fungal isolates while the mixture of them increased this value to 25%. Nevertheless, this bacteria highly increased this %M value in plants colonized by *A. trappei* (641%), by *G. versiforme* (160%), by *P. occultum* (374%) and AMF mixture (by 39%). Nevertheless, the most important mycorrhizal value is the presence of arbuscules within root cells (A%) since they are the fungal structures involved in the bidirectional soil/plant nutrient exchange. The arbuscular production (measured as %A) was the greatest in plants colonized by mixture of fungal strains particularly in interactions with the bacteria. Marulanda et al. [62] and Vivas et al. [59] reported that the beneficial effect of bacterial inoculation was less relevant on AM-colonization than stimulating the active structures

in the AM colonization affecting the metabolic and physiological fungal activities that are considered the most important indexes of effective AM symbiosis [63] which may mitigate the depressive effects of drought.

According to results the plant roots also provided an accurate environment for this endophytic bacterium as microscopic analysis show the bacterial population was enhanced in AM colonized roots. Both microorganisms showing the root niche are also protected from the negative effect of drought allowing a better growth and functioning as results show.

The *B. thuringiensis* ability for IAA production and other beneficial compounds may play important roles in root and microbial growth under stress conditions [64]. In addition, ACC is a precursor of ethylene synthesis and ACC deaminase changes the ACC into ammonia and α-ketobutyrate. Therefore, the lowering of ethylene levels in plants exposed to drought is essential for drought tolerance [65]. This bacteria can be considered a plant-stress homeostasis-regulating rhizobacteria by the biosynthesis of these phytohormones [66]. As well, PHB as carbon storage polymers can support the survival and reproduction of microorganisms under adverse conditions and to improve their tolerance to osmotic stress.

And important result is that *B. thuringiensis* associated to whatever AM fungus alleviates oxidative stress generated in *L. dentata* plants with water deficiency as evidenced by the decreasement of MDA levels. This indicated that the microbial interaction of autochthonous microorganisms (bacteria and AM fungi) may restore the damage in membrane integrity and functionality caused in response of water limitation. Apparently, the dual inoculation of these microorganisms particularly contributed to induce plant drought tolerance.

The modulation of plant antioxidant responses by the microbial treatments applied could be considered one of the most important beneficial effects on the performance of plants grown in arid/semiarid environments. A decreasement of oxidative stress could be considered an indication of induced plant resistance to drought under semiarid conditions. These microorganisms may significantly increase growth and nutritional status under stressed conditions by affecting the antioxidant enzymatic pool and therefore lowering oxidative stress markers.

During periods of drought some metabolic pathways are uncompleted and the electrons, with a high-energy state, are transferred to molecular oxygen to form reactive oxygen species (ROS) that are toxic to molecules and causes oxidative damage to proteins, DNA and lipids [67]. The oxidative stress caused by drought leads to uncontrolled oxidation and radical chain reactions if the scavenging system of a plant does not cope well with the accumulation of ROS [68]. ROS is an early event in stress responses [69-70]. The efficient destruction of O_2^- and

H_2O_2 requires to be modulated by the action of antioxidant enzymes acting in synchrony. In order to understand the antioxidant defense mechanisms, the analyzed results show that plants colonized by the fungus *D. aunantia*, *A. trappei* or the fungal mixture were more capable in combating drought induced oxidative damage, with more effective antioxidant machinery particularly when associated with *B. thuringiensis*. When cytotoxic ROS are produced in excess by drought stress they can destroy the normal cell metabolism through oxidative damage of lipids and antioxidant activities are the form to repair and restore cellular damage. The reduced oxidative damage in inoculated plants indicates a superior capacity to adapt drought stress by developing a highly efficient defense system. These results suggest that inoculated plants have compensatory/adaptive mechanisms of defense against the oxidative stress caused by water deficiency. But, an activation of antioxidant plant apparatus may not be attributed to the regulation of only one particular enzymatic activity but rather to the complex up-regulation of several ROS-scavenging enzymes as SOD, CAT, APX and GR. The antioxidant defense of SOD is made by eliminating ROS that generated H_2O_2 that is removed by CAT, APX and GR. Thus, the role of any of these enzymes is important in protecting plants from drought-induced oxidative stress.

Responses from the individual antioxidant enzymes and their variation with respect to the colonizing fungal species may depend on the availability of plant micronutrients content. Enzymes such as CAT, APX and SOD are metalloenzymes whose activities are determined partly by the availability of the metals that they utilize. The enhancement of Mn, Cu, Fe and Zn in inoculated plants could be involved in the SOD activity observed in these plants since SOD isoenzymes (being Cu-SOD and Zn-SOD the most abundant) were determined in mycorrhizal plants [16, 71-72]. The efficiency of *B. thuringiensis* and the fungal mixture on plant mineral nutrition may be related with the improvement of biochemical metabolism observed.

The effectiveness of inoculated microorganisms is not always recorded here as an stimulation of plant biomass or nutrition but in general, it was evidenced as an improvement of biochemical values related to water status [73].

Results show that each autochthonous AM fungus and the mixture of them showed different levels of effectiveness in enhancing plant nutrition and tolerance to drought.

Differences between the inoculation with single AM fungus versus the complex fungal mixture of this community were expected. Under the severe drought conditions here used, single *A. trappei* resulted to be the most efficient fungus in increasing *L. dentata* growth and in promoting C, N, P, K, Mg and Ca uptake. These results contrasts with the initial hypothesis that the inoculation with a mixture of AM fungal isolated would be better under stress conditions

than a single isolate as was previously suggested by Caravaca et al. [74-76]. Variations among fungal isolates in their ability to produce biochemical changes related with plant drought stress tolerance (antioxidants enzymes, and MDA) were also observed. But the main effects attributed to the microbial ability to alleviate plant drought stress were maximized when the fungal mixture was associated with *B. thuringiensis*.

In conclusion, each autochthonous AM strain has a different inherent potential for improving plant performance under drought conditions. The general results emphasize that the autochthonous bacterial strain (*B. thuringiensis)* increased the mycorrhizal performance of autochthonous mycorrhizal fungi. The bacterium was more efficient at increasing the potential of the mixed fungal community assayed.

Based on results and regarding practical sight the applications of combined microbial treatments involving autochthonous PGPB and AMF seem to be the most suitable procedure to the establishment and restoration of plant cover in degraded arid soils. Nevertheless, the adequate selection of these microorganisms and their combination must be considered as crucial for developing practical methodologies in the restoration programs.

Acknowledgments

E. Armada was financed by Ministry of Science and Innovation (Spain). This work was carried out in the framework of the project reference AGL2009-12530-C02-02. We would like to thank Domingo Álvarez by the morphological identification of autochthonous mycorrhizal fungus and the Service Microscopy (EEZ-CSIC) by the technique of TEM. We thank Dominic Paul Schmid (native English teacher) for their assistance in the revision of this work. And any thanks to Olga López and Francisca González for their collaboration in this work.

References

[1] Y. Bashan, B.G. Salazar, M. Moreno, B.R. Lopez, R.G. Linderman, Restoration of eroded soil in the Sonoran Desert with native leguminous trees using plant growth-promoting microorganisms and limited amounts of compost and water, J. Environ. Manage. 102 (2012) 26-36.

[2] R. Azcón, A. Medina, R. Aroca, J.M. Ruíz-Lozano, Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast interactions, in: F.J. de Bruijn (Ed.) Molecular Microbial Ecology of the Rhizosphere, John Wiley & Sons, Hoboken, New Jersey, USA, 2013, pp. 991-1002.

[3] A. Vivas, R. Azcón, B. Biró, J.M. Barea, J.M. Ruíz-Lozano, Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the

growth of *Trifolium pratense* L. under lead toxicity, Can. J. Microbiol. 49 (2003) 577-588.
[4] J.M. Ruíz-Lozano, R. Porcel, C. Azcón, R. Aroca, Regulation by arbuscular mycorrhizae of the integrated physiological response to salinity in plants: new challenges in physiological and molecular studies, J. Exp. Bot. 63 (2012) 4033-4044.
[5] R. Azcón, J.M. Barea, Mycorrhizosphere interactions for legume improvement, in: M.S. Khan, A. Zaidi, J. Musarrat (Eds.), Microbes for Legume Improvement, Springer-Verlag, Vienna, New York, 2010, pp. 237-271.
[6] A. Marulanda, R. Azcón, J.M. Ruíz-Lozano, Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress, Physiol. Plant. 119 (2003) 526-533.
[7] A. Marulanda, R. Porcel, J.M. Barea, R. Azcón, Drought tolerance and antioxidant activities in lavender plants colonized by native drought-tolerant or drought-sensitive *Glomus* species, Microb. Ecol. 54 (2007) 543-552.
[8] S.F. Wright, A. Upadhyaya, A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi, Plant Soil 198 (1998) 97-107.
[9] M.M. Alguacil, J.A. Hernández, F. Caravaca, B. Portillo, A. Roldán, Antioxidant enzyme activities in shoots from three mycorrhizal shrub species afforested in a degraded semi-arid soil, Physiol. Plant. 118 (2003) 562-570.
[10] P. Gosling, A. Mead, M. Proctor, J.P. Hammond, G.D. Bending, Contrasting arbuscular mycorrhizal communities colonizing different host plants show a similar response to a soil phosphorus concentration gradient, New Phytol. 198 (2013) 546-556.
[11] A. Vivas, A. Vörös, B. Biró, J.M. Barea, J.M. Ruíz-Lozano, R. Azcón, Beneficial effects of indigenous Cd-tolerant and Cd-sensitive *Glomus mosseae* associated with a Cd-adapted strain of *Brevibacillus* sp. in improving plant tolerance to Cd contamination, Appl. Soil Ecol. 24 (2003) 177-186.
[12] R. Hayat, S. Ali, U. Amara, R. Khalid, I. Ahmed, Soil beneficial bacteria and their role in plant growth promotion: a review, Ann. Microbiol. 60 (2010) 579-598.
[13] E. Armada, A. Roldán, R. Azcón, Differential activity of autochthonous bacteria in controlling drought stress in native *Lavandula* and *Salvia* plants species under drought conditions in natural arid soil, Microb. Ecol. 67 (2014) 410-420.
[14] K. Apel, H. Hirt, Reactive oxygen species: Metabolism, oxidative stress, and signal transduction, Annu. Rev. Plant Biol. 55 (2004) 373-399.
[15] N. Ferrol, J. Pérez-Tienda, Coordinated nutrient exchange in arbuscular mycorrhiza, in: C. Azcón-Aguilar, J.M. Barea, S. Gianinazzi, V. Gianinazzi-Pearson (Eds.), Mycorrhizas Functional Processes and Ecological Impact, Springer-Verlag, Berlin, Heidelberg, 2009, pp. 73-87.
[16] M.M. Alguacil, F. Caravaca, R. Azcón, J. Pera, G. Díaz, A. Roldán, Improvements in soil quality and performance of mycorrhizal *Cistus albidus* L. seedlings resulting from addition of microbially treated sugar beet residue to a degraded semiarid Mediterranean soil, Soil Use Manage. 19 (2003) 277-283.
[17] E. Lumini, A. Orgiazzi, R. Borriello, P. Bonfante, V. Bianciotto, Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach, Environ. Microbiol. 12 (2010) 2165-2179.
[18] C. Hazard, P. Gosling, C.J. van der Gast, D.T. Mitchell, F.M. Doohan, G.D. Bending, The role of local environment and geographical distance in determining community composition of arbuscular mycorrhizal fungi at the landscape scale, ISME Journal 7 (2013) 498-508.
[19] H. Maherali, J.N. Klironomos, Influence of Phylogeny on fungal community assembly and ecosystem functioning, Science 316 (2007) 1746-1748.
[20] H. Rees, W. Grant, B. Jones, S. Heaphy, Diversity of Kenyan soda lake alkaliphiles assessed by molecular methods, Extremophiles 8 (2004) 63-71.

[21] J.W. Gerdemann, T.H. Nicolson, Endogone spores in cultivated soils, Nature 195 (1962) 308-309.
[22] E. Sieverding, Vesicular-arbuscular mycorrhiza management in tropical agrosystems, GTZ, Eschborn, Germany, 1991.
[23] R.E. Koske, B. Tessier, A convenient, permanent slide mounting medium, Myc. Soc. Am. Newsl. 34 (1983) 59.
[24] M. Brundrett, L. Melville, R.L. Peterson, Practical Methods in Mycorrhizal Research, Mycologue Publications, Waterloo, Ontario, Canada 1994.
[25] J.L. Spain, Arguments for diagnoses based on unaltered wall structures, Mycotaxon 38 (1990) 71-76.
[26] F. Oehl, G.A. da Silva, B.T. Goto, L.C. Maia, E. Sieverding, Glomeromycota: two new classes and a new order, Mycotaxon 116 (2011) 365-379.
[27] L.S. Bates, R.P. Waldren, I.D. Teare, Rapid determination of free proline for water-stress studies, Plant Soil 39 (1973) 205-207.
[28] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Methods Enzymol. 52 (1978) 302-310.
[29] A.D. Espindola, D.S. Gomes, A.D. Panek, E.C.A. Eleutherio, The role of glutathione in yeast dehydration tolerance, Cryobiology 47 (2003) 236-241.
[30] J.A. Ramsay, E. Berger, R. Voyer, C. Chavarie, B.A. Ramsay, Extraction of poly-3-hydroxybutyrate using chlorinated solvents, Biotechnol. Tech. 8 (1994) 589-594.
[31] I.Y. Lee, H.N. Chang, Y.H. Park, A simple method for recovery of microbial poly-beta-hydroxybutyrate by alkaline-solution treatment, J. Microbiol. Biotechnol. 5 (1995) 238-240.
[32] J.H. Law, R.A. Slepecky, Assay of poly-beta-hydroxybutyric acid, J. Bacteriol. 82 (1961) 33-36.
[33] D.M. Penrose, B.R. Glick, Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria, Physiol. Plant. 118 (2003) 10-15.
[34] M. Honma, T. Shimomura, Metabolism of 1-aminocyclopropane-1-carboxylic acid, Agric. Biol. Chem. 42 (1978) 1825-1831.
[35] M.M. Bradford, Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.
[36] S.A. Gordon, L.G. Paleg, Observations on the quantitative determination of indoleacetic acid, Physiol. Plant. 10 (1957) 39-47.
[37] I. Wöhler, Auxin-indole derivatives in soils determined by a colorimetric method and by high performance liquid chromatography, Microbiol. Res. 152 (1997) 399-405.
[38] R.I. Pikovskaya, Mobilization of phosphorus in soil in connection with vital activity of some microbial species, Mikrobiologya 17 (1948) 362-370.
[39] H. Katznelson, B. Bose, Metabolic activity and phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere, and non-rhizosphere soil, Can. J. Microbiol. 5 (1959) 79-&.
[40] M. Edi-Premono, A.M. Moawad, P.L.G. Vlek, Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere, Indonesian J. Agric. Sci. 11 (1996) 13-23.
[41] J.M. Phillips, D.S. Hayman, Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection, Trans. Br. Mycol. Soc. 55 (1970) 159-161.
[42] M. Giovannetti, B. Mosse, Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots, New Phytol. 84 (1980) 489-500.
[43] A. Trouvelot, J.C. Fardeau, C. Plenchette, S. Gianinazzi, V. Gianinazzi-Pearson, Nutritional balance and symbiotic expression in mycorrhizal wheat, Physiologie Vegetale 24 (1986) 300-300.
[44] G. Forchetti, O. Masciarelli, S. Alemano, D. Álvarez, G. Abdala, Endophytic bacteria in sunflower (*Helianthus annuus* L.): isolation, characterization, and production of

jasmonates and abscisic acid in culture medium, Appl. Microbiol. Biotechnol. 76 (2007) 1145-1152.
[45] H.A. Idris, N. Labuschaghe, L. Korsten, Screening rhizobacteria for biological control of *Fusarium* root and crown rot of sorghum in Ethiopia, Biol. Control 40 (2007) 97-106.
[46] G. Minotti, S.D. Aust, The requirement for iron (III) in the initiation of lipid-peroxidation by iron(II) and hydrogen-peroxide, J. Biol. Chem. 262 (1987) 1098-1104.
[47] B. Halliwell, J. Gutteridge, Free radicals in biology and medicine. 2nd ed, Clarendon Press, Oxford, 1989.
[48] R. Aroca, J.J. Irigoyen, M. Sánchez-Díaz, Drought enhances maize chilling tolerance. II. Photosynthetic traits and protective mechanisms against oxidative stress, Physiol. Plant. 117 (2003) 540-549.
[49] G.I. Burd, D.G. Dixon, B.R. Glick, Plant growth promoting bacteria that decreased heavy metal toxicity in plants, Can. J. Microbiol. 46 (2000) 237-245.
[50] H. Aebi, Catalase *in vitro*, Methods Enzymol. 105 (1984) 121-126.
[51] K. Amako, G.X. Chen, K. Asada, Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants, Plant Cell Physiol 35 (1994) 497-504.
[52] I. Carlberg, B. Mannervik, Glutathione reductase, Methods Enzymol. 113 (1985) 484-489.
[53] D.B. Duncan, Multiple range and multiple F tests, Biometrics 11 (1955) 1-42.
[54] J.M. Barea, J. Palenzuela, P. Cornejo, I. Sánchez-Castro, C. Navarro-Fernández, A. Lopéz-García, B. Estrada, R. Azcón, N. Ferrol, C. Azcón-Aguilar, Ecological and functional roles of mycorrhizas in semi-arid ecosystems of Southeast Spain., J. Arid Environ. 75 (2011) 1292-1301.
[55] C. Mengual, M. Schoebitz, R. Azcon, A. Roldan, Microbial inoculants and organic amendment improves plant establishment and soil rehabilitation under semiarid conditions, J. Environ. Manage. 134 (2014) 1-7.
[56] F. Caravaca, M.M. Alguacil, G. Díaz, P. Marín, A. Roldán, Growth and nitrate reductase activity in *Juniperus oxycedrus* subjected to organic amendments and inoculation with arbuscular mycorrhizae, J. Plant Nutr. Soil Sci. 169 (2006) 501-505.
[57] J. Gale, M. Zeroni, The cost to plants of different strategies of adaptation to stress and the alleviation of stress by increasing assimilation, Plant Soil 89 (1985) 57-67.
[58] A. Vivas, J.M. Barea, R. Azcón, Interactive effect of *Brevibacillus brevis* and *Glomus mosseae*, both isolated from Cd contaminated soil, on plant growth, physiological mycorrhizal fungal characteristics and soil enzymatic activities in Cd polluted soil, Environ. Pollut. 134 (2005) 257-266.
[59] A. Vivas, J.M. Barea, R. Azcón, *Brevibacillus brevis* isolated from Cadmium- or Zinc-contaminated soils improves *in vitro* spore germination and growth of *Glomus mosseae* under high Cd or Zn concentrations, Microb. Ecol. 49 (2005) 416-424.
[60] A. Vivas, A. Marulanda, M. Gómez, J.M. Barea, R. Azcón, Physiological characteristics (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by *Bacillus thuringiensis* inoculation under two phosphorus levels, Soil Biol. Biochem. 35 (2003) 987-996.
[61] A. Vivas, A. Marulanda, J.M. Ruíz-Lozano, J.M. Barea, R. Azcón, Influence of a *Bacillus* sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress, Mycorrhiza 13 (2003) 249-256.
[62] A. Marulanda, J.M. Barea, R. Azcón, Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments. Mechanisms related to bacterial effectiveness, J. Plant Growth Regul. 28 (2009) 115-124.
[63] J.P. Guillemin, M.O. Orozco, V. Gianinazzi-Pearson, S. Gianinazzi, Influence of phosphate fertilization on fungal alkaline-phosphatase and succinate-dehydrogenase activities in arbuscular mycorrhiza of soybean and pineapple, Agric. Ecosyst. Environ. 53 (1995) 63-69.

[64] J. Dobra, V. Motyka, P. Dobrev, J. Malbeck, I.T. Prasil, D. Haisel, A. Gaudinova, M. Havlova, J. Gubis, R. Vankova, Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content, J. Plant Physiol. 167 (2010) 1360-1370.
[65] B.R. Glick, Bacterial ACC deaminase and the alleviation of plant stress, Adv. Appl. Microbiol. 56 (2004) 291-312.
[66] F. Cassán, J. Vanderleyden, S. Spaepen, Physiological and agronomical aspects of phytohormone production by model plant-growth-promoting rhizobacteria (PGPR) belonging to the Genus *Azospirillum*, J. Plant Growth Regul. 33 (2014) 440-459.
[67] G. Miller, N. Suzuki, S. Ciftci-Yilmaz, R. Mittler, Reactive oxygen species homeostasis and signalling during drought and salinity stresses, Plant Cell Environ. 33 (2010) 453-467.
[68] A. Gunes, D.J. Pilbeam, A. Inal, Effect of arsenic-phosphorus interaction on arsenic-induced oxidative stress in chickpea plants, Plant Soil 314 (2009) 211-220.
[69] L.P. Singh, S.S. Gill, N. Tuteja, Unraveling the role of fungal symbionts in plant abiotic stress tolerance, Plant Signal. Behav. 6 (2011) 175-191.
[70] R.K. Singh, O. Dai, G. Nimasow, Effect of arbuscular mycorrhizal (AM) inoculation on growth of Chili plant in organic manure amended soil, Afr. J. Microbiol. Res. 5 (2011) 5004-5012.
[71] J. Arines, M. Quintela, A. Vilariño, J.M. Palma, Protein-patterns and superoxide-dismutase activity in non mycorrhizal and arbuscular mycorrhizal *Pisum sativum* L plants, Plant Soil 166 (1994) 37-45.
[72] J. Arines, A. Vilariño, J.M. Palma, Involvement of the superoxide-dismutase enzyme in the mycorrhization process, Agric. Sci. Finland 3 (1994) 303-306.
[73] J. Kohler, J.A. Hernández, F. Caravaca, A. Roldán, Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress, Environ. Exp. Bot. 65 (2009) 245-252.
[74] F. Caravaca, M.M. Alguacil, R. Azcón, J. Parlade, P. Torres, A. Roldán, Establishment of two ectomycorrhizal shrub species in a semiarid site after in situ amendment with sugar beet, rock phosphate, and *Aspergillus niger*, Microb. Ecol. 49 (2005) 73-82.
[75] F. Caravaca, M.M. Alguacil, J.M. Barea, A. Roldán, Survival of inocula and native AM fungi species associated with shrubs in a degraded Mediterranean ecosystem, Soil Biol. Biochem. 37 (2005) 227-233.
[76] F. Caravaca, M.M. Alguacil, G. Díaz, P. Marín, A. Roldán, Nutrient acquisition and nitrate reductase activity of mycorrhizal *Retama sphaerocarpa* L. seedlings afforested in an amended semiarid soil under two water regimes, Soil Use Manage. 21 (2005) 10-16.

CHAPTER 3.2

Rhizosphere bacterial communities physiological profiles (functional structure) of *Lavandula dentata*, after the inoculation with autochthonous drought tolerant mycorrhizal fungus, and *Bacillus thuringiensis*

E. Armada, R. Azcón, A. Roldán and A. Probanza

1. Introduction

Rhizosphere is an essential part of soil surrounding plant roots that have a strong influence on plant health and ecosystem function. There are a big amount of different types of microorganisms in the rhizosphere interacting with the other soil microbes and with plant roots. The properties of soil rhizosphere make it a unique and active area where the activity and interactions of rhizosphere microorganisms can influence soil conditions and hence plant growth and microorganisms' activities (Zaidi et al., 2003).

Arbuscular mycorrhizal fungi (AMF) are among the most important and influential rhizosphere microbes, significantly affecting the plant growth as far as the other soil microorganisms. The soil environment around plant roots and AM hyphae (where AM fungi and soil bacteria are interactive) is called “mycorrhizosphere” (Linderman, 1988). There are different sorts of bacteria in the soil, interacting with AM fungi, particularly in the rhizosphere. In most cases, the interactions are synergistic (Smith and Read, 2008).

AM fungi develop symbiotic associations with most terrestrial plants (Feddermann et al., 2010), and usually their symbiosis with the host plant is not host-specific. In this symbiosis, the host plant provides to the fungi hydrocarbons, in exchange for nutrients to the plant, especially phosphorous (Hause et al., 2002). The level of specificity is determined by the two symbionts and the ecological parameters. However, some combination of AMF-host plant may result in a more efficient symbiosis under different conditions including stress (Daei et al., 2009; Feddermann et al., 2010).

The plant physiology is affected by AMF symbiosis, and consequently root exudates are also influenced with the production of some new metabolites, resulting in the alteration of microbial populations in the rhizosphere, relative to a nonmycorrhizal plant (Johansson et al., 2004). Also, AM hyphae are able to produce C products as a source of energy for soil microbes

in the mycorrhizosphere, although at a little amount, relative to the rhizosphere (Andrade et al., 1997).

On the other hand, soil microbes are able to produce chemicals that increase the amounts of root exudates resulting in the activation of AM hyphae and thus higher root colonization (Barea et al., 2005b). Additionally, soil microbes produce plant hormones, which can influence AMF establishment as well as spore and hyphal growth (Barea et al., 2005b).

The interactions between AM fungi and soil bacteria are influenced by many different factors. One of them is the ability of bacteria in attachment to the AM hyphae, which differs among different bacteria and is also affected by the hyphal physiological stage (Artursson et al., 2006). Other related parameters are the AMF species and bacterial strains, the plant species, the exudates, and climate features (Sanon et al., 2009).

It has been well stated which bacterial genera are more frequent in the rhizosphere than mycorrhizosphere, indicating that root exudates can be more beneficial to the bacteria than hyphal products (Artursson et al., 2006). There are different examples of positive associations between different bacterial strains including *Bacillus, Paenibacillus, Pseudomonas* and *Rhizobia* with different AMF species including *Glomus calrum, G. intraradices, G. mosseae, and G. versiforme*. These positive effects include the germination and growth of fungi and spores, thus the root colonization of the host plant by AM fungi, the phosphate solubilization, and the pathogens suppression (Artursson et al., 2006).

In the relationship between AM fungi and bacteria at the interface soil-root-hyphae, it is very interesting to consider the role of Plant Growth Promoting Rhizobacteria (PGPR). It has been established that PGPR are able to increase AM fungal development by affecting root colonization as well as by enhancing plant N and P uptake (Marulanda-Aguirre et al., 2008; Marulanda et al., 2009; Richardson et al., 2009). Although it has been indicated that some of the PGPRs are able to perfectly colonize plants roots, data related to the change of inoculation intensity of AM hyphae by PGPR is limited (Hartmann et al., 2009). Investigations carried out since late years of last century, have stated that PGPR can have some stimulatory effects on AM growth (Azcón, 2014; Azcón and Barea, 2010; Linderman, 1997).This indicates that the co-inoculation of AMF and specific PGPR can enhance the activity of AMF during the symbiosis with the host plant (Artursson et al., 2006). Accordingly, it is important to determine the bacterial population with the highest physiological activities, in association with AM fungi. This indicates the bacterial strains that are more efficient, particularly when interacting with AM fungi, and can likely make the use of effective co-inoculation (Barea et al., 2002; Barea et al., 2005a; Franzini et al., 2010; Hartmann et al., 2009).

Obviously, to keep in function this relationship it is necessary a notorious energy support. Thus, the mycorrhizal fungi offer particularly suitable interfaces for heterotrophic soil bacteria thanks to their capacity to continuously shunt part of the photosynthate of the plant partner to its hyphal network. The conditions in the microhabitats that are thus created in the rhizosphere and mycorrhizosphere also imply that different interactive strategies will be required in the bacteria in order to gain a benefit from the newly emerged interfaces (Azcón, 2014; Duponnois et al., 2005; Frey-Klett et al., 2007; Johansson et al., 2004). In that way, has been stated that in a simple (but hostile) substrate such as purified sand, fungal hyphae were found to significantly increase the numbers of associated bacteria (de Boer et al., 2005; Mansfeld-Giese et al., 2002). That finding suggests the acquisition by the bacterial community of nutrients from the fungus (Leveau and Preston, 2008). The fungal exudates may have a qualitative and/or a quantitative impact on the bacterial community. Thus, Filion et al. (1999) founded that the growth of *Pseudomonas chlororaphis* was stimulated in the presence of an extract of a culture of the *G. intraradices*. More recently, Van Hees et al. (2006) reported oxalate and ferricrocin as the main compounds identified in the exudates of the ectomycorrhizal fungus *Hebeloma crustuliniforme* in symbiosis with *Pinus sylvestris.* They also identified malonate and acetate in fungal exudate, albeit in lower amounts than oxalate. Oxalate and acetate were also found, next to carbohydrates and peptides, in material released by the ectomycorrhizal (EM) fungus *Suilus bovines* (Sun et al., 1999). Their analysis showed inositol, xylitol, mannitol and ribose among the main sugars and polyols. Oxalate or oxalic acid may feed bacteria as there are several bacteria reported as oxalotrophs (Sahin, 2003), whereas mannitol-specialized bacteria have also been found, in this case in association with *S. bovinus* growing in soil (Timonen et al., 1998). Glycine, glutamic acid and aspartic acid were the main amino acids present in fungal exudates examined by Sun et al. (1999). But that sort of compounds are not exclusives of EM fungi Toljander et al. (2007) reported formiate, acetate, β glucose and glycogen, along with di- and oligosaccharides and some polymeric compounds, in the exudates of *Glomus sp.* MUCL 43205.

Thus, it can spouse that mycorrizosphere-adapted bacteria utilize a range of specific compounds that are made available by fungal hosts in the vicinity of their hyphae. The mycelial exudates were shown to not only increase bacterial growth and vitality but also influence the bacterial community compositions (Toljander et al., 2007). This suggested that some bacteria preferentially utilized different compounds available in exudates.

But the system considered includes also the plant roots. It is well known that roots exude substantial amounts of low molecular weight organic compounds such as amino acids, sugars and organic acids, resulting in increased microbial populations and activity (Hertenberger et al., 2002; Rovira, 1979). Given the high numbers of fast-growing bacteria, especially Gram

negative strains, in the rhizosphere it has been widely assumed that easily degradable plant exudates are almost exclusively degraded by bacteria (Jones, 1998; Rovira, 1979). Therefore, most publications on dynamics of microorganisms in the rhizosphere deal only with shifts in bacterial communities, but not attend to other parameters such as plant physiological parameters, or AM fungi status as regard root colonization.

Organic acids may also be selective for fungus-associated bacterial strains. External hyphae of various EM fungal species release organic acids, in particular oxalic acid, which are thought to be involved in nutrient withdrawal from solid mineral substrates (Dutton and Evans, 1996; Landeweert et al., 2001). Oxalic acid is also released by white-rot fungi during degradation of lignocellulose (Dutton and Evans, 1996). Although the capacity to degrade the highly oxidized oxalic acid or its salt, oxalate, is not common for soil bacteria, oxalotrophy is taxonomically widespread (Sahin, 2003). The genus *Methylobacterium* is frequently found as the dominant oxalotrophic bacterium on and near oxalate-exuding plants, while the genera *Alcaligenes, Pseudomonas, Ralstonia* and *Streptomyces* have been mentioned as important oxalotrophic bacteria in soils (Sahin, 2003). *Streptomyces* has been found in association with an oxalate-producing Douglas fir mycorrhiza (Knutson et al., 1980). No additional information appears to be available on associations between oxalotrophic bacteria and fungi, an area worthy to further examine in the future.

There is still little information available on the quality and quantity of carbon compounds exuded into the environment by fungi (Johansson et al., 2004; Rangel-Castro et al., 2002) (Finlay and Söderström, 1992). More evidence is clearly required to support the idea that there is a substrate-mediated increase and selection of bacteria by fungi, especially in light of a study finding that, when thymidine incorporation was used to quantify in situ bacterial activity (Olsson et al., 1996), there was no support for the hypothesis that EM mycelia can stimulate bacterial growth via carbon exudation. Furthermore, AM fungi, it has been suggested that the effect of exudates on bacterial populations may be qualitative (relating to species and strain composition) rather than quantitative (Andrade et al., 1997).

The purpose of this study was to examine the influence of five individual strains of autochthonous AM fungi or with the mixture of them and their interactions with *Bacillus thuringiensis* under drought conditions on the functional structure physiological profiles) of rhizosphere bacterial communities of *Lavandula dentata.* The effects of inoculants also were evaluated on plant biometrical parameters (shoot and root dry weight), C plant content, total AMF colonization and percentage of AMF colonization, as far as stable aggregates at root environment. The hypothesis of this work is that the different inoculants could modify plant

growing parameters and change the exudates composition of *Lavandula* roots (and fungi mycelia) under the drought conditions assayed and thus, alter the patterns of use of different C and N sources by bacterial communities of the rhizosphere/mycorrhizosphere.

2. Materials and methods

2.1. Biossay in *Lavandula dentata* plants under greenhouse conditions

2.1.1. Experimental design. Inoculants, soil and plants

The experimental work was based on design with two factors: isolates of arbuscular mycorrhizal fungi species (five different AMF species: *Septoglomus constrictum* EEZ 198; *Diversispora aunantia* EEZ 199; *Archaeospora trappei* EEZ 200; *Glomus versiforme* EEZ 201; *Paraglomus occultum* EEZ 202 and the mixture or consortium of these AMF) and a PGPR strain of *Bacillus thuringiensis* (Bt) (Armada et al., 2014). Thus, there were used 14 treatments: single AMF inoculations (5), Bt plus each AMF (5), a mix of all AMF (1), a mix of all AMF plus Bt (1), single Bt inoculation (1) and an uninoculated control (1).

The soil used in this experiment were taken from the natural park "Vicente Blanes" (Molina de Segura, Murcia, Spain), located at 38° 12' N, 1º 13' W; altitude 393 m. This soil in the experimental area is a Typic Torriorthent (SSS, 2006). The main features of this soil were: low soil organic matter content, and silty-clay texture; pH 8.90, P $1.36 \cdot 10^{-3}$ g kg^{-1} (Olsen Test), organic carbon 0.94%, total nitrogen 0.22%, electrical conductivity of 1.55 dS m^{-1}. The substrate used in this assay consisted in using the previously mentioned Mediterranean soil (sterilized and sieved by 5mm), and mixed with sterile sand to the ratio of (5:2, (v/v)). Substrate was put into pots with a capacity of 0.5 kg. The plant species used in this study was *Lavandula dentata.* The plants were grown for six months at the conditions described below.

Plant inoculations were carried out as follows: 1 mL of pure bacterial culture (10^7 cfu mL^{-1}) grown in nutrient broth for 48 h at 28 ºC, was applied to the appropriate pots at sowing time just below to plant seedlings. A second inoculation was done 15 days later (also using 1 mL, 10^7 cfu mL^{-1}) by applying the bacterial suspension on the soil around the plant on the soil. Corresponding different treatments, 5 g of different isolates of arbuscular mycorrhizal fungi species and consortium of AMF were applied to each one of the appropriate pots at sowing time just below to the seeds. Five replicates were carried out by treatment.

2.1.2. Plant growth conditions

The plants were grown for six months under green-house conditions (temperature ranging from 15 to 21 ºC; 16/8 light/dark photoperiod, and a relative humidity of 50-70%). A photosynthetic photon flux density of 400-700 µmol m^{-2} s^{-1} was applied as supplementary light. Plants were grown along the experiment under drought conditions by keeping soil water capacity to 50% each day after water application but water level decreased along day to nearly 20% water capacity to the next water application.

2.2. Plant determinations

2.2.1. Plant biomass production, nutrients acquisition and rhizosphere processing

After six months of plant growth, plants were harvested (five replicates per each treatment) shoots and roots was weighed and dried for 48 h at 75 ºC to obtain dry weights.

C content in shoot (mg $plant^{-1}$) carried out by the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

Prior to root drying and to obtain rhizosphere samples of the different treatments, fine plant roots were gently shaken, and the soil attached to the roots was considered rhizosphere soil. For each treatment three replicates were used.

2.2.2. Percentage and total of arbuscular mycorrhizal fungal (AMF) root colonization

Arbuscular mycorrhizal fungal (AMF) colonization was assessed after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

2.3. Rhizosphere determinations

2.3.1. Percentage of stable aggregates (SA)

The percentage of stable aggregates (SA) was determined by the method of Benito et al. (1986) modified by Roldán et al., (1994). A portion of soil sample was dry-sieved between 1 and 2 mm. Aliquots of 4 g of the sieved soil were placed on 7.5 cm diameter 250 µm mesh size sieves and subjected to an artificial rainfall of 150 mL. This rainfall was performed by arranging a cylindric reservoir of 6 cm diameter at 1 m height, with the bottom bored by 11 holes, in each one a pipette tip was assembled. The water drops fall on the soil sample distributed in a practically uniform way, with an energy of 270 J m^{-2}. The soil remaining on the sieve was dried

(105 ºC, 5 h) and weighed (P_1). The air-dried soil was moistened and again sieved (250 µm) with the assistance of a small glass stick to disrupt the remaining aggregates. The particles on the sieve coarse sand, small stones and some non-humified organic matter-were dried (105 ºC, 5 h) and weighed (P_2).

The percentage of water-stable aggregates with regard to the total aggregates was calculated by $(P_1 – P_2) \times 100/(4 – P_2)$.

2.3.2. Community physiological profiles

The functional structures of rhizosphere bacterial communities were characterized using Biolog ECO® micro-plates (BIOLOG Inc., Hayward, CA). Carbon/Nitrogen substrates and their biochemical groups of it are summarized in table 1. One gram of rhizosphere soil (wet weight) were homogenized in 10 mL distilled water with an Omnimixer at 16,000 rpm for 1 min. After centrifugation of the soil suspension (750 X g, 10 min), the supernatant was filtered through glass wool. Suspensions were diluted 100-fold in sterile water and were sown in Biolog ECO micro-plates. Plates were incubated in the dark at 25 ºC. Absorbance values at 595 nm were measured each 45 h until 100 h. Absorbance values of the wells were blanked against the control well (corrected o.d.). All negative values were set to zero. The average well colour development (AWCD) was calculated as the mean of the 31 blanked absorbance values. Then, the absorbance value of each well was divided by the AWCD in order to minimize the influence of inoculum density differences between plates (Baudoin et al., 2001). These operations were done with the three replicates of the Biolog ECO micro- plates, and the average AWCD of the three replicates was calculated. At 90 h, substrate Shannon´s diversity index (H), substrate evenness (E´) and substrate richness (S) were calculated, following Zak et al (1994), using the corrected o.d. of each substrate of the Biolog ECO micro- plates.

Table 1. Assignment to biochemical groups of Carbon/Nitrogen substrates in Biolog ECO microplates.

CARBOHYDRATES	ORGANIC ACIDS	AMINO ACIDS	AMINES	OTHERS
D-Cellobiose *(D-Cell)*	Pyruvic Acid Methyl Ester *(Pyru Ac Meth Est)*	L-Arginine *(L-Arg)*	Phenylethyl-amine *(Phen-amine)*	Tween 80 *(Tw 80)*
α-D-Lactose *(a-D-Lact)*	D-Glucosaminic Acid *(D-Glu Ac)*	L-Asparagine *(L-Asp)*	Putrescine *(Putrs)*	Tween 40 *(Tw 40)*
β-Methyl-D-Glucoside *(b-Metl-D-Glu)*	D-Galacturonic Acid *(D-Galac Ac)*	L-Phenylalanine *(L-Phe)*	N-Acetyl-D-Glucosamine *(N-Acet-D-Glu)*	
D-Xylose *(D-Xyl)*	γ-Hydroxybutyric Acid *(g-Hydro but Ac)*	L-Serine *(L-Ser)*		
i-Erythritol *(i-Eryt)*	Itaconic Acid *(Itac Ac)*	L-Threonine *(L-Thr)*		
D-Mannitol *(D-Man)*	α-Ketobutyric Acid *(a-Ketob Ac)*	Glycyl-L-Glutamic Acid *(Gly-L-Glu Ac)*		
Glycogen (*Glyco)*	D-Malic Acid *(D-Mal Ac)*			
Glucose-1-1Phosphate *(Glu-1-1Pho)*	4-Hydroxybenzoic Acid *(4-Hydr benz Ac)*			
D.L-α-Glycerol Phosphate *(D.L-a-Gly Pho)*	2-Hydroxybenzoic Acid *(2-Hydr benz Ac)*			
D-Galactonic Acid γ-Lactone *(D-Galac Ac)*				
α-Cyclodextrin *(a-cyclodex)*				

Acronyms of each one (used in figure 1B) are indicated within parentheses.

2.4. Statistical analyses

The data [biometrical plant parameters, Shannon´s diversity index (H), substrate evenness (E') and substrate richness (S)] were analyzed using SPSS 21 ™ software package for Windows ™, were subjected to one-way general linear model ANOVA (analysis of variance) was used to determine the effect of each treatment. The Duncan's multiple-range test (Duncan, 1955) was used for *post hoc* analysis to determine differences between means. Differences were considered significant at $p \leq 0.05$. Percentage values were ARC-sine-transformed before statistical analysis. Also the Biolog ECO results were subjected to a Principal Component Analysis (PCA) (Harman, 1967)to elucidate the major variation patterns, using SYSTAT ™ v 5.0 (Systat Inc.) for Windows™ program.

3. Results

3.1. Plant biomass production and fungal colonization

L. dentata inoculated plants showed both higher root and shoot biomass under the conditions assayed than non-inoculated plants. As regard AMF colonization of *L. dentata* the single mycorrhizal species (or mixture) inoculated and the particular interaction of each AM fungus with *B. thuringiensis* showed significantly growth difference, but non-significant growth impact of *B. thuringiensis* was detected in most of the cases (Table 2).

Results showed that the most efficient mycorrhizal fungus increasing shoot biomass were *A. trappei* and *P. occultum* which yield 610 and 510 mg plant^{-1} shoot dry weight respectively, while control non-inoculated plants yielded 160 mg plant^{-1}. The AM fungi in addition with *B. thuringiensis* showed significant improvement on *L. dentata* growth, the effectiveness of these fungi as show *B. thuringiensis* associated with *S. constrictum* (Sc+Bt), *G. versiforme* (Gv+Bt) or the mixture of fungi (AMFmix+Bt). The opposite effect was observed when the *B. thuringiensis* was associated to *D. aunantia* (Da+Bt) or *A. trappei* (At+Bt).

Differences in root development between inoculated and non-inoculated plants were also found, and in some cases the combination of AMF with *B. thuringiensis* decreased this value. The double inoculation of *G. versiforme* plus *B. thuringiensis* (Gv+Bt) resulted the most effective treatments increasing 412.5% the root weight in comparison with non-inoculated plants (Table 2).

Root biomass showed higher increase than shoot biomass by AM fungi under the assayed conditions. *D. aunantia* or the mix of fungi resulted in a root/shoot ratio of 1.65 and 1.49 respectively. Nevertheless, inoculation of *B. thuringiensis* decreased this ratio to 0.81 when coinoculated to *D. aunantia* and to 1.16 in coinoculation with the mix of AM fungi (Table 2).

Carbon content of treated plants ranged from 164.11 mg plant^{-1} (*D. aunantia* + *B. thuringiensis*) to 265.16 mg plant^{-1} (mix of AM fungi plus *B. thuringiensis*), being in all cases higher and statistically different than un-inoculated control (Table 2).

Table 2. Biometrical parameters (shoot and root weight), carbon content in shoot, mycorrhizal colonization (% and total), and stable aggregates (SA) in *Lavandula dentata*, inoculated with five arbuscular mycorrhizal fungi species: *Septoglomus constrictum* (Sc); *Diversispora aunantia* (Da); *Archaeospora trappei* (At); *Glomus versiforme* (Gv); *Paraglomus occultum* (Po) and mixture of these AMF (AMF mix) with or without inoculation of *Bacillus thuringiensis* (Bt).

Treatments	**Shoot weight (g)**	**Root weight (g)**	**Root/Shoot ratio**	**C content (mg plant^{-1})**	**AMF (%)**	**Total AMF colonization**	**SA (%)**
Uninoculated control	0.16 a	0.16a	1.14bc	80.0 a	0.0	0	22.80 a
Sc	0.39 c	0.61d	1.56de	176.9 b	22.6 b	454.3 c	64.41 bc
Da	0.43 d	0.72e	1.65e	198.5 c	13.2 a	285.1 b	64.11 b
At	0.61 e	0.74e	1.23c	281.8 d	28.4 c	687.3 de	62.42 b
Gv	0.44 cd	0.73e	1.66e	200.5 bc	27.7 c	704.8 e	63.97 b
Po	0.51 de	0.50c	0.98b	226.8 cd	23.8 bc	442.7 c	63.05 b
AMF mix	0.48 d	0.72de	1.49d	218.6 c	45.6 e	1121.7 f	65.06 b
Bt	0.24 b	0.24b	0.99b	90.0 a	0.0	0	24.70 a
Sc+Bt	0.44 d	0.64d	1.44d	209.5 c	28.0 c	579.6 d	69.72 c
Da+Bt	0.36 c	0.29b	0.81a	164.1 b	16.2 a	175.5 a	66.25 bc
At+Bt	0.53 de	0.66d	1.25c	242.7 cd	33.6 d	725.7 e	68.02 bc
Gv+Bt	0.56 e	0.82f	1.47d	257.9 d	17.6 a	461.1 c	71.18 c
Po+Bt	0.48 de	0.47c	0.98b	220.3 cd	29.4 c	464.5 c	63.94 b
AMF mix+Bt	0.59 e	0.69de	1.16bc	265.2 d	52.2 e	1164.1 f	72.00 c

Within each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

As regards to percentage of AM fungi colonized roots was highly variable depending on the fungal inoculum involved and ranged from 13.2% (*D. aunantia*) to 52.2% (mix of fungi plus *B. thuringiensis*). The bacteria inoculation seems to increase (non-significantly) the symbiotic development and the highest AMF colonization was obtained in plants inoculated with the fungal mixture plus *B. thuringiensis* (Table 2). Total AMF colonization showed almost the same trend than prior described parameter. Highest levels of total AMF colonization were found at AMF mix (alone and plus *B. thuringiensis*) followed by *G. versiforme* and *A. trappei* + *B. thuringiensis* treatments. Lower values were found at both treatments with *D. aunantia.*

Finally, the results of stable aggregates on plants treated roots (Table 2), showed that the treatments AMF mix, *G. versiforme* and *S. constrictum* (in all cases plus *B. thuringiensis*) reached the highest levels. This parameter values were at the other treatments around 63%, and in the cases of un-inoculated control and *B. thuringiensis* treatment were *circa* 23%. As regards controls (un-inoculated and *B. thuringiensis* treatments) in all parameters considered (Table 2) showed lower values (statistically significant) than other treatments and no differences between both controls were found.

3.2. Metabolic profiles, functional structure of rhizosphere bacterial communities and bacterial use of diferent N and C sources

The PCA carried out with Biolog ECO AWCD at 100 h of incubation, in order to elucidate the major variation patterns between treatments are showed in Figure 1A. This PCA explains (considering the two first components) a 42.6% of total variance. This percentage is usually on this range for PCAs carried out with Biolog ECO data. The ordination of samples show differences between the treatments, and a main trend found is a close location (Euclidean distances) between each AMF inoculant and it mirror with *B. thuringiensis*, except for *A. trappei* and *A. trappei* plus *B. thuringiensis* treatments, in which were found higher Euclidean distance than in others couples. Considering the projection of the different samples on the first component of PCA (which explains a 28.7 % of variance), and the overlapping of error bars, it is possible to elucidate three groups (Fig. 1A). The first one located at lower values of axis composed by *S. constrictum, S. constrictum* plus *B. thuringiensis,* the un-inoculated control and *B. thuringiensis* treatment*;* the second group is located at higher values of axis and is formed by *D. aunantia*, *A. trappei* plus *B. thuringiensis*, *G. versiforme* and *G. versiforme* plus *B. thuringiensis*, as far as the AMF mix sample; and finally a third group located at intermediate axis values, which contains the rest of treatments.

As regard the internal variation of the different treatments was also homogenous (as show error bars in both axis), except for *D. aunantia* plus *B. thuringiensis* and for mix of fungi treatment.

Figure 1B shows the projection loading factors of PCA of metabolic profiles on the two first components. These loading factors (the different C and N sources of Biolog Eco micro-plates) appear spread at the Euclidean plan, but are not homogenously spread. Carbohydrates are located mainly at higher values of Principal Component 2, amino acids are located at intermediate positions of that component, and quite spread following the first component, while organic acids are located at lower values of Principal Component 2. It seems that the other two groups (amines and other substances) have not a big effect on samples distribution at PCA. Considering this, it is possible to elucidate which groups of compounds (loading factors) are related with the position of samples at PCA (Fig. 1A). Thus, carbohydrates consumption seem to have more weight on rhizosphere samples *S. constrictum* and *P. occultum* (both alone, and plus *B. thuringiensis*), whereas organic acids predominate in AMF mix and *D. aunantia* (both alone, and plus *B. thuringiensis*). An effect of amino acids consumption can be also observed: the *A. trappei* is located at PCA (Fig. 1A) at a position where (Fig. 1B) is located the centroid of all amino acids; *G. versiforme* and AMF mix with *B. thuringiensis* also seem related with amino acids, specifically L-asparagine and L-arginine; the samples of AMF mix (alone, and plus *B. thuringiensis*) are closely located at PCA to previously cited treatments (Fig. 1A) and also seem to be linked to those amino acids and L-serine. Finally, location of the un-inoculated control and *B. thuring*iensis treatment are related with carbohydrates and organic acids loading factors.

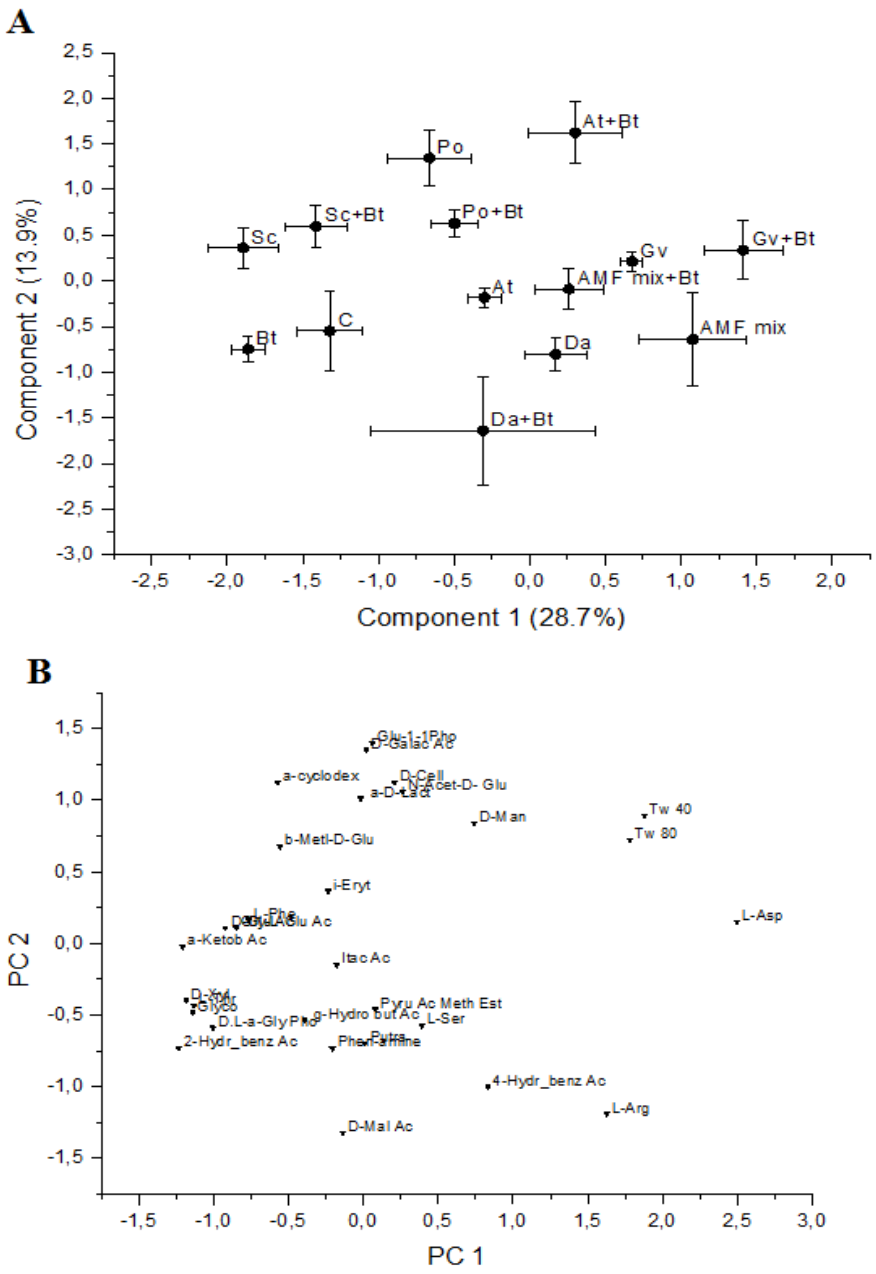


Figure 1. Principal Component Analysis (PCA) analyzing the effect of different inoculants [five arbuscular mycorrhizal fungi species: *Septoglomus constrictum* (Sc); *Diversispora aunantia* (Da); *Archaeospora trappei* (At); *Glomus versiforme* (Gv); *Paraglomus occultum* (Po) and mixture of these AMF (AMF mix) with or without inoculation of *Bacillus thuringiensis* (Bt); C: untreated control] on the rhizosphere metabolic profiles *Lavandula dentata* (Biolog ECO). Values within parentheses in (1A) are the variation explained by the principal component. Bars indicate standard errors (n=3). Projection of loading factors of PCA of metabolic profiles (1B); acronyms of loading factors are indicated at table 1.

Functional diversity can be examined from a variety of perspectives, parallel concepts analogous to those of *taxonomic diversity*. The simplest aspect is substrate richness (S), the number of different substrates that are used by the microbial community. Substrate diversity (H), which encompasses both substrate richness and substrate evenness, may be quantified according to information theory (Magurran, 1988). Similarly, substrate evenness (E´) measures the equitability of activities across all utilized substrates. In figure 2 are shown the results for those parameters on the different treatments. Highest values of diversity (Fig. 2A) were found on *S. constrictum* (alone, and plus *B. thuringiensis*), and *D. aunantia* (alone, and plus *B. thuringiensis*) treatments, reaching 4.45 to 4.55 bits. The lower values were found at *A. trappei* and AMF mix treatments (values around 4.15 bits), and both lower statistically than un-inoculated control diversity value. A general trend found was that for all fungi, the addition in inoculation of the PGPR decreases the functional diversity (not always significantly) except for the AMF mix and *A. trappei* where the co-inoculation with *B. thuringiensis* increased the diversity. Results on substrate richness (S) are shown at figure 2B. Despite the statistical differences between the treatments, the total number of substrates used ranged from 27 in the case of *A. trappei* to 31 in *P. occultum* and un-inoculated control. Finally, figure 2C show substrate evenness (E´), i.e. the equitability of activities across all utilized substrates. The patterns found in this parameter were very similar than those found at diversity: highest values of evenness were found on *S. constrictum* and *D. aunantia*, and lower values appeared at *A. trappei* and AMF mix treatments.

Measures of substrate richness, evenness and diversity do not provide information about the types of substrates that are utilized by each bacterial community. Two treatments could exhibit identical substrate richness, evenness or diversity but still catabolize totally different substrates. To incorporate information about the types of substrates that are used as either the presence or absence of specific catabolic abilities or the activity levels exhibited for particular substrates, figure 3 show a radar diagram with the percentage on AWCD of the biochemical groups of carbon/nitrogen substrates in Biolog ECO micro-plates, in the different treatments. Interestingly, the trends detected considering the loading factors of PCA, are confirm with results show in figure 3. The rhizosphere samples with *S. constrictum* and *P. occultum* (alone, and plus *B. thuringiensis*) used carbohydrates mainly (over 30%), whereas organic acids are the main source used by microbial communities in AMF mix (alone, and plus *B. thuringiensis*) and *D. aunantia* (alone, and plus *B. thuringiensis*) treatments. In the late cases, carbohydrates and amino acids were the second C and N sources used, reaching 23% and 29% respectively. In AMF mix (also plus *B. thuringiensis*) also were found a notorious consumption of amino acids representing between 25% to 30% of total C and N sources used.

Microbial communities of *A. trappei* treated plants showed the highest use of amino acids reaching almost a 40% of total sources used. *G. versiforme* and AMF mix with *B. thuringiensis* rizosphere communities also used amino acids and organic acids (in both cases around 30%).

The controls (un-treated and *B. thuringiensis*) were not different between them, showing *circa* 30% of consumption of carbohydrates, 30% organic acids and 20% of amino acids.

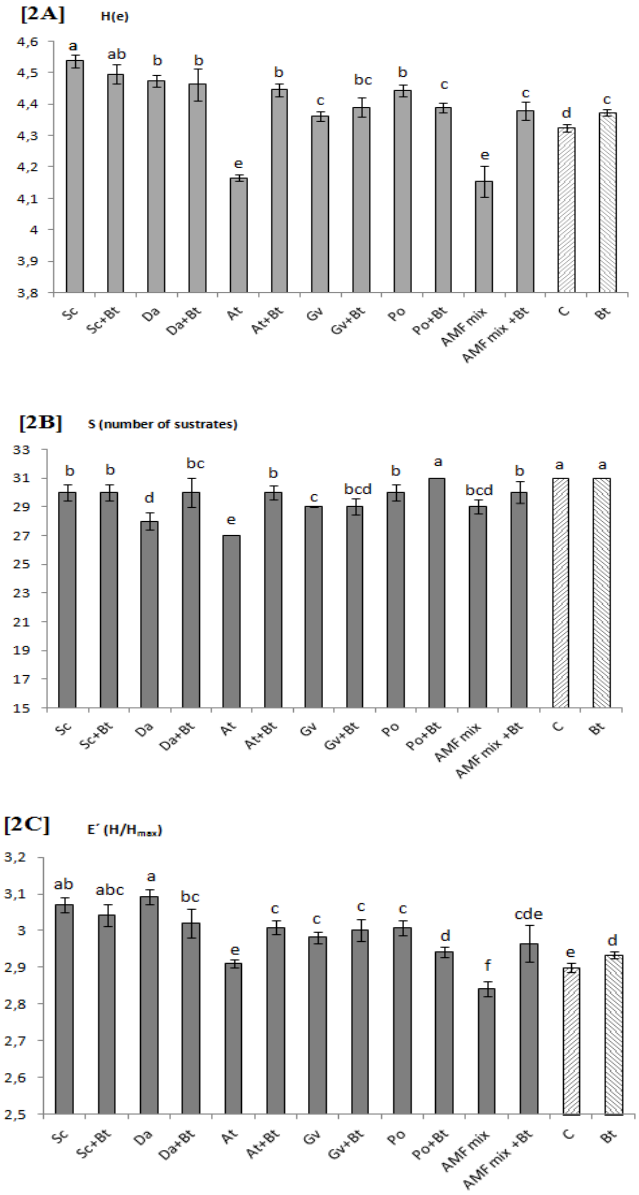


Figure 2. Substrate diversity index (H) [2A], number of substrates used (S) [2B], and substrate evenness (E´) [2C] resulting from Biolog ECO analysis in *Lavandula dentata* rhizosphere inoculated with five arbuscular mycorrhizal fungi species: *Septoglomus constrictum* (Sc); *Diversispora aunantia* (Da); *Archaeospora trappei* (At); *Glomus versiforme* (Gv); *Paraglomus occultum* (Po) and mixture of consortium of these AMF (AMF mix) with or without inoculation

of *Bacillus thuringiensis* (Bt); C: untreated control. Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan'smultiple-range test (n=3).

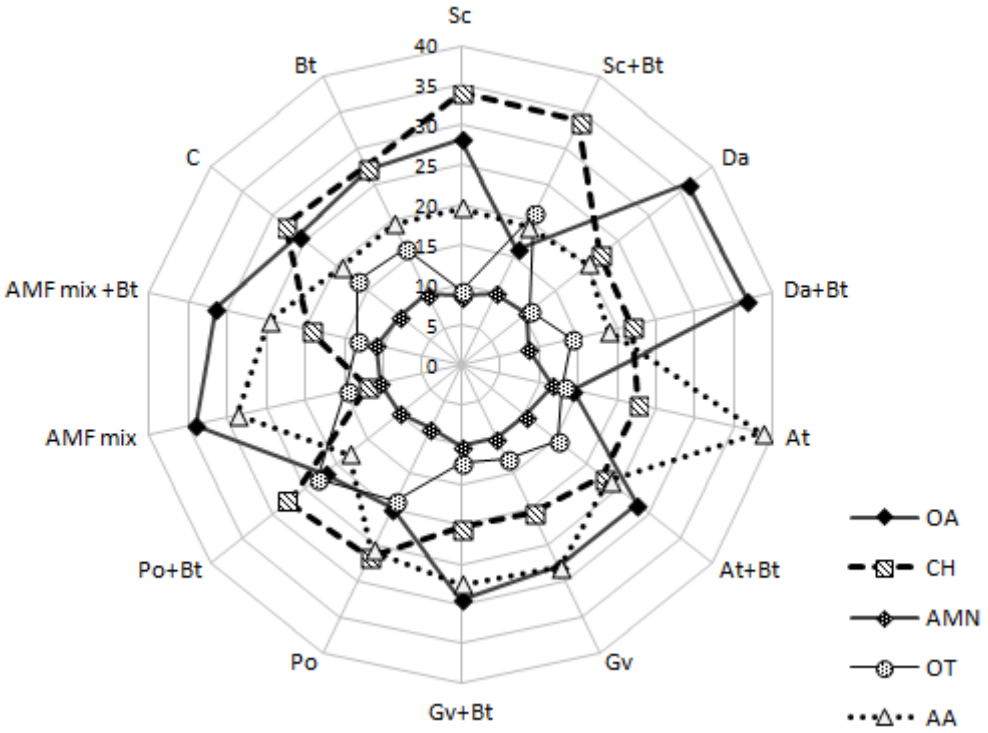


Figure 3. Radar diagram showing the percentage on AWCD of the biochemical groups of Carbon/Nitrogen substrates in Biolog ECO micro-plates, in the different treatments [five arbuscular mycorrhizal fungi species: *Septoglomus constrictum* (Sc); *Diversispora aunantia* (Da); *Archaeospora trappei* (At); *Glomus versiforme* (Gv); *Paraglomus occultum* (Po) and mixture of these AMF (AMF mix) with or without inoculation of *Bacillus thuringiensis* (Bt); C: untreated control]. Biochemical groups (see table 1): CH: carbohydrates; OA: organic acids; AA: Amino acids; AMN: amines; OT: others.

4. Discussion

From the first objective of is study on *L. dentata* (effect of AMF inoculants plus *B. thuringiensis* on plant growing under drought conditions), it had found that the most efficient fungal treatments on C uptake were *A. trappei* or the AMF mix plus *B. thuringiensis.* Additionally these two treatments were similarly effectives in increasing shoot biomass over un-inoculated control. A highest C content in inoculated plants has been reported (Gale and Zeroni, 1985) as one of the mechanisms showed by plants for optimize it survival under drought stress. It seem that the fungal C demand did not results a cost of the symbiosis, as show the highest AMF colonization found in *B. thuringiensis* co-inoculated plants.

The production of IAA-like compounds by *B. thuringiensis* strain used in the present work (Armada et al., 2014) plays an important role in root growth and bacterial stress response (Dobra et al., 2010). ACC (1-aminocyclopropane-1-carboxylic acid) is a precursor of ethylene synthesis, phytohormone which is related with plant stress processes. ACC deaminase (also produced by the *B. thuringiensis* strain here used) degrade the ACC into ammonia and α-ketobutyrate that produce a decrease of ethylene levels in plants exposed to drought, and is essential to it survival (Glick, 2004). This bacteria can be considered as a plant-stress homeostasis-regulating rhizobacteria by the biosynthesis of these phytohormones (Cassán et al., 2014).

Each one of the AM fungus assayed, as far as the mixture of them showed different effectiveness in enhancing plant nutrition (in terms of C content) under the assayed drought conditions. As regards to increases in shoot biomass, the association with *B. thuringiensis* was positive in interaction with *S. constrictum*, *G. versiformis* and the AMF mix, negative with *D. aunantia* and non-relevant with *A. trappei* and *P. occultum*. On the other hand, considering the controls (un-inoculated and *B. thuringiensis*), any effect on biometrical parameters were found on treated plants with bacteria. There suggested that the *B. thuringiensis* bacterial activity increased, at least in part, the beneficial effect of autochthonous mycorrhizal fungi on the nutrition of *L. dentata*, as other authors has recently pointed out (Nadeem et al., 2014), but not by bacteria itself. Previous works (Medina et al., 2003) demonstrated that PGPR plus AMF inoculation shows a synergic effect on plant growth promotion, bigger than the single inoculation with bacteria. Differences found between the inoculation with single AM fungus versus the mix were expected, considering the results of Caravaca et al. (2005a; 2005b; 2005c), which founded that a mixture of AMF isolated would be better under stress conditions than a single isolate. Under the conditions assayed, single *A. trappei* resulted the most efficient fungus in increasing *L. dentata* growth. *A. trappei* was also the most efficient fungus in promoting C uptake but this variation cannot be attributed to the percentage of mycorrhizal colonization reached.

The impact of *B. thuringiensis* assayed in this work in drought tolerance processes, could be associated more than to the root colonization to the proportion of active fungal structures as previously reported (Marulanda et al., 2003; 2005a; Vivas et al., 2005b). This effectiveness of *B. thuringiensis* in enhancing the mycorrhizal functional and metabolic status of whatever AMF here used had a greater relevance on plant physiological status than on growth and nutrition (Vivas et al., 2003a; 2003b).

Regarding AMF colonization, the *B. thuringiensis* bacteria increased this value ranging from 15% (AMF mixture) to 24% (*S. constrictum*). Only in one case the double inoculation (*G. versiforme* + *B. thuringiensis*) decreased the AMF colonization (by 37%). Marulanda et al., (2009) reported that the beneficial effect of bacterial inoculation was less relevant on AM-colonization than on the metabolic and physiological fungal activities that are considered as indexes of effective AM symbiosis (Guillemin et al., 1995). Bearden y Petersen (2000) pointed out the conspicuous role of extraradical mycelium in soil aggregates formation and stabilization. Root development was highly increased in mycorrhizal plants and the exudates (both from roots and hyphae) which can be used by the surrounding bacteria as carbon and energy sources. In fact, root and associated hyphae may form a network that enmeshed fine particles of soil into aggregates, that develops niches (functional opportunities) and microhabitats (spatial opportunities) for bacterial communities (de Boer et al., 2005). In this way, is well known the role of glomalin, a sustance produced by AM fungi as concrete that stabilize aggregates (Wright and Anderson, 2000). The aggregate stabilization is a crucial mechanisms not only affecting available water (Rillig and Mummey, 2006) and reducing the detrimental effects of drought as here we observed, but also in terms of chances (via niche-i.e. use of exudates-, via habitat) for bacterial communities. In this way, in the present study, the effect of *B. thuringiensis* was relevant in stabilizing soil structure of nearly whatever mycorrhizal plants even in those colonized by *G.versiforme* + *B. thuringiensis* that showed lower AM-colonization. We have not determined the develop of extraradical hyphae in co-inoculated plants, but probably may cause the enmeshing soil particles and stabilizing aggregates as previously reported Miller et al. (2010). In the present work, we try to link the previous described aspects with the function of bacterial communities that surround roots and possible extraradical mycelium.

Our results, show clear differences between the *L. dentata* rizosphere samples treated with AMF and PGPR inoculants, in terms of C and N sources utilization using Biolog ECO (Figs. 1A and 1B). This is not the first time that Biolog method is use to relate the utilization of fungus/root exudates released by mycosphere or rhizosphere vs. bacterial communities or populations. Warmink et al. (2009) analyzed the potential use of fungus-related compounds from mycosphere by pseudomonads using the Biolog assay. These authors correlated the utilization of potentially fungal-released compounds as carbon sources with bacterial habitat, and posited that preferential resource utilization might be a key selective mechanism in the fungal niche. This can be extended to the rizosphere niche, which includes the hyphosphere (mycorrhizosphere).

As was pointed out previously, the ordination of samples (Fig. 1A) show differences between the treatments, and an interesting trend found is a close location between each AMF

inoculant and it *mirror* with *B. thuringiensis*, except for *A. trappei* and *A. trappei* plus *B. thuringiensis* treatments. This effect found indicates that the main factor driving the patterns of substrate utilization (under the assayed conditions) is the AMF species, more than the presence of *B. thuringiensis*. This could be related with two processes (which are not mutually excluding, and are probably simultaneous): i) AMF change root exudation patterns, changing (as effect of infection) the plant physiology (Johansson et al., 2004) which alter also root exudates composition (Hage-Ahmed et al., 2013) or ii) exudates shape gen expression of bacterial communities that uses the substrates present in substances released by roots and/or mycelia. Fan et al., (2012) studied the effect of maize root exudates on a *Bacillus amyloliquofaciens* PGPR strain transcriptome. These authors found that the majority of genes with altered expression were up-regulated by exudates, and several groups of these gens strongly induced were involved in metabolic pathways related with nutrient utilization. More recently, Chaparro et al. (2013) combining metatranscriptomics a metabolomics found in *Arabidopsis thaliana* a strong correlation between root exudates (which change in the different stages of plant development) and the expression of microbial gens involved in metabolism of specific compounds. Obviously, we cannot rule out an effect on plant physiology of the PGPR, as far as effect on the function and composition (i.e. structure) of the microbial communities.

The previous described and discussed results suggest that the different AMF inoculants induce different exudation patterns at *L. dentata* under assayed conditions (probably coupled with the induction of changes in gens expression of bacterial communities) that could be related with the substrates present in rhizosphere media and used by bacterial communities. Also, the effect of co-inoculation with the PGPR *B. thuringiensis*, shape this patterns of exudation and thus, the utilization of different substrates change. Indeed, a general trend found was that for all fungi, the addition in inoculation of the PGPR decreases the functional diversity (not always significantly) except for the AMF mix, and *A. trappei* where the co-inoculation with *B. thuringiensis* increased the diversity of substrate utilization (Fig. 2A). Considering the main biochemical groups which are used in the different treatments by rhizosphere bacterial communities, it seem to be strongly related to their capacities to use particular carbonaceous compounds, as evidenced using principal components analyses of Biolog Eco-based substrate utilization tests (Fig. 1B).

This is also consistent with the non multivariated-mediated analysis of use of substrates, represented in figure 3. This radar diagram (with the percentage on AWCD of the biochemical groups of carbon/nitrogen substrates in Biolog ECO micro-plates, in the different treatments) show that *S. constrictum* and *S. constrictum* plus *B. thuringiensis*, as far as *P. occultum* and *P. occultum* plus *B. thuringiensis* rhizosphere samples used carbohydrates mainly (over 30%),

whereas organic acids are the main source used by microbial communities in AMF mix (alone, and plus *B. thuringiensis*) and *D. aunantia* (alone, and plus *B. thuringiensis*) treatments. In the late cases, carbohydrates and amino acids were the second C and N sources used, reaching 23% and 29% respectively. In AMF mix (also plus B. *thuringiensis*) also were found a notorious consumption of amino acids representing between 25% to 30% of total C and N sources used. Microbial communities of *A. trappei* treated plants showed the highest use of amino acids reaching almost a 40% of total sources used. *G. versiforme* and AMF mix with *B. thuringiensis* rizosphere communities also used amino acids and organic acids (in both cases around 30%). In this way is interesting to consider not only the inoculants as the unique driver on exudation by plants of different substances especially under the drought conditions carried out in this experiment. Song et al. (2012) analyzing root exudates of maize plants under water stress founded that osmotic stress increases the organic acid (malic, lactic, acetic citric, succinic and maleic acid) releasing from roots.

On the other hand, we cannot rule out other processes affecting the root exudates composition, and consequently substrate utilization at the different rhizosphere samples. As Scheffknecht et al. (2006) pointed out the release of exudates by mycorrhized plants also depends on the degree of symbiosis. In present experiment we find different levels of root infection (table 2) being the lower 13.2% (*D. aunantia*) and the higher 52.2% (mix of fungi plus *B. thuringiensis*). Nevertheless considering the position of these treatments on PCA (Fig. 1A), as far as use of C and N sources in these samples (Fig. 3) we cannot establish a clear relation between colonization (in that two extreme cases) and exudation (in qualitative terms which are analyzed in this work), since there are slight differences: in rhizosphere of *D. aunantia* treatment are used mainly organic acids (35%) followed by carbohydrates and amino acids (22% and 18% respectively) whereas in mix of fungi plus *B. thuringiensis* treatment are used organic acids (33%) followed by amino acids and carbohydrates (25% and 20% respectively); if we consider the diversity values (Fig. 2A), neither are statistical differences.

It is noticeable the absence of differences between un-inoculated control and *B. thuringiensis* treated plants rhizospheres (Fig. 1A, Fig. 3) in terms of substrate consuming. These differences could be related with the survival ability of the bacteria, after six months of the inoculation. Some previous works where *Bacillus* PGPR strains were inoculated on *Pinus pinea*, *Quercus ilex* and *Alnus glutinosa* demonstrate that the survival of inoculant was lower than 90 days (Domenech et al., 2004; Probanza et al., 2002; Ramos et al., 2003). Thus, the bacterial communities in both cases (un-inoculated control and *B. thuringiensis* treated) could be almost equal, and consequently the use of C and N sources should be equivalent.

Nevertheless it seems that remains slight differences, since values of diversity and evenness are statistically different (Figs. 2A and 2C).

It is well known that low molecular-weight compounds, including amino acids, organic acids, sugars, phenolics and various secondary metabolites, constitute the largest portion of the root exudates (Vivanco et al., 2002). There are previous studies that have stated quantitative and qualitative differences in mycorrhizal root exudates, including differences in amino acids (Harrier and Watson, 2004), flavonoids (Steinkellner et al., 2007), phenolic compounds (McArthur and Knowles, 1992), sugars and organic acids (Lioussanne et al., 2008; 2009; Sood, 2003). Our results indicate that there is an altered exudation, reflected in consumption of different compounds by rizosphere bacterial communities, due to mycorrhizal symbiosis.

In the present work, we have studied the bacterial communities under the functional point of view, and we have demonstrated that different inoculants (more the AMF species than the PGPR) lead to changes in the functional ecology of rhizobacterial communities. The linking between the two nodes (sort of inoculant and functional ecology) seems to be mycorrhizal root exudates. Many studies (Harrier and Watson, 2004; Marschner and Baumann, 2003; Wamberg et al., 2003) have stated that structural ecology of rhizosphere communities (species composition) also are related to mycorrhizal root exudates, that lead alterations in the microbial rhizosphere populations of facultative anaerobic bacteria, fluorescent pseudomonads, *Streptomyces* species and chitinase-producing actinomycetes. Thus, seems to be linked changes on bacterial communities composition, with modification of functional capabilities (i.e. use of substrates) due to root exudation, as we found in the present work.

As regard functional diversity-related parameters (substrate richness, evenness and diversity) at the different treatments, also were found different patterns. Results on substrate richness (Fig. 2B) were not especially informative about differences between the treatments, since the total number of substrates used ranged from 27 to 31 (out of 31). In the case of diversity and evenness (Figs. 2A and 2C) more clear differences were found between the treatments. This slight treatment effect found on richness could be related with the relative small number of substrates tested. In other works were a bigger number of substrates were tested, richness seem to be a parameter more powerful to discriminate communities (Sofo et al., 2014). Highest values of diversity (Fig. 2A) were found on *S. constrictum* (alone, and plus *B. thuringiensis*) and *D. aunantia* (alone, and plus *B. thuringiensis*) treatments. There are few works in literature where *functional* diversity is studied at rhizosphere of mycorrhized plants. On the contrary there are a plethora of *structural* (i.e. taxonomical) diversity studies on roots of mycorrhized plants (Marschner and Baumann, 2003). In this way, Warmink et al. (2009) carried

out an elegant study where they found clear effects of the mycorrhizosphere of diverse fungi both on the bacterial community and on the *Pseudomonas* populations in comparison with those in the corresponding bulk soil using PCR-DGGE analyses. These authors found that structural diversities of the *Pseudomonas* populations increased dramatically in most of the mycospheres tested, which contrasted with a decrease of the diversity of the total bacterial communities in these habitats. Our results (Fig. 2A) all treatments showed statistically higher functional diversity than un-inoculated control, except the AMF mix and the plants inoculated with *A. trappei*. Thus, could be a selection, via exudates of certain bacterial groups. Warmink et al. (2009) named the bacteria adapted/selected to the mycospheres as *fungiphiles.* As in the present work, these authors evidenced (using PCA of Biolog-based substrate utilization tests) a strong relationship between bacteria capacities to use particular carbonaceous (i.e. l-arabinose, l-leucine, m-inositol, m-arabitol, d-mannitol and d-trehalose), were linked to compounds known to occur in exudates.

Interestingly, the lower diversity values were found at *A. trappei* and AMF mix treatments, and both showed lower statistically than un-inoculated control diversity value (Fig. 1A). As previously were pointed out in the present work, *A. trappei* resulted the most efficient fungus in increasing *L. dentata* growth. In addition, *A. trappei* was also the most efficient fungus in promoting C content on plants uptake, but this variation cannot be attributed to the percentage of mycorrhizal colonization reached which were relatively high (Table 2). Results of *A. trappei* inoculated rhizospheres on what substrates are mainly used (Fig. 3) indicate differences respect other treatments: this one have amino acids as main source used by bacterial communities. This fact could be related with a lower exudation of carbon compound, which could explain the bigger C content as far as *L. dentata* growth. Also interestingly when this AMF is inoculated with PGPR strain the function of rhizosphere communities change, which is precisely the contrary found in the other inoculants: the main factor driving the patterns of substrate utilization (under the assayed conditions) is the AMF species more than the presence on inoculation of *B. thuringiensis*, except for *A. trappei*.

In conclusion from the present work, the inoculation of *L. dentata* under drought conditions with the five species of AMF or their mixture (with or without PGPR), increased plant growth parameters and C content but the interaction with bacteria did not always improve the effect of single inoculation. We have found also modifications of the rhizosphere microbial community function, in terms of substrate utilization. Clear differences were found between inoculated plants with different AMF species, which were not substantially altered by co-inoculation with PGPR. This effect indicates that the main factor shaping the patterns of substrate utilization is AMF species, driving plant patterns of exudation. An understanding of

these effects as part of ecosystem processes is essential for obtaining the maximum benefit for plant growth and health in the context of semi-arid zones, where drought effects affect deeply plant fitness.

Acknowledgments

E. Armada was financed by Ministry of Science and Innovation (Spain). This work was carried out in the framework of the project reference AGL2009-12530-C02-02.

References

Andrade G, Mihara KL, Linderman RG, Bethlenfalvay GJ. Bacteria from rhizosphere and hyphosphere soils of different arbuscular-mycorrhizal fungi. Plant Soil 1997;192:71-79.

Armada E, Roldán A, Azcón R. Differential activity of autochthonous bacteria in controlling drought stress in native *Lavandula* and *Salvia* plants species under drought conditions in natural arid soil. Microb Ecol 2014;67:410-420.

Artursson V, Finlay DR, Jansson KJ. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ Microbiol 2006;8:1-10.

Azcón R. Mycorrhizosphere: The Role of PGPR. In: Morte A, Varma A, editors. Root Engineering. 40: Springer Berlin Heidelberg, 2014. p. 107-143.

Azcón R, Barea JM. Mycorrhizosphere interactions for legume improvement. In: Khan MS, Zaidi A, Musarrat J, editors. Microbes for Legume Improvement. Vienna, New York: Springer-Verlag, 2010. p. 237-271.

Barea JM, Azcón R, Azcón-Aguilar C. Mycorrhizosphere interactions to improve plant fitness and soil quality. Anton Leeuw Int J G 2002;81:343-351.

Barea JM, Azcón R, Azcón-Aguilar C. Interactions between mycorrhizal fungi and bacteria to improve plant nutrient cycling and soil structure. In: Buscot F, Varma A, editors. Microorganisms in Soils: Roles in Genesis and Functions. Berlin, Heidelbert: Springer-Verlag, 2005a. p. 195-212.

Barea JM, Pozo MJ, Azcón R, Azcón-Aguilar C. Microbial co-operation in the rhizosphere. J Exp Bot 2005b;56:1761-1778.

Baudoin E, Benizri E, Guckert A. Metabolic fingerprint of microbial communities from distinct maize rhizosphere compartments. Eur J Soil Biol 2001;37:85-93.

Bearden BN, Petersen L. Influence of arbuscular mycorrhizal fungi on soil structure and aggregate stability of a vertisol. Plant Soil 2000;218:173-183.

Benito E, Gómez-Ulla A, Díaz-Fierros F. Descripción de un simulador de lluvia para estudios de erosibilidad del suelo y estabilidad de los agregados al agua. Anal Edafol Agrobiol 1986;45:1115-1126.

Caravaca F, Alguacil MM, Azcón R, Parlade J, Torres P, Roldán A. Establishment of two ectomycorrhizal shrub species in a semiarid site after in situ amendment with sugar beet, rock phosphate, and *Aspergillus niger*. Microb Ecol 2005a;49:73-82.

Caravaca F, Alguacil MM, Barea JM, Roldán A. Survival of inocula and native AM fungi species associated with shrubs in a degraded Mediterranean ecosystem. Soil Biol Biochem 2005b;37:227-233.

Caravaca F, Alguacil MM, Díaz G, Marín P, Roldán A. Nutrient acquisition and nitrate reductase activity of mycorrhizal *Retama sphaerocarpa* L. seedlings afforested in an amended semiarid soil under two water regimes. Soil Use Manage 2005c;21:10-16.

Cassán F, Vanderleyden J, Spaepen S. Physiological and agronomical aspects of phytohormone production by model plant-growth-promoting rhizobacteria (PGPR) belonging to the Genus *Azospirillum*. J Plant Growth Regul 2014;33:440-459.

Chaparro JM, Badri DV, Bakker MG, Sugiyama A, Manter DK, Vivanco JM. Root exudation of phytochemicals in *Arabidopsis* follows specific patterns that are developmentally programmed and correlate with soil microbial functions. Plos One 2013;8.

Daei G, Ardekani MR, Rejali F, Teimuri S, Miransari M. Alleviation of salinity stress on wheat yield, yield components, and nutrient uptake using arbuscular mycorrhizal fungi under field conditions. J Plant Physiol 2009;166:617-625.

de Boer W, Folman LB, Summerbell RC, Boddy L. Living in a fungal world: impact of fungi on soil bacterial niche development. Fems Microbiol Rev 2005;29:795-811.

Dobra J, Motyka V, Dobrev P, Malbeck J, Prasil IT, Haisel D, Gaudinova A, Havlova M, Gubis J, Vankova R. Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content. J Plant Physiol 2010;167:1360-1370.

Domenech J, Ramos-Solano B, Probanza A, Lucas-García JA, Colón JJ, Gutierrez-Mañero FJ. *Bacillus* spp. and *Pisolithus tinctorius* effects on *Quercus ilex* ssp ballota: a study on tree growth, rhizosphere community structure and mycorrhizal infection. For Ecol Manage 2004;194:293-303.

Duncan DB. Multiple range and multiple F tests. Biometrics 1955;11:1-42.

Duponnois R, Colombet A, Hien V, Thioulouse J. The mycorrhizal fungus *Glomus intraradices* and rock phosphate amendment influence plant growth and microbial activity in the rhizosphere of *Acacia holosericea*. Soil Biol Biochem 2005;37:1460-1468.

Dutton MV, Evans CS. Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. Can J Microbiol 1996;42:881-895.

Fan B, Carvalhais LC, Becker A, Fedoseyenko D, von Wiren N, Borriss R. Transcriptomic profiling of *Bacillus amyloliquefaciens* FZB42 in response to maize root exudates. BMC Microbiol 2012;12.

Feddermann N, Finlay R, Boller T, Elfstrand M. Functional diversity in arbuscular mycorrhiza - the role of gene expression, phosphorous nutrition and symbiotic efficiency. Fungal Ecol 2010;3:1-8.

Filion M, St-Arnaud M, Fortin JA. Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. New Phytol 1999;141:525-533.

Finlay R, Söderström B. Mycorrhiza and carbon flow to the soil. In: Allen MJ, editor. Mycorrhizal Functioning. New York: Chapman & Hall, 1992. p. 134-160.

Franzini VI, Azcón R, Latanze-Mendes F, Aroca R. Interaction between *Glomus* species and *Rhizobium* strains affect the nutritional physiology of drought stressed legume hosts. J Plant Physiol 2010;167:614-619.

Frey-Klett P, Garbaye J, Tarkka M. The mycorrhiza helper bacteria revisited. New Phytol 2007;176:22-36.

Giovannetti M, Mosse B. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol 1980;84:489-500.

Glick BR. Bacterial ACC deaminase and the alleviation of plant stress. Adv Appl Microbiol 2004;56:291-312.

Guillemin JP, Orozco MO, Gianinazzi-Pearson V, Gianinazzi S. Influence of phosphate fertilization on fungal alkaline-phosphatase and succinate-dehydrogenase activities in arbuscular mycorrhiza of soybean and pineapple. Agric Ecosyst Environ 1995;53:63-69.

Hage-Ahmed K, Moyses A, Voglgruber A, Hadacek F, Steinkellner S. Alterations in root exudation of intercropped tomato mediated by the arbuscular mycorrhizal fungus *Glomus mosseae* and the soilborne pathogen *Fusarium oxysporum* f. sp lycopersici. J Phytopathol 2013;161:763-773.

Harman H. Modern factor analysis. Chicago: University of Chicago, 1967.

Harrier LA, Watson CA. The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. Pest Manag Sci 2004;60:149-157.

Hartmann A, Schmid M, van Tuinen D, Berg G. Plant-driven selection of microbes. Plant Soil 2009;321:235-257.

Hause B, Maier W, Miersch O, Kramell R, Strack D. Induction of jasmonate biosynthesis in arbuscular mycorrhizal barley roots. Plant Physiol 2002;130:1213-1220.

Hertenberger G, Zampach P, Bachmann G. Plant species affect the concentration of free sugars and free amino acids in different types of soil. J Plant Nutr Soil Sci 2002;165:557-565.

Johansson JF, Paul LR, Finlay RD. Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microb Ecol 2004;48:1-13.

Jones DL. Organic acids in the rhizosphere - a critical review. Plant Soil 1998;205:25-44.

Knutson DM, Hutchins AS, Cromack K. The association of calcium-oxalate utilizing streptomyces with conifer ectomycorrhizae. Anton Leeuw J Microbiol 1980;46:611-619.

Landeweert R, Hoffland E, Finlay RD, Kuyper TW, van Breemen N. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. Trends Ecol Evol 2001;16:248-254.

Leveau JHJ, Preston GM. Bacterial mycophagy: definition and diagnosis of a unique bacterial-fungal interaction. New Phytol 2008;177:859-876.

Linderman RG. Mycorrhizal interactions with the rhizosphere microflora. The mycorrhizosphere effects. Phytopathol 1988;78:366-371.

Linderman RG. Vesicular-Arbuscular Mycorrhizal (VAM) Fungi. In: Carroll GC, Tudzynski P, editors. Plant Relationships Part B. 5B: Springer Berlin Heidelberg, 1997. p. 117-128.

Lioussanne L, Jolicoeur M, St-Arnaud M. Mycorrhizal colonization with *Glomus intraradices* and development stage of transformed tomato roots significantly modify the chemotactic response of zoospores of the pathogen *Phytophthora nicotianae*. Soil Biol Biochem 2008;40:2217-2224.

Lioussanne L, Jolicoeur M, St-Arnaud M. Role of the modification in root exudation induced by arbuscular mycorrhizal colonization on the intraradical growth of *Phytophthora nicotianae* in tomato. Mycorrhiza 2009;19:443-448.

Magurran A. Ecological Diversity and Its Measurement. Princeton: Princeton University Press, 1988.

Mansfeld-Giese K, Larsen J, Bodker L. Bacterial populations associated with mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*. FEMS Microbiol Ecol 2002;41:133-140.

Marschner P, Baumann K. Changes in bacterial community structure induced by mycorrhizal colonisation in split-root maize. Plant Soil 2003;251:279-289.

Marulanda-Aguirre A, Azcón R, Ruíz-Lozano JM, Aroca R. Differential effects of a *Bacillus megaterium* strain on *Lactuca sativa* plant growth depending on the origin of the arbuscular mycorrhizal fungus coinoculated: Physiologic and biochemical traits. J Plant Growth Regul 2008;27:10-18.

Marulanda A, Azcón R, Ruíz-Lozano JM. Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress. Physiol Plant 2003;119:526-533.

Marulanda A, Barea JM, Azcón R. Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments. Mechanisms related to bacterial effectiveness. J Plant Growth Regul 2009;28:115-124.

McArthur DAJ, Knowles NR. Resistance responses of potato to vesicular-arbuscular mycorrhizal fungi under varying abiotic phosphorus levels. Plant Physiol 1992;100:341-351.

Medina A, Probanza A, Manero FJG, Azcón R. Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). Appl Soil Ecol 2003;22:15-28.

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 2010;33:453-467.

Nadeem SM, Ahmad M, Zahir ZA, Javaid A, Ashraf M. The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. Biotechnol Adv 2014;32:429-448.

Olsson PA, Chalet M, Baath E, Finlay RD, Söderström B. Ectomycorrhizal mycelia reduce bacterial activity in a sandy soil. FEMS Microbiol Ecol 1996;21:77-86.

Phillips JM, Hayman DS. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Brit Mycol Soc 1970;55:159-161.

Probanza A, García JAL, Palomino MR, Ramos B, Mañero FJG. *Pinus pinea* L. seedling growth and bacterial rhizosphere structure after inoculation with PGPR *Bacillus* (*B. licheniformis* CECT 5106 and *B. pumilus* CECT 5105). Appl Soil Ecol 2002;20:75-84.

Ramos B, García JAL, Probanza A, Domenech J, Mañero FJG. Influence of an indigenous *European alder* (*Alnus glutinosa* (L.) Gaertn) rhizobacterium (*Bacillus pumilus*) on the growth of alder and its rhizosphere microbial community structure in two soils. New Forests 2003;25:149-159.

Rangel-Castro JI, Danell E, Pfeffer PE. A C-13-NMR study of exudation and storage of carbohydrates and amino acids in the ectomycorrhizal edible mushroom *Cantharellus cibarius*. Mycologia 2002;94:190-199.

Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 2009;321:305-339.

Rillig MC, Mummey DL. Mycorrhizas and soil structure. New Phytol 2006;171:41-53.

Roldán A, García-Orenes F, Lax A. An incubation experiment to determine factors involving aggregation changes in an arid soil receiving urban refuse. Soil Biol Biochem 1994;26:1699-1707.

Rovira A. Biology of the soil–root interface. In: Harley JL, Scott-Russell R, editors. The Soil Root Interface. New York: Academic Press, 1979. p. 145-160.

Sahin N. Oxalotrophic bacteria. Res Microbiol 2003;154:399-407.

Sanon A, Andrianjaka ZN, Prin Y, Bally R, Thioulouse J, Comte G, Duponnois R. Rhizosphere microbiota interfers with plant-plant interactions. Plant Soil 2009;321:259-278.

Scheffknecht S, Mammerler R, Steinkellner S, Vierheilig H. Root exudates of mycorrhizal tomato plants exhibit a different effect on microconidia germination of *Fusarium oxysporum* f. sp lycopersici than root exudates from non-mycorrhizal tomato plants. Mycorrhiza 2006;16:365-370.

Smith SE, Read DJ. Mycorrhizal Symbiosis. New York: Elsevier, Academic Press, 2008.

Sofo A, Ciarfaglia A, Scopa A, Camele I, Curci M, Crecchio C, Xiloyannis C, Palese AM. Soil microbial diversity and activity in a Mediterranean olive orchard using sustainable agricultural practices. Soil Use Manage 2014;30:160-167.

Song F, Han X, Zhu X, Herbert SJ. Response to water stress of soil enzymes and root exudates from drought and non-drought tolerant corn hybrids at different growth stages. Can J Soil Sc 2012;92:501-507.

Sood SG. Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. FEMS Microbiol Ecol 2003;45:219-227.

SSS. Soil Survey Staff (SSS). "Keys to Soil Taxonomy" 10th ed. USDA. Natural Resources, Conservation Service, Washington DC. 2006.

Steinkellner S, Lendzemo V, Langer I, Schweiger P, Khaosaad T, Toussaint J-P, Vierheilig H. Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. Molecules 2007;12:1290-1306.

Sun YP, Unestam T, Lucas SD, Johanson KJ, Kenne L, Finlay R. Exudation-reabsorption in a mycorrhizal fungus, the dynamic interface for interaction with soil and soil microorganisms. Mycorrhiza 1999;9:137-144.

Timonen S, Jorgensen KS, Haahtela K, Sen R. Bacterial community structure at defined locations of *Pinus sylvestris Suillus bovinus* and *Pinus sylvestris Paxillus involutus* mycorrhizospheres in dry pine forest humus and nursery peat. Can J Microbiol 1998;44:499-513.

Toljander JF, Lindahl BD, Paul LR, Elfstrand M, Finlay RD. Influence of arbuscular mycorrhizal mycelial exudates on soil bacterial growth and community structure. FEMS Microbiol Ecol. 2007;61:295-304.

van Hees PAW, Rosling A, Essen S, Godbold DL, Jones DL, Finlay RD. Oxalate and ferricrocin exudation by the extramatrical mycelium of an ectomycorrhizal fungus in symbiosis with *Pinus sylvestris*. New Phytol 2006;169:367-377.

Vivanco J, Guimaraes R, Flores H. Underground plant metabolism: the biosynthetic potential of roots. In: Waisel Y, Eshel A, Kafkafi U, editors. Plant roots, the hidden half, 3rd edn. New York: Marcel Dekker, 2002. p. 1045-1070.

Vivas A, Barea JM, Azcón R. *Brevibacillus brevis* isolated from Cadmium- or Zinc-contaminated soils improves *in vitro* spore germination and growth of *Glomus mosseae* under high Cd or Zn concentrations. Microb Ecol 2005a;49:416-424.

Vivas A, Barea JM, Azcón R. Interactive effect of *Brevibacillus brevis* and *Glomus mosseae*, both isolated from Cd contaminated soil, on plant growth, physiological mycorrhizal fungal characteristics and soil enzymatic activities in Cd polluted soil. Environ Pollut 2005b;134:257-266.

Vivas A, Marulanda A, Gómez M, Barea JM, Azcón R. Physiological characteristics (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by *Bacillus thuringiensis* inoculation under two phosphorus levels. Soil Biol Biochem 2003a;35:987-996.

Vivas A, Marulanda A, Ruíz-Lozano JM, Barea JM, Azcón R. Influence of a *Bacillus* sp on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress. Mycorrhiza 2003b;13:249-256.

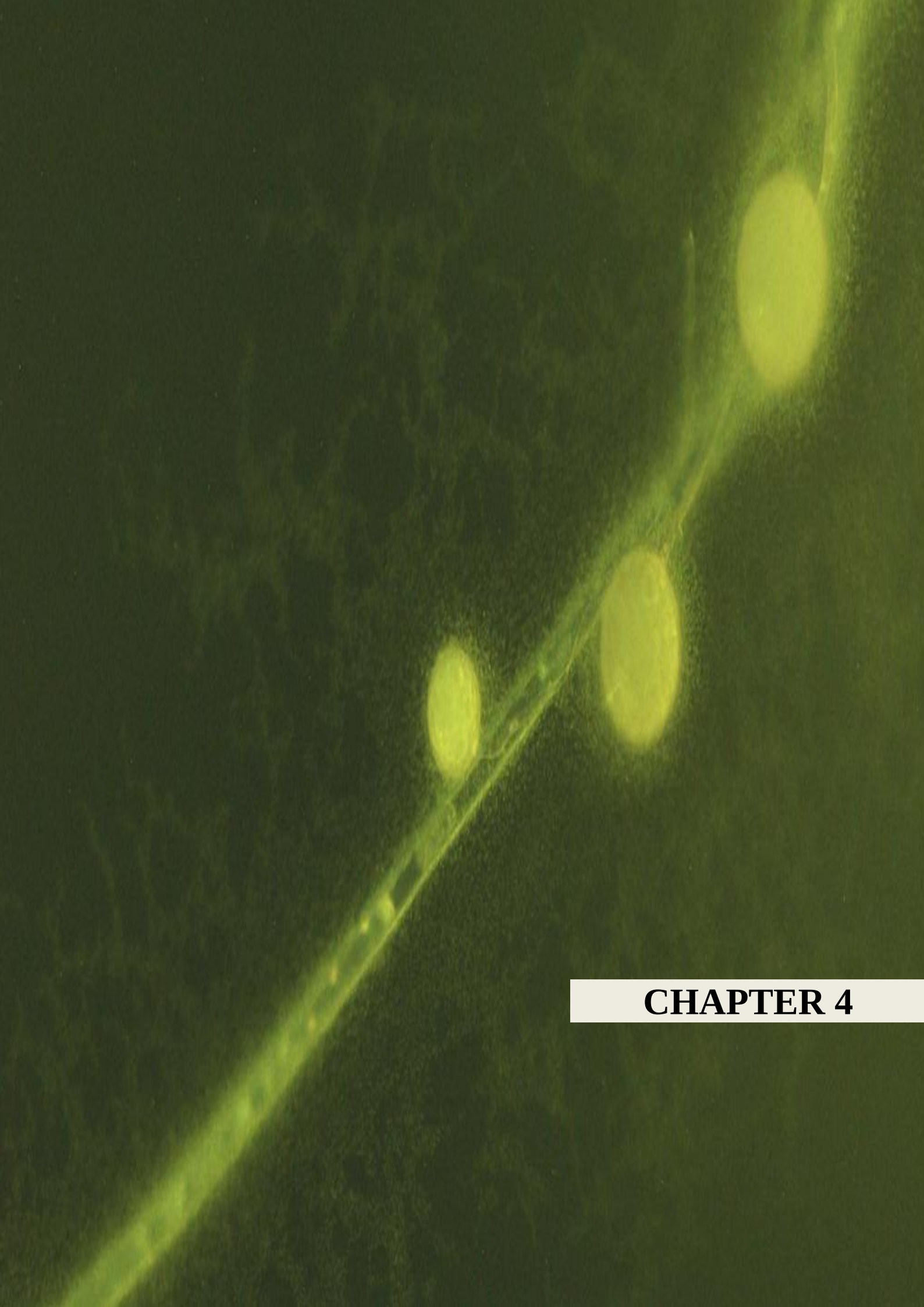
Wamberg C, Christensen S, Jakobsen I, Muller AK, Sorensen SJ. The mycorrhizal fungus (*Glomus intraradices*) affects microbial activity in the rhizosphere of pea plants (*Pisum sativum*). Soil Biol Biochem 2003;35:1349-1357.

Warmink JA, Nazir R, van Elsas JD. Universal and species-specific bacterial 'fungiphiles' in the mycospheres of different basidiomycetous fungi. Environ Microbiol 2009;11:300-312.

Wright SF, Anderson RL. Aggregate stability and glomalin in alternative crop rotations for the central Great Plains. Biol Fertil Soils 2000;31:249-253.

Zaidi A, Khan MS, Amil M. Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (*Cicer arietinum* L.). E J Agron 2003;19:15-21.

Zak JC, Willig MR, Moorhead DL, Wildman HG. Functional diversity of microbial communities - a quantitative approach. Soil Biol Biochem 1994;26:1101-1108.



CHAPTER 4

Potential of mycorrhizal inocula to stimulate growth, nutrition and enzymatic activities in *Retama sphaerocarpa* plants compared with chemical fertilization under drought conditions

Elisabeth Armada, Olga López-Castillo, Antonio Roldán, Rosario Azcón

1. Introduction

Drought and water stress are considered the most important environmental factors limiting plant growth in the world (Panozzo and Eagles, 1999). High temperatures and dry climate have a strong relationship with soil degradation and desertification (Alguacil et al., 2011). The restoration of degraded areas must be started by reclaiming the vegetation as shrubs species and most of the revegetation programs have been developed by using autochthonous plant species that are the most appropriate for reclaiming degraded soils. Restoration of natural vegetation in arid drought zones is quite difficult due to the water and nutrient limitation. There is a strong need for nutrients as C, N and P required for plant nutrition and water but they are frequently limited in semiarid degraded soils. The application of chemical fertilizers can contaminate ground waters or can be converted in insoluble mineral complexes as precipitated forms unavailable for plant acquisition (Rengel and Marschner, 2005). As consequence, a minimum of provided fertilizers can be absorbed by plants. Nevertheless, the establishment of plant cover in these disturbed sites can be facilitated by beneficial soil microorganisms such as arbuscular mycorrhizal (AM) fungi. The interest of use microbial inoculation to recover degraded dry lands has driven to evaluated inocula effectiveness to enhance plant establishment under drought conditions (Armada et al., 2014a). This biotechnology has been proposed to be ecologically important for the plant development in degraded ecosystems (Jeffries and Barea, 2012). Mycorrhizal fungi colonize the roots of more than 90% of plant species having mutual plant and fungus benefit (Smith and Read, 2008) and this symbiosis can improve the nutritional status and growth of plants under both optimal and restricted water levels (Ruíz-Lozano and Azcón, 1995). AM fungi represent an important biological factor for plants to thrive in water-limited conditions not only by increasing the supply of nutrients (Toro et al., 1997) but also by helping plants to support water stress (Ruíz-Lozano et al., 1995a; 1995b; Medina and Azcón, 2012). Selected microorganisms may contribute to plant establishment and growth particularly limited under semiarid conditions (Armada et al., 2014b).

Drought reduces the mycorrhizal colonization but the inoculation of efficient fungi may help in improving colonization and consequently population of AM fungi in this environment. The low microbiological activity of arid soils, due to the low density of microbial propagules, may be critical to the successful reestablishment and recovery of desertified ecosystems. Thus, native strains could be, presumably, the most effective in semiarid sites (Armada et al., 2014b). Among different mycorrhizal species *Rhizophagus intraradices* behaved as one of the most efficient endophyte in taking up soil water (Marulanda et al., 2003) and in increasing plant growth and nutrition under drought conditions (Marulanda et al., 2006).

We hypothesised that under drought conditions autochthonous plant will be particularly benefited from inoculation with a whole native AM fungi consortium due to the diversity and functionality of autochthonous mycorrhizal community than from the inoculation with a single drought adapted mycorrhizal isolate as *R. intraradices* (from our collection). Presumably the inoculation with a complex fungal community would have a greater buffer capacity against the water stress than a single fungal inoculum (Caravaca et al., 2005b).

A general trend in the beneficial effect of mycorrhization is associated with phosphorus acquisition in colonized plants and this is an important mechanism related to plant drought tolerance as was reported (Augé, 2004; Subramanian et al., 2006).

The inoculation with suitable-adapted symbiotic microorganism as autochthonous AM fungi has proved their effectiveness under water stress (Marulanda et al., 2006) and they been proposed with regards the improvement of the performance of plants in these areas (Caravaca et al., 2005b). But there have been no reports on the comparative role of autochthonous AM fungal consortium with fertilizers on plant stress tolerance.

Revegetation with native woody legumes as *Retama sphaerocarpa* are useful colonizer plants for nutrient-deficient arid soils and proved to be more effective than with exotic plants under such unfertile and water limited conditions (Caravaca et al., 2004).

Drought stress can trigger an oxidative burst, induce an array of oxidant enzymes expression (Gururani et al., 2013) and as result, plants are able to counteract drought stress by modulating levels of some antioxidant enzymatic systems (Koussevitzky et al., 2008). Plants have this alternative defence strategy as a tool to overcome the stress constraints and to survive. There are few studies regarding the changes in the activity of antioxidant enzymes in plants modulated by the microbial inoculations under water stress conditions and results reported are highly variable.

To address the mycorrhizal drought tolerance strategies in *R. sphaerocarpa* we selected in a first experiment two types of drought adapted mycorrhizal inocula (autochthonous fungal mixture or the reference *R. intraradices*) and the effect of these biological treatments on drought tolerance were compared with two levels of phosphorus fertilization, 25 and 50 ppm P as H_3PO_4. Drought tolerance of *R. sphaerocarpa* was determined assessing growth, nutrient acquisition, mycorrhizal development and antioxidant enzymatic activities according to the chemical or biological treatments applied using a Mediterranean arid soil under drought conditions.

A second experiment was also carried out using the same plant, soil and environmental conditions. This second experiment was planned based in the well known fact that potassium is the main element related in the alleviation of osmotic stress by being involved in photosynthetic CO_2 fixation and the protection of chloroplasts from photooxidative damage (Romheld and Kirkby, 2010). K^+ as inorganic osmolyte is important in water homeostasis under drought and it is able to regulate osmotic balance, turgor pressure, stomatal opening and transpiration (Loutfy et al., 2012). The objective of this second study was to assess to what extent the effect of the single potassium fertilizer [two levels, 5 or 10 mM K (1K or 2K) as K_2SO_4], may be increased by the addition of autochthonous inocula [*Bacillus thuringiensis* (B) plus the mycorrhizal consortium (M)]. Both inocula were isolated from the same rhizosphere samples as *B. thuringiensis* was selected for exerting a significant effect on drought tolerance in native plants such as *Lavandula*, *Trifolium*, *Salvia* and others (Armada et al., 2014b). Moreover, in a recent study with a plant of agronomic interest as maize the coordinated effect of these autochthonous two microbial groups (*B. thuringiensis* + AM fungi) under drought conditions was evidenced. *B. thuringiensis* increased mainly maize nutrition and AM fungi were more active improving stress tolerance/homeostatic mechanisms (as plant aquaporins and physiological functions) (Armada et al., 2015). Thus, we here hypothesised that these combined biological treatments (MB) could interact with chemical fertilizers (1K or 2K) influencing growth, nutrition, mycorrhizal development and drought tolerance in those drought-adapted Mediterranean plants growing under drought conditions.

Soil enzyme activities are an alternative way of monitoring the soil alterations and perturbations (Naseby and Lynch, 1997). In arid and degraded soils the microbial populations and their activities are low mainly due to the lack of water and suitable substrates (Medina et al., 2004a). Thus, soil enzymatic activities play an important role in the mineralization of the organic products and were assessed as index of microbial soil activity.

The efficacy of native strains from different sites lead to different effects on plant growth and nutrients uptake (Ortiz et al., 2015). The aims of this study is to ascertain i) the comparative effect of mycorrhizal inocula (autochthonous or allochthonous) and P fertilizers (two levels), and to verify the relevance of the AM strain origin or P-fertilizer to the ability to enhance plant growth, nutrition, biochemical antioxidant values related to drought tolerance under semiarid conditions. In addition, to test the impact of dual microbial inoculations (autochthonous AM fungi and the bacteria *B. thuringiensis*) and/or the application of fertilizer potassium (two levels) on *R. sphaerocarpa* growth, nutrition and plant enzymatic activities. In both experiments was analyzed how these treatments change the enzymatic soil activities in this water limited soils.

2. Materials and Methods

2.1. Experimental design

Two independent experiments were carried out in this study. Firstly, in Experiment I, in a microcosm assay under greenhouse conditions, we determined the effectiveness of both a consortium of autochthonous mycorrhizal fungi (M) or *R. intraradices* (RI) compared with two phosphorus levels [25 ppm P (1P) or 50 ppm P (2P) as H_3PO_4] over non-inoculated unfertilized control to increase plant growth, nutrition mycorrhizal development and biochemical values under drought conditions.

In a subsequent study, the Experiment II, once tested (in the experiment I) the mycorrhizal effectiveness of autochthonous fungi in increasing plant drought tolerance we assayed how two levels of K in the growing medium [5 mM K (1K) and 10 mM K (2K) as K_2SO_4], applied as single fertilizer or when these two K levels were dually inoculated with autochthonous microorganism as AM fungi (consortium) plus *B. thuringiensis* affected plant drought tolerance in terms of plant growth, nutrition, mycorrhizal development and biochemical parameters under drought conditions.

In both experiments soil enzymatic activities, as index of soil quality, were determined. Five replicates of each treatment were made resulting a total of 25 pots in experiment I and 20 pots in experiment II.

2.2. Soil characteristics

The experimental soil used was selected from an area located in the Natural Ecological Park "Vicente Blanes" in Molina de Segura, province of Murcia (southeastern Spain) (coordinates 38º12´ N, 1º13´ W, 393 m altitude). The climate is semiarid Mediterranean, with an average annual rainfall lower than 270 mm and the potential evapotranspiration (ETP) reaches approximately 1000 mm. The mean annual temperature is 19.2 ºC with absence of frost period. The soil in the experimental area is a Typic Torriorthent (SSS, 2006) very little developed with a low organic matter content and a silty clay texture that facilitates the degradation of soil structure. The vegetation in the zone was dominated by *Piptatherum miliaceum* L. Cosson., *Trifolium repens* L., some shrubs of *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *R. sphaerocarpa* growing with patchy distribution.

The main soil characteristics were pH 8.90, P $1.36 \cdot 10^{-3}$ g kg^{-1} (Olsen test), organic carbon 0.94%, total N 0.22%, and an electric conductivity of 1.55 dS m^{-1}. Both microcosm experiments were conducted in this soil.

2.3. Isolation, production and identification of drought-tolerant microorganism

The microbial inocula used in these experiments were isolated from the rhizosphere of plants naturally growing in this semiarid soil described above. This rhizosphere soil containing colonized roots, spores and mycelia belonging to the native adapted AM fungi was cultivated for inoculum production (Marulanda et al., 2006).

For inocula production the rhizosphere soil was bulked in an open pot culture of *Zea mays* and *Trifolium repens* with sterile soil/sand (1:1 v/v) mixture. After six months of plant growth the shoots were eliminated and the under-grown part (mycorrhizal roots plus soil possessing fungal spores and mycelium) was maintained by storage for three to six months in polyethylene bags at 4ºC and used as a stock culture. The mycorrhizal fungus *R. intraradices* (EEZ 195) from our collection (Estación Experimental del Zaidín) was also used in the Experiment I as reference.

Plants were inoculated with *R. intraradices* or a consortium of indigenous AM fungi. The fungal spores were isolated by wet-sieving and decanting as described by Ruíz-Lozano and Azcón (1995) and all the spores obtained were morphologically similar to *Septoglomus constrictum* (EEZ 198), *Diversispora aunantia* (EEZ 199), *Archaeospora trappei* (EEZ 200), *Glomus versiforme* (EEZ 201) and *Paraglomus ocultum* (EEZ 202) compared to those from our current EEZ collection. We used as autochthonous mycorrhizal (M) inoculum a mixture of each one of these fungal species.

From the corresponding stock culture, 5 g of this fungal AM consortium (M) or *R. intraradices* (RI) from collection was applied as inocula to the corresponding pots, having both inocula similar average of 80 spores/g of soil and roots with 75% of AM colonization. The M inoculum or the reference *R. intraradices* were applied to each one of the appropriate pots at transplanting time just below the seedlings. Non-mycorrhizal treatments received the same amount of autoclaved inoculum together with a 2 mL aliquot of natural soil filtrate (< 20 μm) containing soil microorganisms with exception of AM fungi.

In the Experiment II an autochthonous bacteria was also used in interaction with autochthonous AM fungi. The bacterium was isolated from the above-mentioned soil (a mixture of rhizospheres from several autochthonous plants species). A homogenate of 1 g soil in 9 mL sterile water was diluted (10^{-2} to 10^{-4}), plated on three different media [Yeast Manitol Agar, Potato Dextrose Agar or Luria-Bertani (LB) Agar] and then incubated at 28 ºC for 48 h, to isolate bacteria from different taxonomic groups.

Identification of isolated autochthonous bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were collected, diluted, lysed and their DNA used as a template in the PCR reactions. All reactions were conducted in 25 μL volume containing PCR buffer 10X, 50 mM $MgCl_2$, 10 μM each primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), 5 U/μL of *Taq* polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with the following conditions: 5 min at 95 ºC, followed by 30 cycles of 45s at 95 ºC, 45s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. The products PCR of were analysed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (PerKin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (NCBI) using BLAST program, unambiguously identified the bacterium as *Bacillus thuringiensis* (Acession NR 043403.1, similarity 98%). The bacteria were grown in 250-mL flasks containing 50 mL of LB medium for 48 h at 28 ºC. In the appropriate pots, plants were inoculated with 1 mL of the bacterial culture (10^8 cfu mL^{-1}). The bacterial inoculum was applied again 15 days later. In control treatments, 1 mL of sterilized bacterial culture was added.

Described soil was sieved (mesh diameter = 2mm) and sterilized by steam (100 ºC for 1h on 3 consecutive days). One month old seedlings of *R. sphaerocarpa* plants were transplanted to pots containing 0.750 kg of a 1:1 mixture of soil:sand (v/v) in Experiment I and 1:2 soil:sand mixture (v/v) in Experiment II. At transplanting time plants were inoculated with the appropriate inoculum.

2.4. Plant growth conditions

Plants (one per pot) were grown for seven and half months in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C and 50% relative humidity. The photosynthetic photon flux density (PPFD) was $503 \cdot 10^{-6}$ mol m^{-2} s^{-1}, as measured with a light-meter (LICOR, model LI-188B). Water loss was compensated by watering every day to reach 50% of water-holding capacity (WHC). During the first 2 weeks of plant growth constant soil water content close to water holding capacity was maintained. After this time, plants were allowed to dry until soil water content was 50% of water holding capacity and maintained under these conditions for additional 30 weeks. To achieve that, the soil moisture in the pots was measured each 24 h and the water was added to reach a maximum of 50% of water holding capacity. However, during the 24-h period between each rewatering the soil water content was progressively decreased to until a minimum value of 40% of water holding capacity. Soil moisture was measured with an ML2 X ThetaProbe (AT Delta-T Devices Ltd, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil (White et al., 1994). This volumetric soil moisture is considered to be a normal environmental condition in dry Mediterranean areas. During the experimental time, a Hewitt's nutritive solution was applied weekly (10 mL pot^{-1}) modified to have ½ N and ¼ P concentrations. In Experiment I, the two P fertilization treatments were applied twice a week (10 mL) of H_3PO_4 solution along the four weeks after transplanting to reach the desired P concentrations (25 or 50 ppm P) in the appropriate pots. Similarly, in Experiment II the two K fertilization treatments were applied twice a week (10 mL) of K_2SO_4 solution along the four weeks after transplanting to reach the desired K concentrations (5 mM or 10 mM K) in the corresponding pots.

2.5. Parameters measured

2.5.1. Biomass production

At the harvest, seven and half months after transplanting in both Experiments, the root system was separated from the shoot and dry weights were measured after drying in a forced drought oven at 75 ºC for 2 days.

2.5.2. Nutrient concentration

Shoot content (mg $plant^{-1}$) of P, K, Ca and Mg as well as of Fe, Mn, Zn and Cu (µg $plant^{-1}$) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Mineral analyses were carried out by the Instrumentation Service (EEZ-CSIC), Granada, Spain.

2.5.3. Specific absorption rate

Specific absorption rate (SAR) is defined as the amount of nutrients or metal absorbed per unit of root biomass (Gray and Schlesinger, 1983). It was calculated as follows:

SAR= Plant nutrient (μg) / Root mass (μg)

2.5.4. Mycorrhizal development and glomalin content

Fungal colonization was assessed after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Philips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method Giovanneti and Mosse (1980) after counting 150 intersections. Mycorrhizal development was evaluated by the method of Trouvelot et al. (1986) using MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The parameters measured according to this method were the frequency of AM colonization in the sample (%F), intensity of AM colonization (%m) in the whole root system (%M), and relative and absolute arbusculum richness (%a and %A) referred to the calculated whole root system respectively.

The extraradical mycelium was evaluated in rhizosphere soil as glomalin content in soil as described by Wright and Upadhyaya (1996).

2.5.5. Antioxidant enzymatic activities

Shoot tissues were homogenized (Aroca et al., 2003) in a cold mortar with 4 mL 100 mM phosphate buffer (pH 7.2) containing 60 mM KH_2PO_4, 40 mM K_2HPO_4, 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 18,000 *g* for 10 min at 4 ºC, and the supernatant was used for enzyme activity determination. Total SOD activity (EC 1.15.1.1) (Burd et al., 2000) was measured on the basis of SOD's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 ºC. CAT activity (EC 1.11.1.6) was measured as described by Aebi (1984) conducted in 2 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 μL of enzyme extract. It was determined the consumption of H_2O_2 and followed by decrease in absorbance at 240 nm for 1 min (extinction coefficient (ε_{240}) of 39.6 mM^{-1} cm^{-1}). APX activity (EC 1.11.1.11) was measured in a 1 mL reaction volume containing 80 mM potassium phosphate buffer (pH 7.0), 2.5 mM hydrogen peroxide and 0.5 mM sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the

oxidation rate for ascorbate (Amako et al., 1994). GR activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH (Carlberg and Mannervik, 1985). The reaction mixture (1 mL) contained 50 mM Tris buffer 3 mM $MgCl_2$ (pH 7.5), 1 mM oxidized glutathione, 50 µL enzyme extract, and 0.3 mM NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in µmol NADPH oxidized mg^{-1} protein, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH (ε_{340}=6.22 mM^{-1} cm^{-1}). Total soluble protein amount was determined using the Bradford (Bradford, 1976) method and bovine serum albumin (BSA) as standard.

2.5.6. Soil enzymatic activities

In rhizosphere soil samples enzymatic activities were determined in both experiments.

Dehydrogenase activity was determined following Skujins' method (Skujins, 1976), as modified by García et al. (1997). For this, 1 g of soil at 60% of its field capacity was exposed to 0.2 mL of 0.4% INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h, at 22 ºC in darkness. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 mL of methanol, by shaking vigorously for 1 min and filtering through a Whatman N° 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Alkaline phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP) 0.115 M as substrate. Two milliliters of 0.5 M sodium acetate buffer adjusted to pH 5.5 using acetic acid (Naseby and Lynch, 1997) and 0.5 mL of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 mL of 0.5 M $CaCl_2$ and 2 mL of 0.5 M NaOH were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969). In controls, the substrate was added before the $CaCl_2$ and NaOH addition.

β-glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG), 0.05 M (Masciandaro et al., 1994) as substrate. This assay is also based on the release and detection of PNP. Two milliliters of 0.1 M maleate buffer (pH 6.5) and 0.5 mL of substrate were added to 0.5 g of sample and incubated at 37 ºC for 90 min. The reaction was stopped with tris-hydroxymethyl aminomethane (THAM) according to Tabatabai (1982). The amount of PNP was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969).

Urease activity was determined by the method of Nannipieri et al. (1980), and expressed as µmol $N\text{-}NH_3$ g^{-1} soil $\cdot h^{-1}$.

2.6. Statistical analyses

Data from both experiments were analyzed using SPSS 21 software package for Windows, were subjected to one-way general linear model ANOVA (analysis of variance) was used to determine the effect of each treatment. The Duncan's multiple-range test (Duncan, 1955) was used for post hoc analysis to determine differences between means. Differences were considered significant at $p \leq 0.05$. Percentage values were arc-sine-transformed before statistical analysis.

3. Results

3.1. Experiment I

Regarding results of this experiment, whichever mycorrhizal inocula applied behaved similarly to P fertilization (1P or 2P) on shoot dry mass production. The four treatments applied similarly enhanced shoot dry biomass compared to control plants but root development was lower in RI colonized plants (Fig. 1). In spite of non-significant differences in growth of P treated or mycorrhizal inoculated plants the highest shoot biomass (79% over control) was obtained in plants colonized by the consortium of autochthonous fungi (Fig. 1).

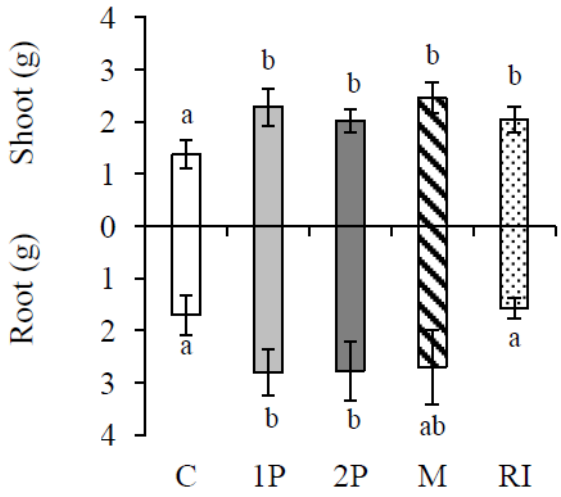


Fig.1. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], autochthonous AM fungal consortium (M) or the reference R. intraradices (RI), over control (C) on the dry weight of shoot and root (g) of R. sphaerocarpa under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple range test (n= 5).

Shoot and root development of P-fertilized plants as well as some of the analyzed nutrients (P, K, Ca, Fe, Mn, Zn and Cu) reached similar values irrespective of P level applied (Figs. 1, 2 and 3). Thus, the application of 25 ppm P (1P), under these drought environmental conditions, may be considered as the optimum amount of P-fertilization to reach the maximum plant growth and nutrition.

Regarding P and K content no-differences were observed in plants P fertilized, irrespective of P level and M inoculated having similar root development (Figs. 1 and 2). Nevertheless, the uptake of P, K, Ca or Mg was significantly higher in *R. intraradices*-colonized plants than in control plants having similar root biomass (Figs. 1 and 2). Consequently, the specific absorption rate for these macronutrients were highly enhanced in *R. intraradices* colonized plants (Table 1).

The root development in *R. intraradices* colonized plants was more reduced than in the rest of treated plants (Fig. 1). As a result of lower root growth, less nutrients transport to the above grown parts is expected. However, the opposite effect was observed in *R. intraradices* inoculated plants since the uptake of macronutrients was higher in these plants. Results show that this fungus play a significant role in the extra acquisition of some nutrients (P, K, Ca and Mg) and their specific absorption rate (SAR) under drought conditions (Fig. 2, Table 1).

Moreover, in the case of P, *R. intraradices*-colonized plants acquired the highest proportion of this nutrient, even more than plants P-fertilized with 50 ppm P (Fig. 2). Regarding K, Ca and Mg plant content both mycorrhizal inocula (M or RI) resulted as active as the highest P-fertilization in the uptake these nutrients (Fig. 2).

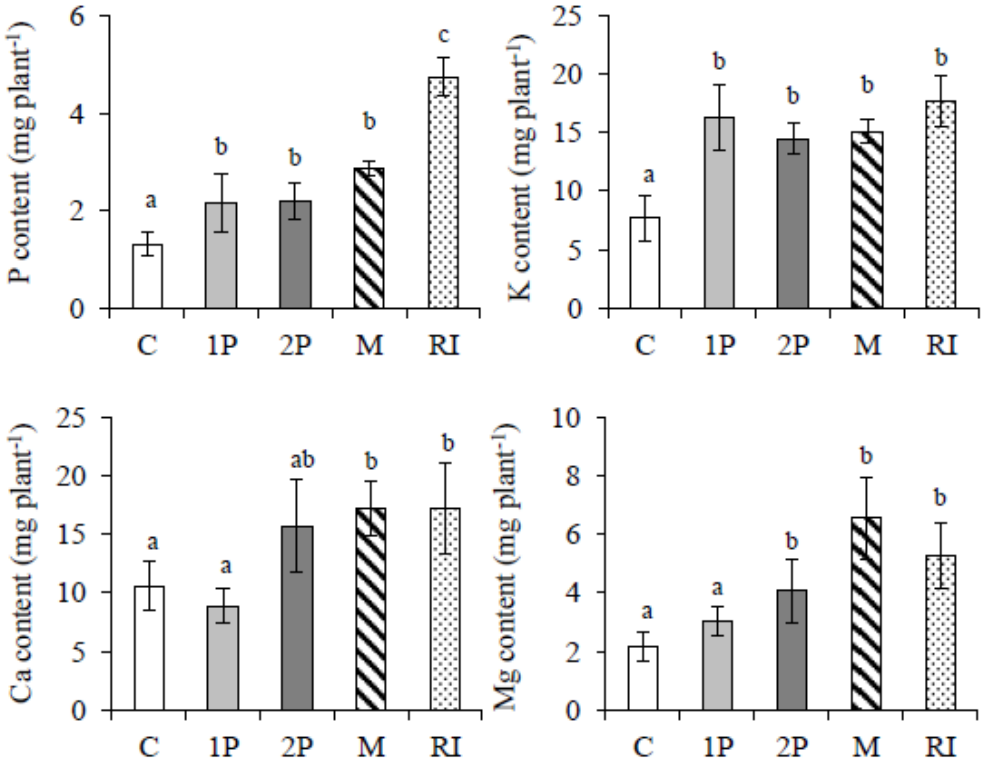


Fig.2. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], autochthonous AM fungal consortium (M) or the reference *R. intraradices* (RI), over control (C) on P, K, Ca and Mg content (mg plant^{-1}) of *R. sphaerocarpa* under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple range test (n= 5).

Micronutrients as Fe, Mn, Zn and Cu content did not significantly change irrespective of applied treatment with the exception of *R. intraradices* that highly depressed the content of whatever micronutrient here analyzed (Fig. 3).

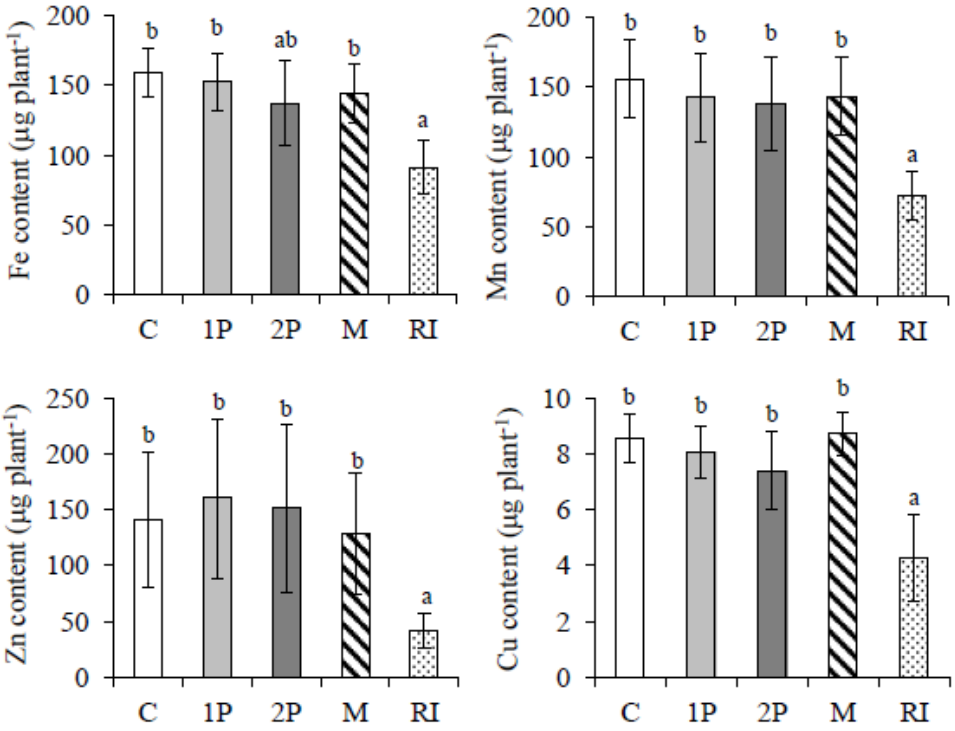


Fig.3. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], autochthonous AM fungal consortium (M) or the reference R. intraradices (RI), over control (C) on Fe, Mn, Zn and Cu content (µg plant-1) of R. sphaerocarpa under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple range test (n= 5).

Table 1. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], authochthonous AM fungal consortium (M) or the reference *R. intraradices* (RI), over control (C) on the specific absorption rate (SAR) of P, K, Ca, Mg, Fe, Mn, Zn and Cu by *R. sphaerocarpa*.

	P	K	Ca	Mg	Fe	Mn	Zn	Cu
C	0.8 a	4.5 a	6.2 b	1.2 a	92.9 b	91.4 b	82.3 c	5.0 b
1P	0.8 a	5.8 a	3.2 a	1.1 a	54.4 a	50.9 a	57.0 b	2.9 a
2P	0.8 a	5.2 a	5.6 ab	1.5 a	49.3 a	49.7 a	54.6 b	2.7 a
M	1.1 a	5.6 a	6.4 b	2.4 b	53.5 a	53.1 a	47.7 b	3.2 a
RI	3.0 b	11.2 b	11.0 c	3.3 c	58.2 a	46.0 a	26.5 a	2.7 a

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

There were no-significant differences in the percentage of mycorrhizal root length produced by both mycorrhizal inocula. The intraradical mycorrhizal development was analyzed in terms of frequency (%F), intensity (%M and %m) and relative and absolute arbusculum richness (%a and %A) in colonized roots. The extraradical mycelium was evaluated as glomalin content in soil (Table 2). But similar intra and extra mycorrhizal colonization levels were found irrespective of the mycorrhizal inoculum applied.

No nodule production was observed in spite of the application of an extract from non-sterilized natural soil presumably having *Rhizobium*.

Table 2. Mycorrhizal symbiotic development by the authochthonous AM fungal consortium (M) or the reference *R. intraradices* (RI) on colonization frequency (%F), intensity (%M), intensity of colonization (%m), arbuscule abundance (%a), richness of arbuscules (%A) and glomalin (μg g^{-1}soil).

	%F	%M	%m	%a	%A	μg glomalin g^{-1}soil
M	100 a	28.9 a	28.9 a	56.6 a	20.6 a	1.2 a
RI	100 a	30.4 a	30.4 a	64.9 a	21.1 a	1.0 a

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

Antioxidant activities were here analyzed as an index of the ability of plants to counteract the oxidative damage caused by the drought imposed. Results show that the APX activity was the highest in P-fertilized plants and the lowest in mycorrhizal inoculated plants of similar size (Figs. 1 and 4). For SOD and CAT activities the lowest values were observed in M-colonized plants that decreased these both activities compared to whatever level of P fertilization while *R. intraradices* reduced GR activity (Fig. 4).

The β-glucosidase activity increased by 176% in response to M inoculum (Fig. 5) and dehydrogenase activity was enhanced by both inocula but only 10%. But non-significant differences on these enzymatic activities as result of the P fertilization were observed. Treatments applied did not change phosphatase and urease activities (Fig. 5).

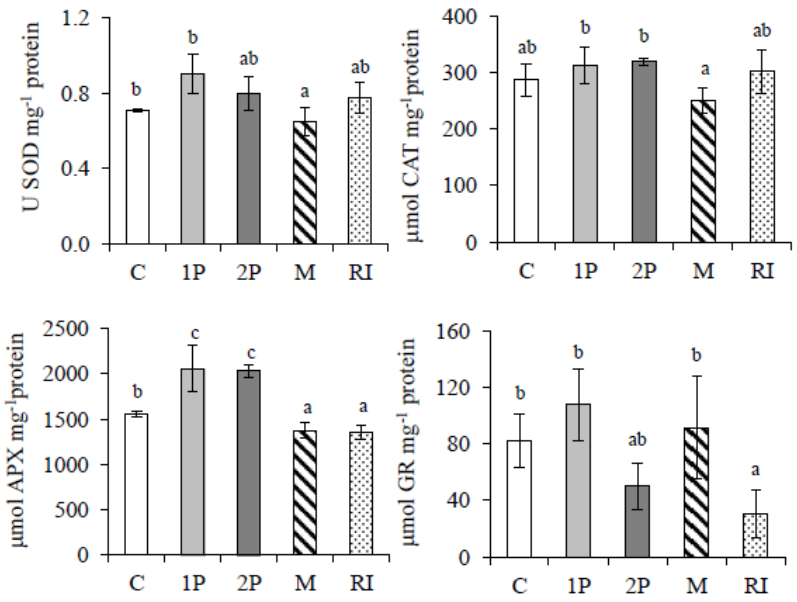


Fig.4. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], autochthonous AM fungal consortium (M) or the reference *R. intraradices* (RI), over control (C) on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidise (APX) and glutathione reductase (GR) antioxidant activities in shoot of *R. sphaerocarpa* under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple range test (n= 3).

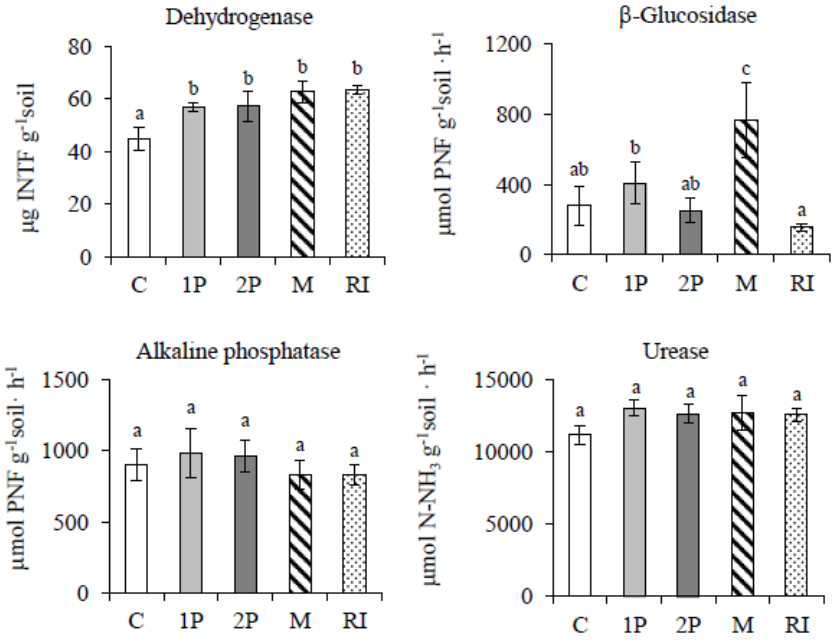


Fig.5. Comparative effect of fertilizers [1P (25 ppm P); 2P (50 ppm P)], autochthonous AM fungal consortium (M) or the reference R. intraradices (RI), over control (C) on dehydrogenase, β-glucosidase, alkaline phosphatase and urease enzymatic activities in soil under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple range test (n= 3).

3.2. Experiment II

Growth of untreated control plants was not represented since it resulted negligible in this experiment. The shoot biomass of *R. sphaerocarpa* plants was the highest in 1K fertilized plants irrespective of inocula applied (Fig. 6). The highest K level (2K) has a reducing, but non-significant effect on shoot growth particularly in inoculated plants. The highest level of K-fertilization (2K) increased root growth but the inocula reduced this value (Fig. 6). The comparative effect on plant biomass production shows that inoculation was not important for plant growth promotion irrespective of a lower (5 mM K) or a higher (10 mM K) K level in the growing medium. Regarding plant nutrition a significant increase of P (by 583.3%), K (by 78.6%), Zn (by 90.8%) and Cu (by 219%) content was obtained in inoculated plants compared to the respective 1K fertilized non-inoculated plants (Figs. 7 and 8) under drought conditions. Inocula also enhanced specific absorption rate (SAR) of these plants (Table 3). In contrast, the microbial inocula interaction with 2K decreased the plant acquisition of K, Ca, Fe and Mn compared to single 2K fertilized plants. Nevertheless, 2K fertilization enhanced P, K and Mn contents compared to the lowest K level (1K) (Figs. 7 and 8).

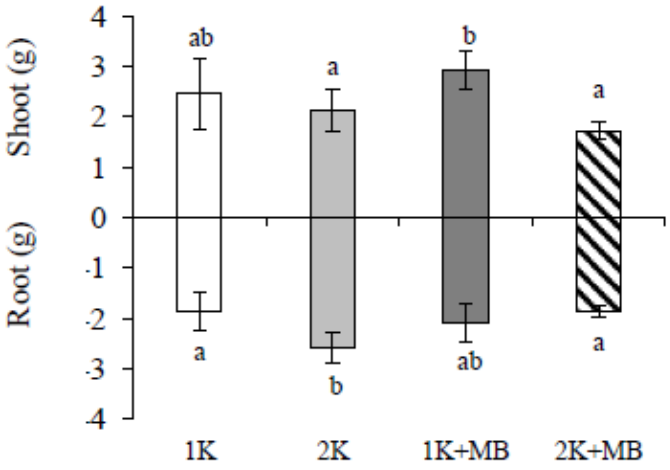


Fig.6. Comparative effect of fertilizers [1K (5mM K) and 2K (10 mM K)] in interaction or not with authochthonous mycorrhyzal fungal consortium (M) and *B. thuringiensis* (B), on the dry weight of shoot and root (g) of *R. shpaerocarpa* under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

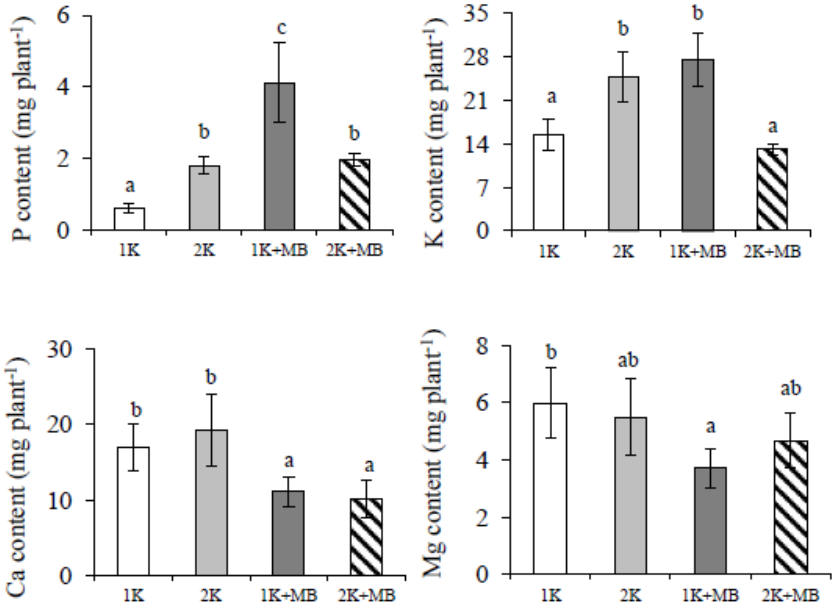


Fig.7. Comparative effect of fertilizers [1K (5mM K) and 2K (10 mM K)] in interaction or not with authochthonous mycorrhyzal fungal consortium (M) and *B. thuringiensis* (B), on P, K, Ca and Mg content (mg plant^{-1}) of *R. shpaerocarpa* under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

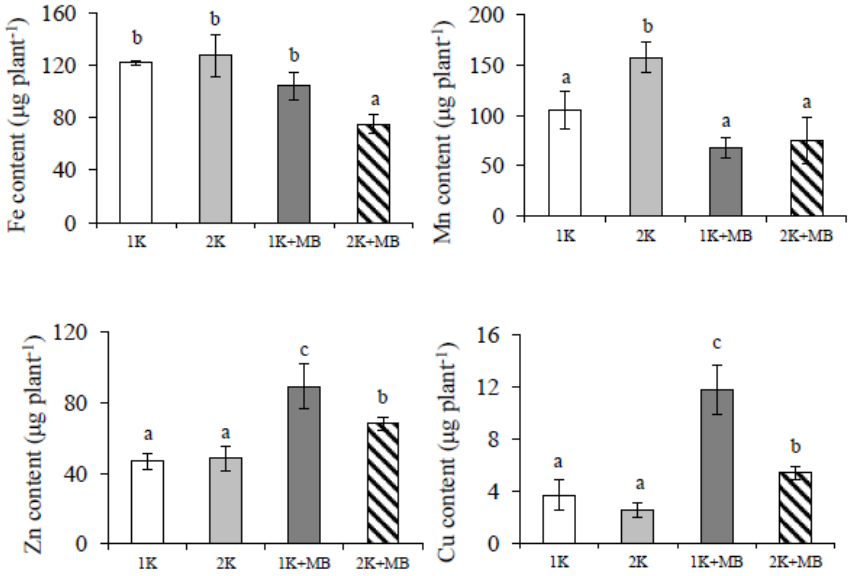


Fig.8. Comparative effect of fertilizers [1K (5mM K) and 2K (10 mM K)] in interaction or not with authochthonous mycorrhyzal fungal consortium (M) and *B. thuringiensis* (B), on Fe, Mn, Zn and Cu content (µg plant^{-1}) of *R. shpaerocarpa* under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

Table 3. Comparative effect of fertilizers [1K (5 mM K) and 2K (10 mM K)] in interaction or not with autochthonous fungal consortium (M) and *B. thuringiensis* (B) on the specific absorption rate (SAR) of P, K, Ca, Mg, Fe, Mn, Zn and Cu by *R. sphaerocarpa.*

	P	K	Ca	Mg	Fe	Mn	Zn	Cu
1K	0.3 a	8.2 ab	9.0 b	3.2 c	64.7 b	55.7 b	24.9 a	2.0 a
2K	0.7 ab	9.6 b	7.5 b	2.1 ab	49.3 a	61.0 b	18.7 a	1.0 a
1K + MB	2.0 c	13.2 c	5.3 a	1.8 a	50.1 a	32.4 a	42.9 b	5.6 b
2K + MB	1.0 b	7.0 a	5.4 a	2.5 b	40.2 a	40.4 a	36.5 b	2.9 a

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

The impact of each K level on the development of the mycorrhizal colonization was only relevant regarding values as intensity (%M and %m) and richness of arbuscules (%a and %A). Both mycorrhizal parameters were highly depressed by 2K fertilization (Table 4).

Table 4. Mycorrhizal symbiotic development by the authochthonous AM fungal consortium (M) plus *B. thuringiensis* (B) with whatever of K fertilizers [1K (5 mM) and 2K (10 mM)] on colonization frequency (%F), intensity (%M), intensity of colonization (%m), arbuscule abundance (%a), richness of arbuscules (%A) and glomalin (μg g^{-1}soil).

	%F	%M	%m	%a	%A	μg glomalin g^{-1}soil
1K + MB	74.7 a	8.4 b	10.5 b	46.2 b	4.7 b	0.7 a
2K + MB	83.3 a	1.4 a	1.6 a	34.8 a	0.5 a	0.2 a

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=5).

Whichever of the antioxidant activities here determined were enhanced by the highest K (2K) level applied (Fig. 9). But strongest depressing effect in the antioxidant activities was observed as result of K-fertilization and inocula interaction. The lowest values of whatever antioxidant activity was measured in inoculated plants irrespective of K level being CAT and GR totally depressed. None whatever CAT and GR activities were determined in these dual

treated plants. The SOD and APX activities also decreased by the microbial inoculation applied being SOD 1.8 (1K) and 2.4 (2K) times lower while APX was reduced by 2.33 (1K) and 5.92 (2K) times (Fig. 9).

The treatments applied did not change dehydrogenase and urease enzymatic activities. Regarding β-glucosidase, this activity increased with the higher level of K and also as result of microbial inoculation. In fact, inoculated plants with 2K resulted the most effective in increasing β-glucosidase activity in rhizosphere soil (Fig. 10). Nevertheless, the inocula behaved in a different way regarding the alkaline phosphatase activity since the inocula highly enhanced this activity associated to 1K. But non-significant differences between 1K and 2K and between 2K with and without inocula were found (Fig. 10).

Here, as in the Experiment I, no nodules were formed by native rhizobial population in roots of *R. sphaerocarpa* plants in despite an extract of natural soil being added, after transplanting, in all the pots.

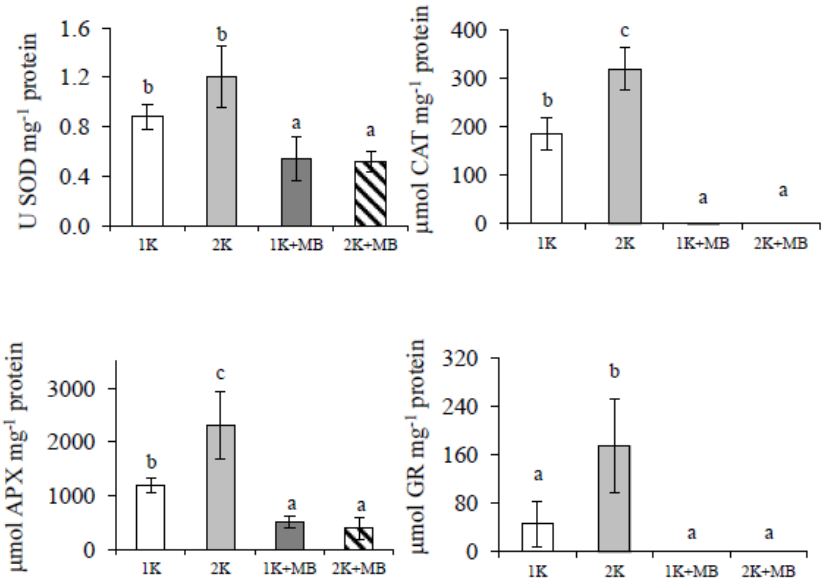


Fig.9. Comparative effect of fertilizers [1K (5mM K) and 2K (10 mM K)] in interaction or not with authochthonous mycorrhyzal fungal consortium (M) and *B. thuringiensis* (B), on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) antioxidant activities in shoot of *R. shpaerocarpa* under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

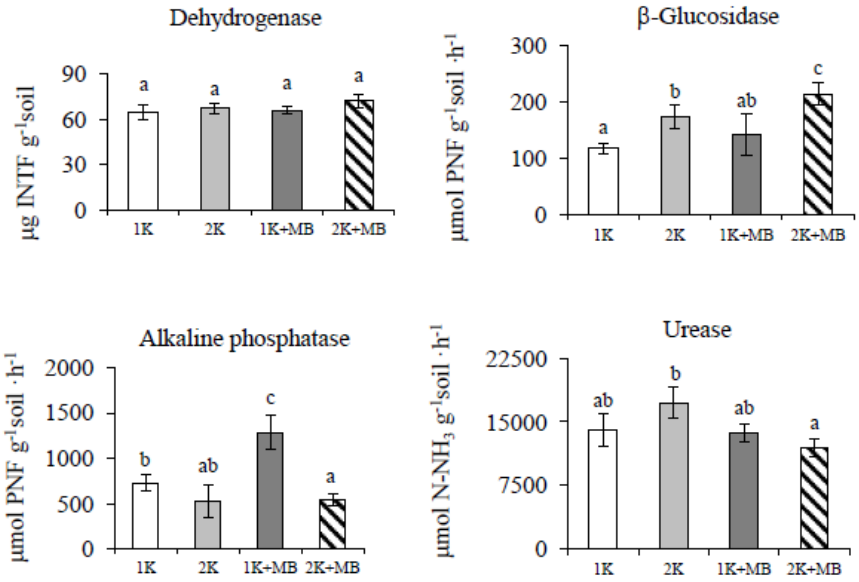


Fig.10. Comparative effect of fertilizers [1K (5mM K) and 2K (10 mM K)] in interaction or not with authochthonous mycorrhyzal fungal consortium (M) and *B. thuringiensis* (B), on dehydrogenase, β-glucosidase, alkaline phosphatise and urease enzymatic activities in soil under drought conditions. Within each value bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n=3).

4. Discussion

Here we addressed the comparative role of P fertilization rate (25 or 50 ppm) and of mycorrhizal symbiosis (from allochthonous and autochthonous strains) in the drought tolerance of *R. sphaerocarpa* plants growing in an arid Mediterranean soil.

The comparative effect of single *R. intraradices* from collection vs. the whole autochthonous AM fungal community evidenced non-significant differences in terms of mycorrhizal colonization, growth and nutritional content (K, Ca and Mg). Moreover, the fungus *R. intraradices* resulted to be more efficient on P acquisition and less on Fe, Mn, Zn and Cu. Our initial hypothesis was that the autochthonous fungal community ought to be the most efficient inocula under these experimental conditions as suggested by Caravaca et al. (2005a). But here, the allochthonous *R. intraradices* was the most effective treatment in promoting P uptake, more than the autochthonous AM consortium or whatever level of P fertilizer applied (25 or 50 ppm). Previously was reported by Marulanda et al., (2003) that the different impact of mycorrhizal inocula to improve plant growth under drought stress may be attributed to the differences in intra and/or extraradical colonization and proportion of active structures as

arbuscules. But in this study the mycorrhizal inoculated plants did not vary in their mycorrhizal development.

Results show that the mycorrhizal inoculation irrespective of fungal origin resulted as positive as phosphorus fertilization in improving *R. sphaerocarpa* growth. In fact, both mycorrhizal inocula behaved differently regarding *R. sphaerocarpa* nutrient acquisition in spite of similar frequency/intensity and arbuscular development of these mycorrhizal colonizations. No correlation between mycorrhizal root colonization and P nutrition was found since no differences in terms of intra and extraradical mycorrhizal development were observed between the two mycorrhizal inocula used.

In general, this symbiosis can increase plant biomass due to the highest acquisition of nutrients specially those having low mobility. The content of P was maximized in *R. sphaerocarpa* plants colonized by the reference *R. intraradices* and this nutritional effect could be associated with a greater ability for P transference from soil to the plant for the colonizing RI fungus. The allochthonous RI maintained its positive effect under water stress when inoculated in soil different from their isolation source.

Results of SAR also suggest the highest ability and efficiency of *R. intraradices* colonized roots to the P, K, Ca and Mg uptake. The nutritional results are not related to the growth effect when comparing chemical (1P or 2P) and biological (M or RI) fertilization. The increased K and P content in inoculated plants could enhanced some physiological and biochemical plant parameters which may be even more relevant than the nutrition in the tolerance under drought associated with the mycorrhizal activity. As it is known K has an important role as inorganic osmolyte which in turn increases the osmotic potential within the cell, while Ca is important in membrane protection and Mg modulates ionic currents across the chloroplasts and vacuole membranes (regulating stomatal opening and ion balance in cells) under dry conditions (Parida and Jha, 2013). The enhancement of Mg content in mycorrhizal plants suggests that the functioning of photosynthetic apparatus was not affected by drought in mycorrhizal colonized *R. sphaerocarpa* but drought lends to severe damage to membrane integrity in many plants (Silva et al., 2010).

Antioxidant processes reflect the modified redox status of the stressed cells in plants (Gururani et al., 2013). Thus, specific antioxidant activities, particularly APX, were the highest in P fertilized plants and the lowest in those mycorrhizal inoculated. These results suggest that water seems to be less limited in mycorrhizal plants and thus, these plants did not require to increase APX and CAT activities to counteract ROS production by drought stress (Armada et al., 2014b). Results indicate that P-fertilized plants suffer a stronger drought stress than these

mycorrhizal colonized having similar growth and nutrition. Antioxidant activities have been proposed as stress indexes since they are highly sensitive to the metabolic and physiological status of plants (Ortiz et al., 2015). The efficient destruction of O^{2-} and H_2O_2 generated under water stress requires the action of antioxidant enzymes acting in synchrony to minimize these toxic radicals. But the lowest activities found in mycorrhizal plants preclude a direct role of these enzymes on such process. Particularly APX activity is the main antioxidant related with the maintenance of osmotic balance and also it may facilitate the nutrient uptake in colonized plants (Azcón et al., 2013). Different roles of this symbiosis in drought alleviation in relation to changes in particular antioxidant activities have been reported. They ranged from increase (Garg and Kaur, 2013) to decrease (Gunes et al., 2009; Yang et al., 2009). Alternatively, different changes on such activities in plants colonized by different fungi have been found (Marulanda et al., 2007). The lowest APX activity in mycorrhizal plants than in P-fertilized plants of similar development supports the view of the crucial role of this symbiosis in alleviating drought stress. The lowest CAT and SOD activities (in AM-colonized plants) and GR activity (in *R. intraradices* inoculated plants) are also indication of such drought-tolerance in mycorrhizal plants. These results may be regarded as greater protective capacity and as a proof of maintenance water uptake under low water availability. The autochthonous fungi (M) and also RI resulted an essential component to protect plants against stress conditions (Azcón et al., 2009). But the combinations of nutritional, physiological and biochemical mechanisms seem to play a crucial role in drought tolerance. These results suggest that to successfully establishment plant in drought arid areas it is important to use efficient and adapted mycorrhizal fungi.

The comparisons of autochthonous mycorrhizal (M) and P-fertilized plants having similar P nutrition and root dry weight but different antioxidant activities and elements such as Fe, Mn, Zn and Cu have relevance and important impact in plant physiological and biochemical processes related to the drought tolerance (Ruíz-Lozano et al., 1995a; 1995b). These results allow to conclude that independent and additional mechanisms are involved in the drought tolerance of mycorrhizal plants (Augé, 2004). Nevertheless, the differences in these parameters between both mycorrhizal inocula preclude any broader generalization.

From experiment II we evidenced that the combination of autochthonous microorganisms applied (AM fungi consortium plus *B. thuringiensis*) resulted highly effective in improving P, K, Zn and Cu content only at the lowest K level applied (5 mM K) but not at the highest K level (10 mM) in the growing medium. According to these results the potential of inocula to alleviate drought stress was limited beyond a certain level of K.

The 2K level (10 mM K) increased P, K and Mn over 1K (5 mM K) in *R. sphaerocarpa* shoots. But at this highest level of K the AM colonization was highly depressed (particularly intensity and arbuscule abundance).

The inocula did not effect *R. sphaerocarpa* growth but in contrast, pronounced differences in nutrients assimilation was shown according to the inocula/K level interaction. Here, the highest P and K plant content in 1K inoculated plants correlated with the greatest intraradical mycorrhizal colonization determined. High differences in these values were observed among plants inoculated under each one of these two K levels. Differences in the amount of active fungal structures as arbuscules, is an explanation for the better fungal performance and functioning in arid environment as have suggested by Marulanda et al., (2003).

In the experiment I, whatever chemical or biological treatment applied increased dehydrogenase activity particularly each one of mycorrhizal inocula. This enzymatic activity reflected soil microbial community. The reactivation of the rhizosphere microbial populations by the inocula is indication of rehabilitation of degraded soils. β-glucosidase activity was only increased in soil inoculated with the autochthonous mycorrhizal consortium (M) that increased by 2.76 times such enzymatic value which indicates carbohydrates transformation that is important as energy source for microorganism. Consequently, mycorrhizal inoculation not only increased plant characteristics but also the microbial properties and quality of arid soils. In a previous study (Azcón et al., 2013) reported that autochthonous mycorrhizal fungi not only affected the bacterial microbial structure but also increased the microbial diversity (by 233%) compared to P fertilization.

Similarly, in the Experiment II, the inocula significantly increased the phosphatase activity (in 1K fertilized soil) and β-glucosidase activity (in 2K fertilized soil). Measurement of these soil hydrolases are indication of changes in soil fertility since they are involved in the mineralization of compounds that provide nutrients as N, P and C. The effectiveness of inocula in this experiment was based on a direct improvement of nutrient status particularly P, K, Zn and Cu. The highest P shoot content in these plants could be explained by the PGPR abilities of inocula applied and also by the highest value of phosphatase activity in the rhizosphere of these plants. The main role of this phosphatase is to catalyze the hydrolysis of organic phosphates increasing the P available to plants and thus improving plant P uptake. The enhancement of soil enzyme activities, particularly β-glucosidase, by the inocula may be related to the reactivation of the rhizosphere microbial population by increasing water soluble C. Carbohydrates are also involved in aggregate stabilization and soil water retention. Glomalin has a high C content [until 50% as Rillig et al., (2004) reported] and it also acts in the soil aggregation (Wright and

Upadhyaya, 1996). Thus, these values indicated that the applied inocula may enhance rehabilitation of arid degraded soils contributing to soil fertility and quality (Alguacil et al., 2003; Medina et al., 2004b).

As results show, the higher performance of inoculated plants than those fertilized reaffirm the important role of inocula applied in sustaining the plant cover under drought in these nutrient deficient arid soils.

In conclusion, the mycorrhizal effect in enhancing shoot biomass and growth was similar to this produced by P fertilization however a drop in particular antioxidant activities in mycorrhizal colonized plants as APX (by M and RI inocula) CAT (by M inoculum) and GR (by RI inoculum) may indicate the highest potential of mycorrhizal colonization to alleviate drought stress in these plants.

Inocula (M+B) positively interacted on nutrients acquisition with the lowest K fertilization (5 mM K) and negatively with the highest (10 mM K). But the decreasing SOD and APX activities and the suppression of CAT and GR in inoculated K-fertilized plants may indicate the highest ability of inoculated plants to cope with drought independently of nutritional status.

Mycorrhizal inoculants may be more important than chemical fertilization orchestrating antioxidant activities along the process of drought tolerance.

Soil enzymatic activities, as β-glucosidase, increased in inoculated plants indicating the improvement of physic-chemical soil characteristics.

Acknowledgments

E. Armada was financed by Ministry of Science and Innovation (Spain). This work was carried out in the framework of the project reference AGL2009-12530-C02-02. We thank the Instrumentation Service (EEZ-CSIC) for the plant analysis. We thank Shaun Smith (native English teacher) for their assistance in the revision of this article.

References

Aebi, H., 1984. Catalase *in vitro*. Methods in Enzymology 105, 121-126.

Alguacil, M.D., Roldán, A., Salinas-García, J.R., Querejeta, J.I., 2011. No tillage affects the phosphorus status, isotopic composition and crop yield of *Phaseolus vulgaris* in a rain-fed farming system. Journal of the Science of Food and Agriculture 91, 268-272.

Alguacil, M.M., Hernández, J.A., Caravaca, F., Portillo, B., Roldán, A., 2003. Antioxidant enzyme activities in shoots from three mycorrhizal shrub species afforested in a degraded semi-arid soil. Physiologia Plantarum 118, 562-570.

Amako, K., Chen, G.X., Asada, K., 1994. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant and Cell Physiology 35, 497-504.

Armada, E., Azcón, R., López-Castillo, O.M., Calvo-Polanco, M., Ruiz-Lozano, J.M., 2015. Autochthonous arbuscular mycorrhizal fungi and Bacillus thuringiensis from a degraded Mediterranean area can be used to improve physiological traits and performance of a plant of agronomic interest under drought conditions. Plant Physiology and Biochemistry 90, 64-74.

Armada, E., Portela, G., Roldán, A., Azcón, R., 2014a. Combined use of beneficial soil microorganism and agrowaste residue to cope with plant water limitation under semiarid conditions. Geoderma 232, 640-648.

Armada, E., Roldán, A., Azcón, R., 2014b. Differential activity of autochthonous bacteria in controlling drought stress in native *Lavandula* and *Salvia* plants species under drought conditions in natural arid soil. Microbial Ecology 67, 410-420.

Aroca, R., Irigoyen, J.J., Sánchez-Díaz, M., 2003. Drought enhances maize chilling tolerance. II. Photosynthetic traits and protective mechanisms against oxidative stress. Physiologia Plantarum 117, 540-549.

Aroca, R., Vernieri, P., Ruíz-Lozano, J.M., 2008. Mycorrhizal and non-mycorrhizal *Lactuca sativa* plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. Journal of Experimental Botany 59, 2029-2041.

Augé, R.M., 2004. Arbuscular mycorrhizae and soil/plant water relations. Canadian Journal of Soil Science 84, 373-381.

Azcón, R., Medina, A., Aroca, R., Ruíz-Lozano, J.M., 2013. Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast interactions, in: de Bruijn, F.J. (Ed.), Molecular Microbial Ecology of the Rhizosphere. John Wiley & Sons, Hoboken, New Jersey, USA, pp. 991-1002.

Azcón, R., Medina, A., Roldán, A., Biró, B., Vivas, A., 2009. Significance of treated agrowaste residue and autochthonous inoculates (arbuscular mycorrhizal fungi and *Bacillus cereus*) on bacterial community structure and phytoextraction to remediate soils contaminated with heavy metals. Chemosphere 75, 327-334.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Burd, G.I., Dixon, D.G., Glick, B.R., 2000. Plant growth promoting bacteria that decreased heavy metal toxicity in plants. Canadian Journal of Microbiology 46, 237-245.

Caravaca, F., Alguacil, M.M., Azcón, R., Parlade, J., Torres, P., Roldán, A., 2005a. Establishment of two ectomycorrhizal shrub species in a semiarid site after in situ amendment with sugar beet, rock phosphate, and *Aspergillus niger*. Microbial Ecology 49, 73-82.

Caravaca, F., Alguacil, M.M., Barea, J.M., Roldán, A., 2005b. Survival of inocula and native AM fungi species associated with shrubs in a degraded Mediterranean ecosystem. Soil Biology and Biochemistry 37, 227-233.

Caravaca, F., Figueroa, D., Barea, J.M., Azcon-Aguilar, C., Roldan, A., 2004. Effect of mycorrhizal inoculation on nutrient acquisition, gas exchange, and nitrate reductase activity of two Mediterranean-autochthonous shrub species under drought stress. Journal of Plant Nutrition 27, 57-74.

Carlberg, I., Mannervik, B., 1985. Glutathione reductase. Methods in Enzymology 113, 484-489.

Duncan, D.B., 1955. Multiple range and multiple F tests. Biometrics 11, 1-42.

García, C., Hernández, M.T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Communication in Soil Science and Plant Nutrition 28, 123-134.

Garg, N., Kaur, H., 2013. Response of antioxidant enzymes, phytochelatins and glutathione production towards Cd and Zn stresses in *Cajanus cajan* (L.) Millsp genotypes colonized by arbuscular mycorrhizal fungi. Journal of Agronomy and Crop Science 199, 118-133.

Giovannetti, M., Mosse, B., 1980. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytologist 84, 489-500.

Gray, J.T., Schlesinger, W.H., 1983. Nutrient use by evergreen and deciduous shrubs in Southern-California .2. Experimental investigations of the relationship between growth, nitrogen uptake and nitrogen availability. Journal of Ecology 71, 43-56.

Gunes, A., Pilbeam, D.J., Inal, A., 2009. Effect of arsenic-phosphorus interaction on arsenic-induced oxidative stress in chickpea plants. Plant and Soil 314, 211-220.

Gururani, M.A., Upadhyaya, C.P., Baskar, V., Venkatesh, J., Nookaraju, A., Park, S.W., 2013. Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance. Journal of Plant Growth Regulation 32, 245-258.

Jeffries, P., Barea, J.M., 2012. Arbuscular Mycorrhiza – a key component of sustainable plant-soil ecosystems, in: Hock, B. (Ed.), The Mycota, a comprehensive treatise on fungi as experimental systems for basic and applied research. Springer, Berlin, Heidelberg, pp. 51-75.

Kessler, B., Lorenzo, V.d., Timmis, K.N., 1992. A general system to integrate *lacZ* fusions into the chromosome of gram-negative eubacteria: regulation of the *Pm* promoter in the *TOL* plasmid studied with all controlling elements in monocopy. Molecular & General Genetics 233, 293-301.

Koussevitzky, S., Suzuki, N., Huntington, S., Armijo, L., Sha, W., Cortes, D., Shulaev, V., Mittler, R., 2008. Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. Journal of Biological Chemistry 283, 34197-34203.

Lambertsen, L., Sternberg, C., Molin, S., 2004. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. Environmental Microbiology 6, 726-732.

Loutfy, N., El-Tayeb, M.A., Hassanen, A.M., Moustafa, M.F.M., Sakuma, Y., Inouhe, M., 2012. Changes in the water status and osmotic solute contents in response to drought and salicylic acid treatments in four different cultivars of wheat (*Triticum aestivum*). Journal of Plant Research 125, 173-184.

Marulanda, A., Azcón, R., Ruíz-Lozano, J.M., 2003. Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress. Physiologia Plantarum 119, 526-533.

Marulanda, A., Barea, J.M., Azcón, R., 2006. An indigenous drought-tolerant strain of *Glomus intraradices* associated with a native bacterium improves water transport and root development in *Retama sphaerocarpa*. Microbial Ecology 52, 670-678.

Marulanda, A., Porcel, R., Barea, J.M., Azcón, R., 2007. Drought tolerance and antioxidant activities in lavender plants colonized by native drought-tolerant or drought-sensitive *Glomus* species. Microbial Ecology 54, 543-552.

Masciandaro, G., Ceccanti, B., García, C., 1994. Anaerobic digestion of straw and piggery wastewater: II. Optimization of the process. Agrochimica 3, 195-203.

Medina, A., Azcón, R., 2012. Reclamation strategies of semiarid mediterranean soil: improvement of the efficiency of arbuscular mycorrhizal fungi by inoculation of plant growth promoting microorganisms and organic amendments, in: Hafidi, M., Duponnois, R. (Eds.), The Mycorrhizal Symbiosis in Mediterranean Environment: Importance in Ecosystem Stability and in Soil Rehabilitation Strategies. Nova Science Publishers New York, pp. 87-106.

Medina, A., Vassilev, N., Alguacil, M.M., Roldán, A., Azcón, R., 2004a. Increased plant growth, nutrient uptake, and soil enzymatic activities in a desertified mediterranean soil amended with treated residues and inoculated with native mycorrhizal fungi and a plant growth-promoting yeast. Soil Science 169, 260-270.

Medina, A., Vassileva, M., Caravaca, F., Roldán, A., Azcón, R., 2004b. Improvement of soil characteristics and growth of *Dorycnium pentaphyllum* by amendment with agrowastes and inoculation with AM fungi and/or the yeast *Yarowia lipolytica*. Chemosphere 56, 449-456.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of phosphatase, urease, proteases, organic-carbon, and nitrogen from soil. Soil Science Society of America Journal 44, 1011-1016.

Naseby, D.C., Lynch, J.M., 1997. Rhizosphere soil enzymes as indicators of perturbations caused by enzyme substrate addition and inoculation of a genetically modified strain of *Pseudomonas fluorescens* on wheat seed. Soil Biology & Biochemistry 29, 1353-1362.

Ortiz, N., Armada, E., Duque, E., Roldán, A., Azcón, R., 2015. Contribution of arbuscular mycorrhizal fungi and/or bacteria to enhancing plant drought tolerance under natural soil conditions: Effectiveness of autochthonous or allochthonous strains. Journal of Plant Physiology 174, 87-96.

Panozzo, J.F., Eagles, H.A., 1999. Rate and duration of grain filling and grain nitrogen accumulation of wheat cultivars grown in different environments. Australian Journal of Agricultural Research 50, 1007-1015.

Parida, A.K., Jha, B., 2013. Physiological and biochemical responses reveal the drought tolerance efficacy of the halophyte *Salicornia brachiata*. Journal of Plant Growth Regulation 32, 342-352.

Phillips, J.M., Hayman, D.S., 1970. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society 55, 159-161.

Rengel, Z., Marschner, P., 2005. Nutrient availability and management in the rhizosphere: exploiting genotypic differences. New Phytologist 168, 305-312.

Rillig, M.C., 2004. Arbuscular mycorrhizae, glomalin, and soil aggregation. Canadian Journal of Soil Science 84, 355-363.

Romheld, V., Kirkby, E.A., 2010. Research on potassium in agriculture: needs and prospects. Plant and Soil 335, 155-180.

Ruíz-Lozano, J.M., Azcón, R., 1995. Hyphal contribution to water uptake in mycorrhizal plants as affected by the fungal species and water status. Physiologia Plantarum 95, 472-478.

Ruíz-Lozano, J.M., Azcón, R., Gómez, M., 1995a. Effects of arbuscular-mycorrhizal Glomus species on drought tolerance: Physiological and nutritional plant-responses. Applied and Environmental Microbiology 61, 456-460.

Ruíz-Lozano, J.M., Gómez, M., Azcón, R., 1995b. Influence of different *Glomus* species on the time-course of physiological plant-responses of lettuce to progressive drought stress periods. Plant Science 110, 37-44.

Silva, E.N., Ferreira-Silva, S.L., Fontenele, A.d.V., Ribeiro, R.V., Viégas, R.A., Silveira, J.A.G., 2010. Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in *Jatropha curcas* plants. Journal of Plant Physiology 167, 1157-1164.

Skujins, J., 1976. Extracellular enzymes in soil. CRC critical reviews in microbiology 4, 383-421.

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis, 3rd ed. Elsevier, Academic Press, New York.

SSS, 2006. Soil Survey Staff (SSS). "Keys to Soil Taxonomy" 10th ed. USDA. Natural Resources, Conservation Service, Washington DC.

Subramanian, K.S., Santhanakrishnan, P., Balasubramanian, P., 2006. Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress. Scientia Horticulturae 107, 245-253.

Tabatabai, M.A., 1982. Soil enzymes, in: Page, A.L., Miller, E.M., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2 2nd ed. Agron Monogr 9. ASA and SSSA, Madison, Wisconsi, pp. 501-538.

Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology & Biochemistry 1, 301-307.

Toro, M., Azcón, R., Barea, J.M., 1997. Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (P^{32}) and nutrient cycling. Applied and Environmental Microbiology 63, 4408-4412.

Trouvelot, A., Fardeau, J.C., Plenchette, C., Gianinazzi, S., Gianinazzi-Pearson, V., 1986. Nutritional balance and symbiotic expression in mycorrhizal wheat. Physiologie Vegetale 24, 300-300.

White, I., Knight, J.H., Zegelin, S.J., Topp, G.C., 1994. Comments to 'considerations on the use of time-domain reflectometry (TDR) for masuring soil water content' by WR Whalley. Journal of Soil Science 45, 503-508.

Wright, S.F., Upadhyaya, A., 1996. Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Science 161, 575-586.

Wu, H., Wu, X., Li, Z., Duan, L., Zhang, M., 2012. Physiological evaluation of drought stress tolerance and recovery in cauliflower (*Brassica oleracea* l.) seedlings treated with methyl jasmonate and coronatine. Journal of Plant Growth Regulation 31, 113-123.

Yang, J., Kloepper, J.W., Ryu, C.-M., 2009. Rhizosphere bacteria help plants tolerate abiotic stress. Trends in Plant Science 14, 1-4.

CHAPTER 5

Autochthonous arbuscular mycorrhizal fungi and *Bacillus thuringiensis* from a degraded Mediterranean area can be used to improve physiological traits and performance of a plant of agronomic interest under drought conditions

Elisabeth Armada, Rosario Azcón, Olga M. López-Castillo, Mónica Calvo-Polanco, Juan Manuel Ruiz-Lozano

Plant Physiology and Biochemistry (2015) 90: 64-74

1. Introduction

Plants are constantly confronted with environmental constraints of both biotic and abiotic origin. In particular, drought one of the most common environmental stresses experienced by soil plants (Shinozaki et al., 2003). Drought stress affects plant-water relations, as well as, specific and nonspecific physiological responses (Beck et al., 2007), causing an important detrimental effect on plant growth and nutrition and, thus, limiting crop production. In fact, drought is considered as major cause of declining crop productivity worldwide (Vinocur and Altman, 2005). There is consensus that global climate change is actually occurring and that its negative effects will probably increase in the coming years, imposing significant difficulties to plant and crop development in many areas of the world. These difficulties will be particularly important in current semi-arid agricultural zones (Denby and Gehring, 2005).

Plants usually interact with soil microorganisms that make them more efficient in coping with environmental limitations such as drought. Several strategies have been suggested to overcome the negative effects of drought (Warren, 1998). The most explored approaches have been the breeding for tolerant varieties and the use of genetic engineering. However, an alternative strategy is to induce drought stress tolerance by using beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR). There is ample information about the interactions occurring among AM fungi and PGPR, resulting in the promotion of key processes for plant nutrition, growth and health, particularly in stressed environment (Armada et al., 2014b; Marulanda-Aguirre et al., 2008; Vivas et al., 2006). Moreover, several studies have shown that using native AMF and PGPR, which appear to be physiologically and genetically adapted to the stress conditions of the environment of origin,

provides a higher benefit for plant performance than non-native isolates (Armada et al., 2014b; Oliveira et al., 2005; Querejeta et al., 2006).

Plants can tolerate severe environmental conditions such as drought, and for that they need to adapt several physiological, biochemical and cellular/molecular processes in order to maintain cell homeostasis (Urano et al., 2010). Osmotic stress is a frequent consequence of plant tissues exposed to drought that induces plant water imbalance (Beck et al., 2007). The accumulation of some metabolites in plant tissues is an important mechanism to overcome the osmotic stress (Armada et al., 2014b; Bárzana et al., 2014). Several authors have reported that PGPR inoculation provides a better plant water balance under osmotic stress (Pereyra et al., 2012). Bacteria have developed mechanisms to cope with drought stress such as the ability to enhance indole-3-acetic acid (IAA) synthesis (Marulanda et al., 2009). Moreover, activities of several bacterial enzymes involved in the ascorbate-glutathione cycle are related with the severity of the stress (Kasim et al., 2013). In many cases, inoculated drought-stressed plants showed lower antioxidant activities than non-inoculated plants. These results are indicative of the bacterial capacity to reduce reactive oxygen species (ROS) levels in drought stressed plants and were also correlated with increased physiological parameters such as photosynthesis (Armada et al., 2014a; 2014b; Kasim et al., 2013; Rueda-Puente et al., 2010).

Under drought conditions, plants have to face with the problem of acquiring sufficient amount of water from the soil (Ouziad et al., 2006), and aquaporins participate in this process (Maurel et al., 2008). Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient (Maurel et al., 2008). These proteins are present in all kingdoms and belong to the major intrinsic protein (MIP) family of transmembrane proteins. In maize two major classes of plant aquaporins are located in the plasma membrane (PIPs) and in the tonoplast (TIPs). PIPs and TIPs isoforms have been recognized as central pathways for transcellular and intracellular water transport (Maurel et al., 2008).

In the last few years, much effort has been concentrated on investigating the function and regulation of aquaporins. High levels of aquaporin expression were shown in tissues with high water fluxes across membranes (Maurel et al., 2008; Otto and Kaldenhoff, 2000). Thus, aquaporins seem to play a specifically important role in controlling transcellular water transport in plant tissues (Javot and Maurel, 2002). In any case, the relationship between aquaporins and plant responses to water deficit is still elusive and with contradictory results (Aharon et al., 2003; Lian et al., 2004). In addition, although many aquaporins are highly selective for water, uptake experiments with *Xenopus laevis* oocytes clearly showed that certain aquaporins are

permeable to small solutes such as glycerol, urea, amino acids, CO_2 and/or NH_3/NH_4 or even small peptides and ions (Kaldenhoff et al., 2007; Uehlein et al., 2007), which opens many questions about the physiological roles of aquaporins, especially in AM plants (Maurel and Plassard, 2011). Interestingly, several maize aquaporins have been shown to be regulated by the AM symbiosis under different drought scenarios, and their regulation has been related with the exchange of water and other molecules of physiological importance between the host plant and the AM fungus (Bárzana et al., 2014).

In a previous study we have shown that several native PGPRs from an arid and degraded Mediterranean area were effective in promoting plant growth and development in *Lavandula dentata* and *Salvia officinalis* growing under drought conditions in a natural soil containing also the native AM fungal population (Armada et al., 2014b). However, the question remains if these microorganisms can be also used to promote plant growth in a non-native plant of agronomic interest such as maize (*Zea mays* L.).

Maize is one of the most important crops both for human and animal consumption. According to the Maize CRP Annual Report (2013) (http://maize.org/wp-content/uploads/sites/5/2014/07/MAIZE-CRP-Annual-Report-2013-web.pdf), maize is cultivated on more than 142 million ha worldwide and it is estimated to produce around 913 million tonnes of grain per year, accounting for one third of the total global grain production. Although maize is originally from Mesoamerica, nowadays it is the third most important cereal crop and ranks first in countries with developing economies (Mejía, 2003). However, in arid and semi-arid regions and, particularly in Mediterranean areas, maize is vulnerable to adverse environmental conditions due to limited rainfall, high evapotranspiration, and high temperature (Azevedo Neto et al., 2006).

Thus, the aim of the present study was to analyse the effectiveness of drought-adapted autochthonous microorganisms (*Bacillus thuringiensis* and a consortium of AM fungi) to improve plant growth and physiology under two watering conditions of a non-native plant species which is an important cereal crop. The bacterium *B. thuringiensis* was selected as it was the most effective bacterial strain in the previous study (Armada et al., 2014b).

2. Materials and methods

2.1. Experimental design

The experiment had a 3x2 factorial design with four inoculation treatments: (1) non-inoculated control plants (C), (2) plants inoculated with *Bacillus thuringiensis* (Bt), (3) plants inoculated with a consortium of AM fungi (AMF) and (4) plants dually inoculated with AMF+Bt. In addition, plants were cultivated either under well-watered conditions throughout the entire experiment, or were subjected to drought stress for 8 weeks. Each treatment had ten replicates to give a total of 80 pots.

2.2. Molecular identification of the bacterial strain

The autochthonous bacterium, identified as *Bacillus thuringiensis,* was isolated from a semiarid soil at the Natural Ecological Park "Vicente Blanes" in Molina de Segura, (Murcia, Spain) (Armada et al., 2014b). This area suffers from drought and low nutrients availability and, as a result, desertification. Bt was the most abundant cultivable bacterial type in such arid soil. The bacterium was isolated from the above-mentioned soil (a mixture of rhizospheres from several autochthonous plant species). A homogenate of 1 g soil in 9 mL sterile water was diluted (10^{-2} to 10^{-4}), plated on three different media [Agar Yeast Mannitol, Dextrose Potato agar or Luria-Bertani agar (LB)] and then incubated at 28 ºC for 48 h, to isolate bacteria from different taxonomic groups.

Identification of isolated bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were collected, diluted, lysed and their DNA used as a template in the PCR reactions. All reactions were conducted in 25 µL volume containing PCR buffer 10X, 50 mM $MgCl_2$, 10 µM each primers: 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), (Rees et al., 2004) and 5 U/µL of *Taq* polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with the following conditions: 5 min at 95 ºC, followed by 30 cycles of 45s at 95 ºC, 45s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. The products of PCR were analysed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (NCBI) using BLAST program (Altschul et al., 1990).

2.3. Isolation and identification of the arbuscular mycorrhizal fungi (AMF)

AM fungal spores were separated from the soil samples by a wet sieving process (Sieverding, 1991). The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG) (Koske and Tessier, 1983); a mixture of PVLG and Melzer's reagent (Brundrett et al., 1994); a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain, 1990). For identification of the AM fungi species, spores were then examined using a compound microscope at up to 400-fold magnification as described for glomeromycotean classification (Oehl et al., 2011).

2.4. Soil Characteristics and inocula multiplication

The soil used was selected from an area located at the Natural Ecological Park "Vicente Blanes" in Molina de Segura, Murcia (southeastern Spain) (coordinates 38º12' N, 1º13' W, 393 m altitude). The climate is semiarid Mediterranean, with an average annual rainfall lower than 270 mm and the potential evapotranspiration (ETP) reaches approximately 1000 mm. The mean annual temperature is 19.2 ºC with absence of frost period. The soil in the experimental area is a Typic Torriorthent, very little developed, with low organic matter content and a silty clay texture that facilitates the degradation of soil structure. The vegetation in the zone is dominated by *Piptatherum miliaceum* L. Cosson., *Trifolium repens* L., with some shrubs of *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *Retama sphaerocarpa* growing in a patchy distribution.

The main soil characteristics were pH 8.90, P $1.36 \cdot 10^{-3}$ g kg^{-1} (Olsen test), organic carbon 0.94%, total N 0.22%, and an electric conductivity of 1.55 dS m^{-1}. Soil was sieved (mesh diameter = 2mm) and sterilized by steaming (100 ºC for 1 h on 3 consecutive days). Sand and vermiculite were autoclaved. *Zea mays* seeds were sown in pots containing 1.5 kg of a 1:2:2 mixture of soil: sand: vermiculite (v/v/v). Plants were inoculated with the appropriate inocula at sowing time.

One millilitre of pure bacterial culture (10^7 cfu mL^{-1}), grown in LB medium for 48 h at 28 ºC, was applied to the appropriate pots four days after sowing. The bacterial inoculum was applied again 15 days later. In control treatments, 1 mL of sterilized bacterial culture was added. The AM fungal consortium was multiplied in an open pot culture with sorghum. Five grams of AM fungal consortium, containing soil, root fragments and fungal spores and mycelia were applied to each one of the appropriate pots at sowing time, just below the maize seeds. Non-inoculated control plants received the same amount of autoclaved mycorrhizal inoculum

together with a 3 ml aliquot of a filtrate (<20 μm) from the AM inoculum in order to provide a general microbial population free of AM propagules.

2.5. Plant growth conditions

Plants were grown for 2.5 months in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C and 50% relative humidity. The photosynthetic photon flux density (PPFD) was $700 \cdot 10^{-6}$ mol m^{-2} s^{-1}, as measured with a light-meter (LICOR, model LI-188B). During the first 2 weeks of plant growth, water was supplied daily to reach 100% of water-holding capacity. After this time, plants from the drought treatment were allowed to dry until soil water content was 50% of water holding capacity, and maintained under these conditions for additional 8 weeks. However, during the 24-h period comprised between each rewatering the soil water content was progressively decreasing until a minimum value of 30% of water holding capacity. Soil moisture was measured with ML2X ThetaProbe (AT Delta-T Devices Ltd, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil (Roth et al., 1992; White et al., 1994). During the growing period, Hewitt´s nutrient solution was applied weekly (10 mL pot^{-1}) modified to have ½ N and ¼ P concentrations.

2.6. Parameters measured

2.6.1. Biomass production and nutrients acquisition

At harvest time, shoots were excised from the roots, and both shoots and roots were weighted to record fresh weights (ten replicates per treatment, n=10) and root length measured. After that, they were dried for 2 days at 75 ºC to obtain dry weights.

Shoot mineral analysis of N, C, P, K, Mg and Ca (mg $plant^{-1}$), as well as, of B, Fe, Zn and Cu (μg $plant^{-1}$) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Mineral analyses were carried out by the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

2.6.2. Symbiotic development

Roots were carefully washed and stained as described in Phillips and Hayman (1970). The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples, using the gridline intersect method (Giovannetti and Mosse, 1980).

2.6.3. Photosynthetic efficiency

Photosystem II efficiency was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll *a* fluorescence. FluorPen quantifies the quantum yield of photosystem II as the ratio between the actual fluorescence yield in the light-adapted state (F_v) and the maximum fluorescence yield in the light-adapted state (F_m), according to Oxborough and Baker (1997). Measurements of photosynthetic efficiency were taken in the second youngest leaf of each plant.

2.6.4. Stomatal conductance

Stomatal conductance was determined 2 h after the light turned on by using a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the second youngest leaf from each plant.

2.6.5. Shoot water potential

Mid-day leaf water potential (Ψ) was determined one day before harvest with a C-52 thermocouple psychrometer chamber and a HR-33T microvoltmeter (Wescor Inc., Logan, UT, USA). Leaf discs were cut, placed inside the psychrometer chamber and allowed to reach temperature and water vapour equilibrium for 15 min before measurements were made by the dew point method.

2.6.6. Electrolyte leakage

Leaf electrolyte leakage was determined in six plants per treatment (n=6). Leaf samples were washed with deionized water to remove surface-adhered electrolytes. The samples were placed in closed vials containing 10 mL of deionized water and incubated at 25 ºC on a rotary shaker for 24 h, and the electrical conductivity of the solution (L_0) was determined using a conductivity meter (Metler Toledo AG 8603, Switzerland). Samples were then autoclaved at 120 ºC for 20 min and the final electrical conductivity (L_f) was obtained after cooling at 25 ºC. The electrolyte leakage was defined as follows: $(L_o\text{-}L_{water})/(L_f\text{-}L_{water})$ X 100, where L_{water} is the conductivity of the deionized water used to incubate the samples.

2.6.7. Leaf Photosynthetic Pigment Contents

Photosynthetic pigments were extracted in 100% methanol from leaf samples (0.2 g). Extinction coefficients and equations reported by Lichtenthaler (1987) were used to calculate the pigment concentrations.

2.6.8. Oxidative damage to lipids and hydrogen peroxide content

Lipid peroxides were extracted by grinding 0.5 g of shoots and roots with an ice-cold mortar and 5 mL of trichloroacetic acid (TCA) 5%. Homogenates were centrifuged at 12,290 *g* for 10 min. The chromogen was formed by mixing 0.5 mL of supernatant with 1.5 mL of a reaction mixture containing 20% (w/v) TCA, 0.5% (w/v) 2-thiobarbituric acid (TBA), and by incubating the mixture at 95 ºC for 30 min (Minotti and Aust, 1987). After cooling at room temperature, absorbance was measured at 532 nm. Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge (1989). The calibration curve was made using MDA in the range of 0.1-100 µmol. The blank for all samples was prepared by replacing the sample with extraction medium.

Hydrogen peroxide content in shoots and roots was determined by Patterson's method (1984), with slight modifications as described by Aroca et al. (2003). Five hundred milligrams of shoot fresh weight was homogenized in a cold mortar with 5 mL 5% (w/v) TCA containing 0.01 g of activated charcoal and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 3,070 *g* for 10 min. The supernatant was filtered through a Millipore filter (0.22 µm). A volume of 1.0 mL of 100 mM potassium phosphate buffer (pH 8.4) and 1.0 mL of the colorimetric reagent were added to 100 µL of the supernatant. The colorimetric reagent was freshly made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4-2 (2-pyridylazo) resorcinol (disodium salt). The samples were incubated at 45 ºC for 1 h and the absorbance at 508 nm was recorded. The calibration curve was made using H_2O_2 in the range of 50-1000 µmol. The blank was made by replacing plant extract by TCA 5%.

2.6.9. Shoot proline content

The proline was extracted in 100 mM phosphate buffer (pH 7.8) from 0.5 g of fresh shoots and roots. Proline was determined by spectrophotometric analysis at 520 nm using the ninhydrin reaction according to Bates et al. (1973).

2.6.10. Total ascorbate and glutathione content

Total ascorbate was quantified photometrically by the reduction of 2,6-dichlorophenolindophenol (DCPIP) as described by Leipner et al. (1997). Five hundred milligrams of the youngest fully developed leaves of each plant group were homogenized in 5 mL ice-cold 2% (w/v) metaphosphoric acid in the presence of 1 g NaCl. The homogenate was filtered through a filter paper. An aliquot of 3 µL was mixed with 20 µL 45% (w/v) K_2HPO_4 and 10 µL homocysteine 0.1%. After 15 min incubation at 25 ºC, 100 µL citrate-phosphate buffer 2

M (pH 2.3) and 100 µL DCPIP 0.003% (w/v) were added. The absorbance was measured at 524 nm. Total ascorbate is expressed in mmol ascorbate g^{-1} shoot or root dry weight.

Glutathione content was measured as described by Smith (1985). Five hundred milligrams of the youngest fully developed leaves of each plant group were homogenized in a cold mortar with 5 mL 5% (w/v) sulfosalicylic acid and the homogenate was filtered and centrifuged at 10,000 rpm for 10 min. One milliliter of supernatant was mixed with 1.5 mL 0.5 M K-phosphate buffer (pH 7.5). The standard incubation medium was a mixture of: 0.5 mL 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.2 mL 6 mM 5, 5'-dithiobis-(2-nitrobenzoic acid), 0.1 mL 2 mM NADPH, and 0.1 mL (1 unit) glutathione reductase. The reaction was initiated by the addition of 0.1 mL glutathione standard or of extract. The absorbance was measured at 412 nm and expressed mmol glutathione g^{-1} shoot or root dry weight.

2.6.11. Root hydraulic conductivity (Lpr)

The L_{pr} was determined in six plants per treatment (n=6), using a high pressure flow meter (HPFM, Dynamax, Inc.), between 3 and 4 h after sunrise. The roots were detached from the shoot with a razor blade and, immediately after excision, connected to the HPFM. Water was pressurized into the roots from 0 to 0.5 MPa in the transient mode to calculate root hydraulic conductance (K_r). L_{pr} was determined by dividing K_r by the root fresh weight (Calvo-Polanco et al., 2014).

2.6.12. Molecular analyses

Total RNA was isolated from maize shoots and roots by a phenol/chloroform extraction method, followed by LiCl precipitation (Kay et al., 1987). DNase treatment of total RNA and cDNA synthesis were done with Quantitec Reverse Transcription kit (Qiagen, Hilden, Germany). The expression of the PIP aquaporin subfamily from maize was determined by means of real-time quantitative RT-PCR (iCycler system Bio-Rad, Hercules, CA, U.S.A.), adjusting protocols to optimize the PCR reaction to each gene. The primer sets used to amplify each gene in the synthesized cDNAs were designed in the 3' and 5' untranslated regions of each gene (the less conserved regions) in order to avoid unspecific amplifications of the different PIPs (Hachez et al., 2006). The efficiency of the primer sets was evaluated as described by Bárzana et al. (2014).

Standardization was carried out by measuring the expression levels of four different housekeeping genes from maize: poliubiquitin (gi:248338), tubulin (gi:450292), GAPDH (gi:22237) and elongation factor 1 (gi:2282583). After analyses, the best scoring genes were

selected. Thus, poliubiquitin gene was chosen in shoots and GAPDH gene was chosen for roots, as the most stable genes in all the treatments. Real-time PCR experiments were carried out in three independent RNA samples and at least three times for each sample, with the threshold cycle (CT) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Negative controls without cDNA were used in all PCR reactions.

2.6.13. Statistical analyses

Data were analyzed using SPSS 21 software package for Windows and subjected to one-way general linear model ANOVA (analysis of variance). The Duncan's (Duncan, 1955) multiple-range test was used for post-hoc comparisons to determine differences between means. Differences were considered significant at $p \leq 0.05$. Percentage values were arc-sine transformed before statistical analysis.

3. Results

3.1. Identification and characteristics of microorganisms used as inocula

Each bacterial sequence was compared with the 16S rDNA database. Similarity searches at NCBI using BLAST program, unambiguously identified the bacterium as *Bacillus thuringiensis* (Acession NR 043403.1, similarity >98%).

The more predominant AMF species identified in the native consortium used in this study area were: *Septoglomus constrictum*, *Diversispora aunantia*, *Archaespora trappei*, *Glomus versiforme*, and *Paraglomus ocultum*, which were catalogued and included in the collection of EEZ (codes EEZ 198 to EEZ 202, respectively).

B. thuringiensis (Bt) grown under osmotic stress [induced with 40% polyethylene glycol (PEG) (equivalent to -3.99 MPa)], decreased cell growth and certain plant growth promoting abilities (data not shown). In fact, the stress increased proline and ACC production, but did not change the levels of IAA and reduced slightly the phosphate solubilisation ability. Thus, the stress applied in the culture medium to test the bacterial stress tolerance and its PGPR abilities did not reduce significantly the bacterial potential to improve plant growth by mechanisms such as IAA and ACC production or phosphate solubilisation.

3.2. Plant growth and symbiotic development

Under well-watered conditions, the applied microbial treatments did not affect significantly shoot or root biomass but affect significantly in root length (single inoculation of Bt or AM increased in a 13% and dually inoculated increased by 20% compared with non-inoculated control plants). Nevertheless, under drought conditions the greatest maize shoot development were achieved in plants inoculated with Bt (30% of increase in shoots over non-inoculated control plants) and dually inoculated with AM fungi plus Bt (32.5% of increase in shoots over non-inoculated control plants). Drought stress had a negative effect in reducing shoot and root growth (Table 1). The lower negative effect of drought was observed in plants dually inoculated, where only a slight reduction of shoot and root dry weights were found. Plants inoculated with AM+Bt showed the highest root length under drought stress conditions (Table 1).

AM-colonization was not observed in non-inoculated plants at harvest time, 75 days after inoculation. No differences in the percentage of root colonization were observed between well-watered and drought-stressed maize plants (Table 1). Similarly, no significant differences on this parameter were observed in dually inoculated plants.

Table 1 Shoot and root dry weights, root length and AMF colonization in non-inoculated maize plants (C), plants singly inoculated with Bacillus thuringiensis (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		Shoot dry weight (g)	Root dry weight (g)	Root length (cm)	AMF (%)
Well-watered	C	1.00 ± 0.05 a	0.71 ± 0.03 a	391 ± 20.4 a	0 ± 0.00 a
	Bt	1.02 ± 0.07 a	0.74 ± 0.04 a	442 ± 20.9 ab	0 ± 0.00 a
	AM	0.97 ± 0.06 a	0.65 ± 0.03 a	441 ± 10.7 ab	22 ± 0.02 b
	AM + Bt	0.94 ± 0.07 a	0.64 ± 0.05 a	470 ± 20.5 b	23 ± 0.05 b
Drought	C	0.77 ± 0.04 a	0.66 ± 0.04 a	410 ± 20.4 b	0 ± 0.00 a
	Bt	1.00 ± 0.08 b	0.62 ± 0.07 a	342 ± 30.1 a	0 ± 0.00 a
	AM	0.87 ± 0.05 ab	0.64 ± 0.04 a	411 ± 20.2 b	16 ± 0.02 b
	AM + Bt	1.02 ± 0.07 b	0.74 ± 0.07 a	480 ± 20.4 c	23 ± 0.04 b

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (for shoot and root dry weights and AMF colonization n = 10. For root length n = 5).

3.3. Accumulation of macro and micronutrients

The different treatments applied had an important effect on plant nutrients acquisition. Results showed that microbial treatments were the main source of variation in the uptake of nutrients. Plants inoculated with microorganisms increased nutrients acquisition as compared to control plants, both under well-watered and under drought conditions (Table 2 and 3). Drought reduced the uptake of N, C, P, K, Mg and Ca as well as B, Zn and Cu. In general, under drought conditions the inoculation of *B. thuringiensis* induced an increase in the tissue contents of nutrients. The single inoculation of this microorganism in drought-stressed plants increased N, C, P and K by 51%, 23.6%, 37% and 38%, respectively (Table 2 and 3). A similar trend was observed for micronutrients, which were maximized by the bacterial inoculation and increased by 58% (Mg), 35% (Ca), 43% (B), 95% (Fe), 65.8% (Zn) and 76% (Cu) (Table 2 and 3). Under well-watered conditions the bacterium did not significantly affect these nutritional values.

Table 2 Contents of N and C in shoots and total chlorophylls and carotenoids in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		N (mg plant^{-1})	C (mg plant^{-1})	Total chlorophylls (mg g^{-1} DW)	Total carotenoids (mg g^{-1} DW)
Well-watered	C	12.57 ± 1.4 a	392.4 ± 17.8 a	4.08 ± 1.1 a	285.08 ± 73.8 a
	Bt	13.27 ± 1.0 a	414.4 ± 21.2 a	4.07 ± 0.7 a	289.10 ± 53.3 a
	AM	11.13 ± 1.2 a	395.3 ± 16.8 a	4.93 ± 1.4 a	337.47 ± 92.6 a
	AM + Bt	11.66 ± 0.7 a	398.6 ± 34.9 a	4.47 ± 0.9 a	308.26 ± 65.6 a
Drought	C	9.23 ± 1.2 a	320.9 ± 15.4 a	2.61 ± 0.4 a	186.36 ± 40.8 a
	Bt	13.95 ± 0.7 b	396.7 ± 19.5 b	3.35 ± 0.7 a	223.41 ± 46.2 a
	AM	8.18 ± 0.5 a	366.0 ± 13.6 ab	3.19 ± 0.8 a	227.89 ± 63.4 ab
	AM + Bt	8.64 ± 0.4 a	410.2 ± 20.6 b	4.04 ± 1.2 a	275.27 ± 83.2 b

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 5).

Table 3 Contents of P, K, Mg, Ca, B, Fe, Zn and Cu in shoots in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		P (mg plant^{-1})	K (mg plant^{-1})	Mg (mg plant^{-1})	Ca (mg plant^{-1})	B (µg plant^{-1})	Fe (µg plant^{-1})	Zn (µg plant^{-1})	Cu (µg plant^{-1})
Well-watered	C	0.53 ± 0.02 a	39.81 ± 2.5 a	4.40 ± 0.4 a	5.62 ± 0.6 a	7.54 ± 1.3 b	35.79 ± 8.1 a	11.69 ± 1.1 a	6.17 ± 0.5 b
	Bt	0.57 ± 0.02 a	42.01 ± 2.2 a	5.62 ± 0.4 b	6.48 ± 0.6 a	8.79 ± 0.5 b	30.82 ± 4.3 a	13.82 ± 1.5 ab	7.42 ± 0.5 b
	AM	0.74 ± 0.04 b	39.36 ± 2.5 a	3.96 ± 0.3 a	4.81 ± 0.4 a	4.45 ± 0.5 a	26.57 ± 3.5 a	15.13 ± 1.4 ab	3.67 ± 0.3 a
	AM + Bt	0.80 ± 0.03 b	35.37 ± 1.7 a	4.23 ± 0.4 a	5.01 ± 0.4 a	5.05 ± 0.5 a	29.10 ± 4.6 a	17.79 ± 1.5 b	4.74 ± 0.4 a
Drought	C	0.35 ± 0.02 a	27.81 ± 1.8 a	3.39 ± 0.3 a	4.53 ± 0.5 a	5.75 ± 0.5 a	42.08 ± 5.9 a	9.87 ± 1.1 a	4.48 ± 0.5 a
	Bt	0.48 ± 0.03 b	38.42 ± 1.3 b	5.35 ± 0.4 c	6.17 ± 0.6 a	8.21 ± 0.6 b	82.14 ± 3,2 b	16.37 ± 0.8 b	7.88 ± 0.6 b
	AM	0.65 ± 0.03 c	31.91 ± 1.5 a	4.12 ± 0.4 ab	5.40 ± 0.5 a	4.89 ± 0.4 a	33.67 ± 3.3 a	13.90 ± 0.9 b	4.02 ± 0.4 a
	AM + Bt	0.73 ± 0.05 c	37.13 ± 1.8 b	4.96 ± 0.4 bc	5.86 ± 0.5 a	6.05 ± 0.7 a	36.94 ± 9.7 a	13.92 ± 0.7 b	4.66 ± 0.7 a

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 5).

3.4. Leaf photosynthetic efficiency, stomatal conductance, water potential, electrolyte leakage and photosynthetic pigments

The effectiveness of AM fungi in increasing photosynthetic efficiency and stomatal conductance was more relevant under well-watered than under drought conditions (Table 4). Microbial treatments did not significantly affect shoot water potential at any water level applied (Table 4).

Regarding the membrane electrolyte leakage, the drought stress treatment increased this value in control plants, while AM colonization highly reduced this value regardless of the watering regime. Such decrease was more important under drought than under well-watered conditions (Table 4).

The leaf content of carotenoids was reduced by drought conditions. However, under stress conditions, plants dually inoculated with AM+Bt had the highest carotenoids content (Table 2). The chlorophyll content was not significantly enhanced by the inoculation with microorganisms, and it decreased as a consequence of drought.

Table 4 Photosynthetic efficiency, stomatal conductance, shoot water potential, root hydraulic conductivity (L_{pr}) and leaf electrolyte leakage in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		Photosynthetic efficiency (Fv/Fm)	Stomatal conductance (mmol H_2O m^{-2} s^{-1})	Shoot water potential (MPa)	L_{pr} (mg H_2O g^{-1}RFW MPa^{-1} $h^{-1}x10^{-6}$)	Leaf electrolyte leakage (%)
Well-watered	C	0.22 ± 0.03 a	14.80 ± 0.8 ab	-3.31 ± 0.3 a	3.22 ± 6.9 a	6.41 ± 0.5 a
	Bt	0.21 ± 0.04 a	14.20 ± 0.8 a	-3.43 ± 0.2 a	2.99 ± 5.0 a	13.43 ± 3.1 b
	AM	0.35 ± 0.02 b	19.08 ± 2.1 b	-3.72 ± 0.1 a	3.49 ± 3.7 a	4.49 ± 0.8 a
	AM + Bt	0.39 ± 0.02 b	23.57 ± 1.8 c	-3.35 ± 0.1 a	2.14 ± 2.4 a	3.16 ± 0.4 a
Drought	C	0.26 ± 0.02 a	12.80 ± 0.4 a	-3.77 ± 0.4 a	2.38 ± 7.2 a	8.46 ± 0.9 b
	Bt	0.33 ± 0.03 a	14.47 ± 2.3 a	-3.69 ± 0.1 a	2.58 ± 1.4 a	8.10 ± 1.7 b
	AM	0.34 ± 0.02 a	17.52 ± 1.5 a	-4.26 ± 0.5 a	6.95 ± 9.4 b	3.57 ± 0.5 a
	AM + Bt	0.30 ± 0.02 a	18.23 ± 2.1 a	-3.26 ± 0.2 a	5.18 ± 1.3 ab	4.05 ± 0.8 ab

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 10). For L_{pr}: n = 6.

3.5. Root hydraulic conductivity (Lpr)

We measured root hydraulic conductivity (L_{pr}) in order to analyse the influence of the different treatments on root water transport capacity (Table 4). Under well-watered conditions inoculation of microorganisms did not significantly affect the hydraulic conductivity of maize root. In contrast, under drought conditions, AM plants increased L_{pr} by 192% and AM+Bt plants by 117%, when compared to non-inoculated control plants. The single inoculation with Bt did not significantly affect this parameter.

3.6. Oxidative damage to lipids, H_2O_2, proline, ascorbate and glutathione accumulation in shoot and root tissues

The most significant effect of the inocula applied on these parameters was found in root tissue under drought conditions (Table 5 and 6).

In roots under drought stress, MDA, H_2O_2, proline and glutathione accumulation were considerably lower in the inoculated treatments than in the control one (Table 6). The mycorrhizal association highly decreased the values of MDA by 71% and H_2O_2 by 58%. Mycorrhizal plants decreased proline in shoots (by 36%) compared with control ones, but such decrease was greater in roots (88.6%), and dual inoculation reduced proline by 55%. In root of Bt-inoculated plants proline was lowered by 79% as compared to control plant.

Glutathione accumulation resulted more affected by inoculants than ascorbate. In shoots, inoculation of AM and AM+Bt increased glutathione levels by 63% and by 54% under well-watered conditions. In roots it decreased by 20% and by 37%, respectively. Under drought conditions glutathione decreased in roots, particularly after dual AM+Bt inoculation (by 56%) (Table 5 and 6).

Table 5 Shoot oxidative damage to lipids (measured as malondialdehyde equivalents, MDA) and contents of hydrogen peroxide, proline, ascorbate and glutathione in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		MDA ($\mu mol\ g^{-1}$ DW)	H_2O_2 ($\mu mol\ g^{-1}$ DW)	Proline ($\mu mol\ g^{-1}$ DW)	Ascorbate ($mmol\ g^{-1}$ DW)	Glutathione ($mmol\ g^{-1}$ DW)
Well-watered	C	193.5 ± 5.9a	319.0 ± 11.1 a	553.5 ± 142.9 a	292.2 ± 12.7 ab	130.3 ± 7.8 a
	Bt	184.3 ± 17.0a	321.6 ± 11.3 a	784.3 ± 165.3 a	300.5 ± 10.9 ab	136.0 ± 16.1 a
	AM	194.8 ± 19.5a	330.1 ± 36.8 a	520.4 ± 58.5 a	324.6 ± 15.9 b	213.7 ± 14.8 b
	AM + Bt	260.3 ± 54.5a	393.4 ± 46.8 a	491.4 ± 200.3 a	286.3 ± 11.4 a	200.4 ± 10.7 b
Drought	C	611.3 ± 140.0a	395.4 ± 83.2 a	889.2 ± 64.8 b	364.3 ± 28.6 a	179.6 ± 39.3 ab
	Bt	315.2 ± 53.7a	273.2 ± 66.1 a	982.1 ± 122.9 b	373.0 ± 22.2 a	132.1 ± 7.1 a
	AM	365.2 ± 69.9a	287.1 ± 70.9 a	571.7 ± 82.0 a	381.4 ± 12.4 a	241.7 ± 20.8 b
	AM + Bt	447.5 ± 129.1a	348.3 ± 97.5 a	524.0 ± 45.1 a	384.4 ± 8.9 a	187.3 ± 36.6 b

For each paramenter the means ± standard errors ate given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 4).

Table 6 Root oxidative damage to lipids (measured as malondialdehyde equivalents, MDA) and contents of hydrogen peroxide, proline, ascorbate and glutathione in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		MDA ($\mu mol\ g^{-1}$ DW)	H_2O_2 ($\mu mol\ g^{-1}$ DW)	Proline ($\mu mol\ g^{-1}$ DW)	Ascorbate ($mmol\ g^{-1}$ DW)	Glutathione ($mmol\ g^{-1}$ DW)
Well-watered	C	36.3 ± 10.4 a	173.1 ± 45.8 a	457.9 ± 123.8 a	926.2 ± 104.9 a	1318.6 ± 94.6 c
	Bt	22.9 ± 5.2 a	364.3 ± 71.2 ab	682.3 ± 101.7 a	848.6 ± 53.6 a	1368.2 ± 63.5 c
	AM	16.8 ± 2.1 a	496.4 ± 100.8 ab	561.4 ± 96.7 a	838.3 ± 55.1 a	1060.4 ± 54.2 b
	AM + Bt	21.4 ± 3.8 a	540.1 ± 170.5 b	662.2 ± 141.1 a	800.3 ± 30.1 a	827.9 ± 22.4 a
Drought	C	113.7 ± 8.6 bc	560.9 ± 122.1 b	1754.7 ± 129.8 c	572.6 ± 86.3 a	1623.5 ± 250.5 c
	Bt	153.0 ± 39.4 c	406.3 ± 61.2 ab	366.6 ± 141.2 ab	423.7 ± 166.7 a	1116.7 ± 113.0 b
	AM	33.3 ± 20.4 a	237.1 ± 45.4 a	200.1 ± 0.0 a	511.5 ± 84.8 a	1141.2 ± 90.9 b
	AM + Bt	48.1 ± 11.5 ab	272.4 ± 57.7 a	782.0 ± 263.9 b	541.6 ± 24.8 a	714.4 ± 155.0 a

For each parameter the means ± standard error are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 4).

3.7. Aquaporin gene expression

The maize PIP aquaporin subfamily was analyzed both in shoots and in roots. Several of these genes resulted unaffected by the treatments applied (data not shown). The expression of genes presented in Figures 1 to 4 resulted regulated by the treatments applied. Thus, under well-watered conditions, the expression of *ZmPIP1;2*, *ZmPIP1;5* and *ZmPIP1;6* was inhibited in shoot tissues by inoculation with Bt, while the gene *ZmPIP2;1* was up-regulated (Fig.1). The genes *ZmPIP2;4* and *ZmPIP2;6* were inhibited by either AM, Bt or their combination (AM+Bt).

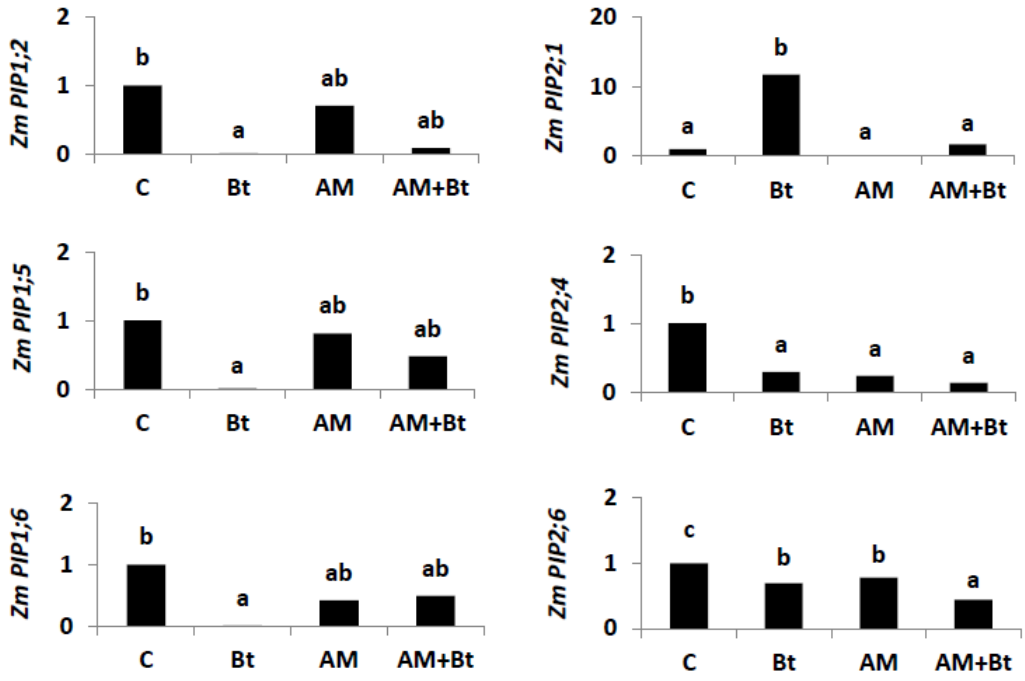


Fig 1. Shoot gene expression (in relative units) of maize aquaporins in non-inoculated plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered conditions. Values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test.

In roots, a different effect was observed, since AM inoculation alone or in combination with Bt enhanced the expression of *ZmPIP1;2*, *ZmPIP2;1*, *ZmPIP2;2*, *ZmPIP2;5* and *ZmPIP2;6* (Fig.2). Inoculation with Bt alone inhibited the expression of several aquaporins, while it increased the expression of *ZmPIP2;3* (both alone and in combination (AM+Bt)).

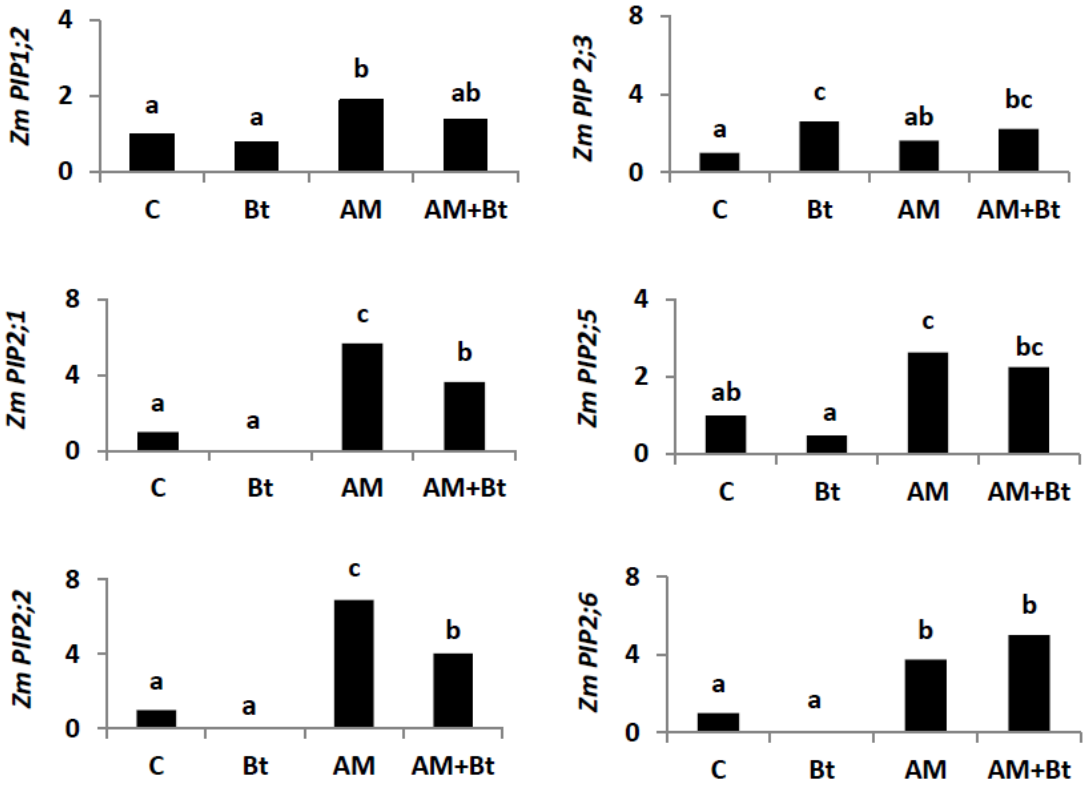


Fig 2. Root gene expression (in relative units) of maize aquaporins. See legend for Fig. 1.

Under drought stress conditions, the inoculation with the AM fungi up-regulated the expression of *ZmPIP1;6* and *ZmPIP2;5* in shoots, but such up-regulation was abolished when Bt was co-inoculated with the fungi (Fig.3). The genes *ZmPIP2;4* and *ZmPIP2;6* were down regulated by either Bt or AM inoculation. In roots, the inoculation of the AM fungi up-regulated the expression of *ZmPIP2;3* and *ZmPIP2;4* and the double inoculation AM+Bt up-regulated *ZmPIP1;1*, *ZmPIP1;3*, and *ZmPIP2;3* (Fig.4).

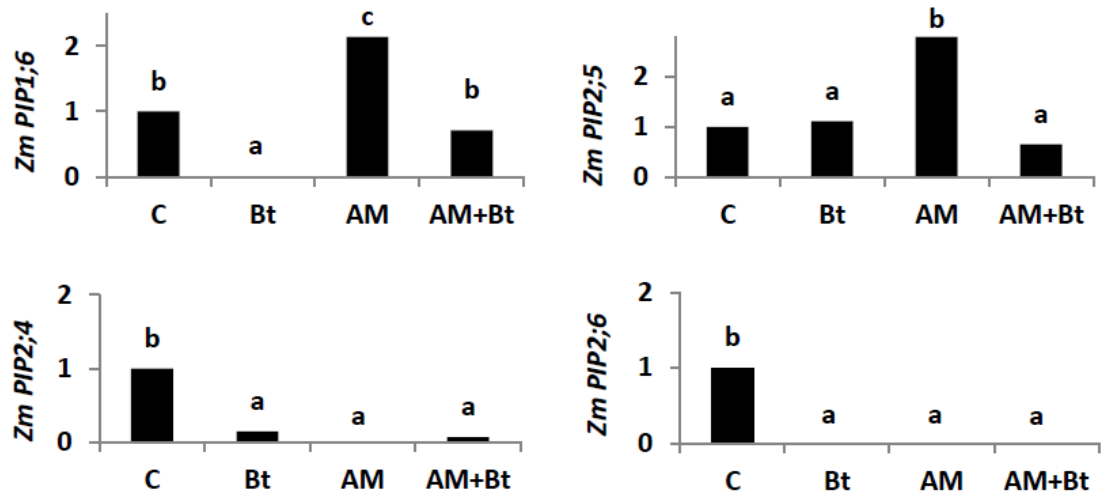


Fig 3. Shoot gene expression (in relative units) of maize aquaporins in non-inoculated plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under drought conditions. Values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test.

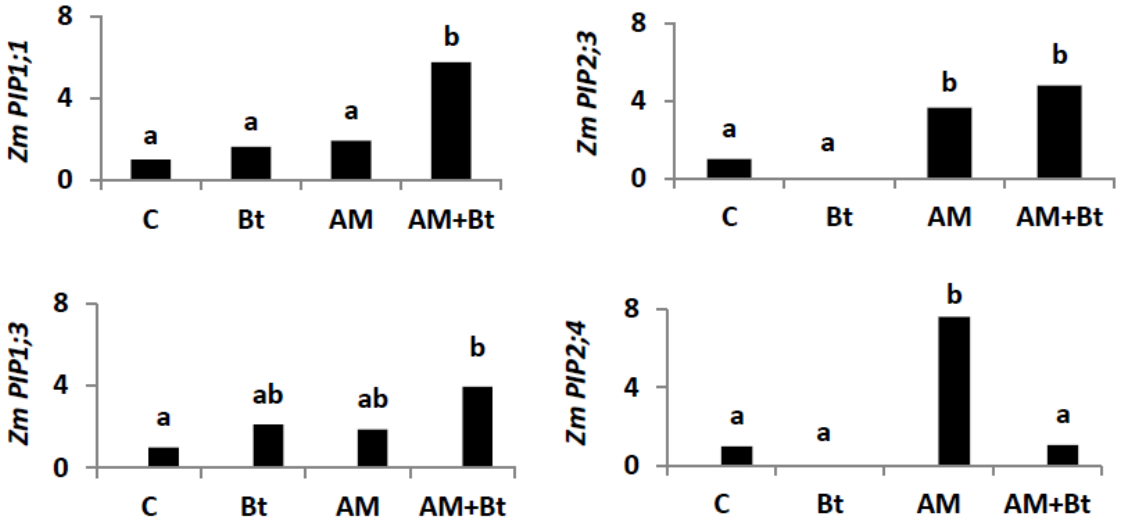


Fig 4. Root gene expression (in relative units) of maize aquaporins. See legend for Fig. 3.

4. Discussion

The microbial inoculants applied affected several physiological and molecular processes such as nutrients acquisition, plant biomass production, antioxidative plant responses and expression of aquaporins genes. Indeed, these processes could be particularly regulated according to the inoculant abilities, resulting in a better root development and enhanced nutrition and physiological/biochemical plant values, which represent adaptations to support and counteract the water limiting conditions (Aroca et al., 2012).

In this study, drought highly reduced growth in non-inoculated maize plants but such biomass reduction was smaller in inoculated plants, particularly in those dually inoculated. Also, C, P, K, Ca and Mg were not reduced by drought in these AM+Bt inoculated plants. The non-significant reduction of C in inoculated plants under drought means that the bacterium had a non-limited energy source for its growth and for its activities, allowing its maximum potential. As well, the C fungal requirements resulted compensated by the benefits provided by the symbiotic association (Gianinazzi et al., 2010).

The enhancement of P nutrition is considered an important mechanism of AM-colonized plants to improve growth and water status (Marulanda-Aguirre et al., 2008). As data show, P was the only nutrient increased in shoots by AMF under well-watered conditions (7% by AM and 51% by AM+Bt). Nevertheless, under drought stress such mycorrhizal effect increased up to 86% (AM) and 108% (AM+Bt). Bt inoculation alone also enhanced the uptake of this nutrients by 37%. This mycorrhizal effect on P acquisition could be linked to a lower electrolyte leakage and higher glutathione accumulation in these colonized plants (Ruíz-Sánchez et al., 2011). These treatments stimulated root growth and, probably the lateral root formation, thus increasing the water uptake capacity of inoculated plants. This may also affect the switching of water flow through apoplastic or symplastic pathways, thereby improving plant stress tolerance (Bárzana et al., 2012).

Maize is sensitive to water shortages and maize plants are able to maintain C assimilation during drought by decreasing transpiration (Ghannoum, 2009). To improve maize productivity under drought, it may be advantageous to consider whole plant strategies such as root traits, uptake and distribution of nutrients into plant tissues, accumulation of compatible solutes, reduction of oxidative damage, and regulation of water uptake and transport by means of aquaporins (Boomsma and Vyn, 2008). In addition, the maintenance of physiological values such as photosynthesis is also important (Türkan and Demiral, 2009). Photosynthesis performance is the most important factor influencing the plant growth and survival. In this study, dually inoculated plants showed the greatest stomatal conductance and a better

functioning of the photosynthetic machinery, which may explain why dually inoculated plants were less affected by drought stress in spite of the higher plant biomass (increased by 111%) of these plants.

The photosynthetic efficiency values increased by microbial inoculation under well-watered conditions, and can be considered as an important process to increase physiological status in these non-stressed plants. Drought stressed maize plants synthesized more proline in shoot and root tissues than well-watered plants. Proline enables the plant to maintain an osmotic balance under low water potential by adjusting osmotic potential and stabilizing membranes and proteins (Mäkelä et al., 2000; Yoshiba et al., 1997). Concomitantly, stressed plants showed the highest oxidative damage to lipids and glutathione accumulation (in shoots and roots) and ascorbate in shoots. Curiously, the lowest enhancements in proline (shoots and roots) and glutathione in roots were found in mycorrhizal plants. The results obtained evidenced that the PGP microorganisms applied alleviated the oxidative stress generated in maize plants by the water limitation, causing a decrease of MDA and H_2O_2 levels. These effects may contribute to maintain membrane integrity and function (Evelin et al., 2009). Changes in physiological plant parameters seem more important in AMF-colonized plants, while those related to nutrition were more affected by Bt inoculation. Thus, dual inoculation was highly effective exerting beneficial effects related to drought tolerance in maize.

B. thuringiensis was the most effective treatment increasing Fe, Zn and Cu and particularly Fe, probably through production of siderophores (Dimkpa et al., 2009a; 2009b). It is well known that PGPR may improve the plant growth by several mechanisms such as stimulating the synthesis of phytohormones, solubilizing non-available nutrients, optimizing the supply of nutrients and by reducing the levels of ethylene in plants (ACC deaminase production) under stress conditions (Azcón et al., 2013; Yang et al., 2009).

Roots play an important role in drought adaptation and modifying their anatomical and morphological characteristics can contribute to drought tolerance (Kashiwagi et al., 2005). Here mycorrhizal plants had a better root development under drought, which may be further enhanced by the associated extraradical fungal mycelia, allowing the plant to take up more nutrients from deeper soil layers and helping the plant to cope with drought. These nutrients acquisition may be a useful trait for plant resistance to drought.

In this study, the bacterial IAA production did not improve the root development. Thus, the enhanced nutrients uptake by bacterial inoculation under drought conditions cannot be attributed to this cause. However, microorganisms in soil play a major role enhancing the availability of nutrients in the rooting medium.

Regarding L_{pr} values, drought reduced this parameter in control plants, but it was increased in the presence of microorganisms used as inoculants [by 192% (AM) and by 117% (AM+Bt)]. Regulation of aquaporins expression may play important roles to compensate drought effects on root hydraulic conductivity (Bárzana et al., 2014). Indeed, aquaporins provide a low resistance pathway for the movement of water across membranes and their gating ability provides greater control for the movement of water along plant tissues (Maurel et al. 2008). Thus, in this study we analyzed the expression pattern of the whole PIP aquaporin subfamily, as the most relevant for regulation of water transport in maize (Chaumont and Tyerman, 2014). Some of these PIP genes resulted regulated by the microorganisms used as inoculant, but results varied in shoots and roots and also depending on the watering conditions. In shoot tissues, most of the PIPs were down-regulated under well watered conditions, except *ZmPIP2;1* that was up-regulated by Bt application. Under drought stress conditions, single AMF inoculation up-regulated *ZmPIP1;6* and *ZmPIP2;5,* while inoculation with Bt or AM+Bt inhibited the expression of most of the aquaporins.

In root tissues most of the PIPs were up-regulated in AMF-inoculated plants (singly or dually inoculated) under well-watered conditions. This includes *ZmPIP2;5*, which is one of the most expressed aquaporins in maize roots (Hachez et al., 2006). Under drought stress conditions, two PIP genes were up-regulated by single AMF inoculation and three PIP genes by dual AM+Bt inoculation. Curiously, ZmPIP2;4 was only up-regulated by single AMF inoculation, in agreement with recent results by Bárzana et al. (2014), but this effect disappeared when the AMF was co-inoculated with Bt. This suggests that the function that this specific aquaporin plays *in planta* may be compensated by the bacterial activity.

The function and regulation of aquaporins is quite intensively integrated to explain the remarkable hydraulic properties of plants (Maurel et al., 2008). According to the composition of their selectivity filters (Hove and Bhave, 2011), all the PIPs genes regulated by the microbial inoculants applied in this study have the potential for water transport. Moreover, *ZmPIP2;2* and *ZmPIP2;5* have been shown to transport high amounts of water (Bárzana et al., 2014; Hachez et al., 2008). Thus, the effects of the microbial inoculants on the PIP genes in maize may be related to a possible role of these aquaporins in root water uptake from soil. Indeed, under drought stress conditions the root hydraulic conductivity of AM- or AM+Bt-inoculated plants was significantly higher than that of uninoculated control plants, and this correlated with the up-regulation of several PIP genes in roots of these plants. Nevertheless, it has become increasingly clear that some aquaporins (including PIPs) do not exhibit a strict specificity for water and can transport also other small neutral molecules such as glycerol, urea, carbon dioxide (CO_2), hydrogen peroxide (H_2O_2) or boric acid (Bienert et al., 2014; Fitzpatrick and Reid, 2009; Heinen

et al., 2014; Uehleln et al., 2003), highlighting the potential relevance of aquaporins for plant physiology (Li et al., 2014).

The ability of aquaporins to transport urea has pointed to important roles for aquaporins in nitrogen metabolism. The diffusion of CO_2 through aquaporins suggests their involvement in carbon fixation and photosynthesis. The ability of aquaporins to transport H_2O_2 points to important roles in stress signalling and responses. Silicon seems to be crucial for responses to biotic and abiotic stresses (Maurel et al., 2008; Miwa et al., 2009). Indeed, (Li et al., 2014) have recently shown that silicon induced an up-regulation of certain aquaporins in sorghum and this translated into higher root hydraulic conductivity under osmotic stress. Thus, it is possible that the regulation of several PIP aquaporins by the microbial inoculants used in this study may also affect the uptake and/or transport *in planta* of these compounds, with subsequent effects on plant physiology (Li et al., 2014). For instance, *ZmPIP1;6* and *ZmPIP2;5* were considerably induced in shoots of AMF-inoculated plants under drought stress. *ZmPIP1;6* can transport CO_2 (Heinen et al., 2014) and *ZmPIP2;5* has been shown to be involved in leaf radial water movement (Hachez et al., 2008) and can also transport H_2O_2 (Bienert et al., 2014). Thus, their activity may have contributed to a high transpiration and photosynthetic rates in these plants, as well as, to a better signalling of the drought stress responses, resulting in enhanced growth.

In conclusion, results show that the bacterium used (*B. thuringiensis*) has a strong impact on plant nutrition, while the AM fungi were more active improving stress tolerance/homeostatic mechanisms, including regulation of plant aquaporins with several putative physiological functions. Thus, the combination of morphological, metabolic and physiological effects obtained using both microorganisms (AM+Bt) allowed maize plants to gain tolerance against drought.

This study demonstrated that the use of beneficial microorganisms is a promising approach to alleviate drought stress damage in maize plants. The present results support those reported in previous studies, using shrubs exposed to different environmental conditions (natural soil) but the same bacterial strain (Armada et al., 2014b). In addition, our results validate the benefits of using autochthonous plant growth promoting microorganisms from a degraded Mediterranean area not only to protect native plants against drought, but also an agronomically important plant such as maize.

Acknowledgments

E. Armada was financed by Ministry of Science and Innovation (Spain) (BES-2010-042736). This work was carried out in the framework of the project reference AGL2012-39057-C02. We thank Domingo Álvarez for the morphological identification of autochthonous mycorrhizal fungus.

References

Aharon, R., Shahak, Y., Wininger, S., Bendov, R., Kapulnik, Y., Galili, G., 2003. Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. Plant Cell 15, 439-447.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Armada, E., Portela, G., Roldán, A., Azcón, R., 2014a. Combined use of beneficial soil microorganism and agrowaste residue to cope with plant water limitation under semiarid conditions. Geoderma 232, 640-648.

Armada, E., Roldán, A., Azcón, R., 2014b. Differential activity of autochthonous bacteria in controlling drought stress in native *Lavandula* and *Salvia* plants species under drought conditions in natural arid soil. Microb. Ecol. 67, 410-420.

Aroca, R., Porcel, R., Ruíz-Lozano, J.M., 2012. Regulation of root water uptake under abiotic stress conditions. J. Exp. Bot. 63, 43-57.

Aroca, R., Vernieri, P., Irigoyen, J.J., Sánchez-Díaz, M., Tognoni, F., Pardossi, A., 2003. Involvement of abscisic acid in leaf and root of maize (*Zea mays* L.) in avoiding chilling-induced water stress. Plant Sci. 165, 671-679.

Azcón, R., Medina, A., Aroca, R., Ruíz-Lozano, J.M., 2013. Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast interactions, in: de Bruijn, F.J. (Ed.), Molecular Microbial Ecology of the Rhizosphere. John Wiley & Sons, Hoboken, New Jersey, USA, pp. 991-1002.

Azevedo Neto, A.D., Prisco, J.T., Eneas-Filho, J., de Abreu, C.E.B., Gomes-Filho, E., 2006. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ. Exp. Bot. 56, 87-94.

Bárzana, G., Aroca, R., Bienert, G.P., Chaumont, F., Ruíz-Lozano, J.M., 2014. New insights into the regulation of aquaporins by the arbuscular mycorrhizal symbiosis in maize plants under drought stress and possible implications for plant performance. Mol. Plant-Microbe Interact. 27, 349-363.

Bárzana, G., Aroca, R., Paz, J.A., Chaumont, F., Martínez-Ballesta, M.C., Carvajal, M., Ruíz-Lozano, J.M., 2012. Arbuscular mycorrhizal symbiosis increases relative apoplastic water flow in roots of the host plant under both well-watered and drought stress conditions. Ann. Bot. 109, 1009-1017.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205-207.

Beck, E.H., Fettig, S., Knake, C., Hartig, K., Bhattarai, T., 2007. Specific and unspecific responses of plants to cold and drought stress. J. Biosci. 32, 501-510.

Bienert, G.P., Heinen, R.B., Berny, M.C., Chaumont, F., 2014. Maize plasma membrane aquaporin *ZmPIP2;5*, but not *ZmPIP1;2*, facilitates transmembrane diffusion of hydrogen peroxide. Biochim. Biophys. Acta-Biomembr. 1838, 216-222.

Boomsma, C.R., Vyn, T.J., 2008. Maize drought tolerance: Potential improvements through arbuscular mycorrhizal symbiosis? Field Crops Res. 108, 14-31.
Brundrett, M., Melville, L., Peterson, R.L., 1994. Practical Methods in Mycorrhizal Research. Mycologue Publications, Waterloo, Ontario, Canada
Calvo-Polanco, M., Sánchez-Romera, B., Aroca, R., 2014. Mild salt stress conditions induce different responses in root hydraulic conductivity of *Phaseolus vulgaris* over-time. Plos One 9, e90631 doi:90610.91371/journal.pone.0090631.
Chaumont, F., Tyerman, S.D., 2014. Aquaporins: highly regulated channels controlling plant water relations. Plant Physiol. 164, 1600-1618.
Denby, K., Gehring, C., 2005. Engineering drought and salinity tolerance in plants: lessons from genome-wide expression profiling in *Arabidopsis*. Trends Biotechnol. 23, 547-552.
Dimkpa, C.O., Merten, D., Svatos, A., Buechel, G., Kothe, E., 2009a. Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. Soil Biol. Biochem. 41, 154-162.
Dimkpa, C.O., Merten, D., Svatos, A., Buechel, G., Kothe, E., 2009b. Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively. J. Appl. Microbiol. 107, 1687-1696.
Duncan, D.B., 1955. Multiple range and multiple F tests. Biometrics 11, 1-42.
Evelin, H., Kapoor, R., Giri, B., 2009. Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. Ann. Bot. 104, 1263-1280.
Fitzpatrick, K.L., Reid, R.J., 2009. The involvement of aquaglyceroporins in transport of boron in barley roots. Plant, Cell Environ. 32, 1357-1365.
Ghannoum, O., 2009. C4 photosynthesis and water stress. Ann. Bot. 103, 635-644.
Gianinazzi, S., Gollotte, A., Binet, M.N., van Tuinen, D., Redecker, D., Wipf, D., 2010. Agroecology: The key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20, 519-530.
Giovannetti, M., Mosse, B., 1980. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84, 489-500.
Hachez, C., Heinen, R.B., Draye, X., Chaumont, F., 2008. The expression pattern of plasma membrane aquaporins in maize leaf highlights their role in hydraulic regulation. Plant Mol. Biol. 68, 337-353.
Hachez, C., Moshelion, M., Zelazny, E., Cavez, D., Chaumont, F., 2006. Localization and quantification of plasma membrane aquaporin expression in maize primary root: A clue to understanding their role as cellular plumbers. Plant Mol. Biol. 62, 305-323.
Halliwell, B., Gutteridge, J., 1989. Free radicals in biology and medicine. 2nd ed. Clarendon Press, Oxford.
Heinen, R.B., Bienert, G.P., Cohen, D., Chevalier, A.S., Uehlein, N., Hachez, C., Kaldenhoff, R., Le Thiec, D., Chaumont, F., 2014. Expression and characterization of plasma membrane aquaporins in stomatal complexes of *Zea mays*. Plant Mol. Biol.
Hove, R.M., Bhave, M., 2011. Plant aquaporins with non-aqua functions: Deciphering the signature sequences. Plant Mol. Biol. 75, 413-430.
Javot, H., Maurel, C., 2002. The role of aquaporins in root water uptake. Ann. Bot. 90, 301-313.
Kaldenhoff, R., Bertl, A., Otto, B., Moshelion, M., Uehlein, N., 2007. Characterization of plant aquaporins. Methods Enzymol. 428, 505-531.
Kashiwagi, J., Krishnamurthy, L., Upadhyaya, H.D., Krishna, H., Chandra, S., Vadez, V., Serraj, R., 2005. Genetic variability of drought-avoidance root traits in the mini-core germplasm collection of chickpea (*Cicer arietinum* L.). Euphytica 146, 213-222.
Kasim, W.A., Osman, M.E., Omar, M.N., Abd El-Daim, I.A., Bejai, S., Meijer, J., 2013. Control of drought stress in wheat using plant-growth-promoting bacteria. J. Plant Growth Regul. 32, 122-130.
Kay, R., Chan, A., Daly, M., McPherson, J., 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236, 1299-1302.

Koske, R.E., Tessier, B., 1983. A convenient, permanent slide mounting medium. Myc. Soc. Am. Newsl. 34, 59.
Leipner, J., Fracheboud, Y., Stamp, P., 1997. Acclimation by suboptimal growth temperature diminishes photooxidative damage in maize leaves. Plant Cell Environ. 20, 366-372.
Li, G., Santoni, V., Maurel, C., 2014. Plant aquaporins: Roles in plant physiology. Biochimica Et Biophysica Acta-General Subjects 1840, 1574-1582.
Lian, H.L., Yu, X., Ye, Q., Ding, X., Kitagawa, Y., Kwak, S.S., Su, W.A., Tang, Z.C., 2004. Erratum: The role of aquaporin RWC3 in drought avoidance in rice. Plant Cell Physiol 45, 481-489.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350-382.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402-408.
Mäkelä, P., Kärkkäinen, J., Somersalo, S., 2000. Effect of glycinebetaine on chloroplast ultrastructure, chlorophyll and protein content, and RuBPCO activities in tomato grown under drought or salinity. Biol. Plant. 43, 471-475.
Marulanda-Aguirre, A., Azcón, R., Ruíz-Lozano, J.M., Aroca, R., 2008. Differential effects of a *Bacillus megaterium* strain on *Lactuca sativa* plant growth depending on the origin of the arbuscular mycorrhizal fungus coinoculated: Physiologic and biochemical traits. J. Plant Growth Regul. 27, 10-18.
Marulanda, A., Barea, J.M., Azcón, R., 2009. Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments. Mechanisms related to bacterial effectiveness. J. Plant Growth Regul. 28, 115-124.
Maurel, C., Plassard, C., 2011. Aquaporins: for more than water at the plant-fungus interface? New Phytol. 190, 815-817.
Maurel, C., Verdoucq, L., Luu, D.-T., Santoni, V., 2008. Plant aquaporins: Membrane channels with multiple integrated functions. Annu. Rev. Plant Biol. 59, 595-624.
Mejía, D., 2003. Maize: Post-Harvest Operations. FAO-AGST, Rome.
Minotti, G., Aust, S.D., 1987. The requirement for iron (III) in the initiation of lipid-peroxidation by iron(II) and hydrogen-peroxide. J. Biol. Chem. 262, 1098-1104.
Miwa, K., Kamiya, T., Fujiwara, T., 2009. Homeostasis of the structurally important micronutrients, B and Si. Curr. Opin. Plant Biol. 12, 307-311.
Oehl, F., Sieverding, E., Palenzuela, J., Ineichen, K., Silva, G., 2011. Advances in *Glomeromycota* taxonomy and classification. IMA Fungus 2, 191-199.
Oliveira, R.S., Vosátka, M., Dodd, J.C., Castro, P.M.L., 2005. Studies on the diversity of arbuscular mycorrhizal fungi and the efficacy of two native isolates in a highly alkaline anthropogenic sediment. Mycorrhiza 16, 23-31.
Otto, B., Kaldenhoff, R., 2000. Cell-specific expression of the mercury-insensitive plasma-membrane aquaporin NtAQP1 from *Nicotiana tabacum*. Planta 211, 167-172.
Ouziad, F., Wilde, P., Schmelzer, E., Hildebrandt, U., Bothe, H., 2006. Analysis of expression of aquaporins and Na+/H+ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. Environ. Exp. Bot. 57, 177-186.
Oxborough, K., Baker, N.R., 1997. Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components - calculation of qP and *Fv'/Fm'* without measuring *Fo'*. Photosynth. Res. 54, 135-142.
Patterson, B.D., Macrae, E.A., Ferguson, I.B., 1984. Estimation of hydrogen-peroxide in plant-extracts using titanium (IV). Anal. Biochem. 139, 487-492.
Pereyra, M.A., Garcia, P., Colabelli, M.N., Barassi, C.A., Creus, C.M., 2012. A better water status in wheat seedlings induced by *Azospirillum* under osmotic stress is related to morphological changes in xylem vessels of the coleoptile. Appl. Soil Ecol. 53, 94-97.
Phillips, J.M., Hayman, D.S., 1970. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 159-161.

Querejeta, J.I., Allen, M.F., Caravaca, F., Roldán, A., 2006. Differential modulation of host plant delta C-13 and delta O-18 by native and nonnative arbuscular mycorrhizal fungi in a semiarid environment. New Phytol. 169, 379-387.

Rees, H.C., Grant, W.D., Jones, B.E., Heaphy, S., 2004. Diversity of Kenyan soda lake alkaliphiles assessed by molecular methods. Extremophiles 8, 63-71.

Roth, C.H., Malicki, M.A., Plagge, R., 1992. Empirical evaluation of the relationship between soil dielectric constant and volumetric water content as the basis for calibrating soil moisture measurements. J. Soil Sci. 43, 1-13.

Rueda-Puente, E.O., Murillo-Amador, B., Castellanos-Cervantes, T., García-Hernández, J.L., Tarazòn-Herrera, M.A., Moreno Medina, S., Gerlach Barrera, L.E., 2010. Effects of plant growth promoting bacteria and mycorrhizal on *Capsicum annuum* L. var. aviculare ([Dierbach] D'Arcy and Eshbaugh) germination under stressing abiotic conditions. Plant Physiol. Biochem. 48, 724-730.

Ruíz-Sánchez, M., Armada, E., Muñoz, Y., García de Salamone, I.E., Aroca, R., Ruíz-Lozano, J.M., Azcón, R., 2011. *Azospirillum* and arbuscular mycorrhizal colonization enhance rice growth and physiological traits under well-watered and drought conditions. J. Plant Physiol. 168, 1031-1037.

Shinozaki, K., Yamaguchi-Shinozaki, K., Seki, M., 2003. Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant Biol. 6, 410-417.

Sieverding, E., 1991. Vesicular-arbuscular mycorrhiza management in tropical agrosystems. GTZ, Eschborn, Germany.

Smith, I.K., 1985. Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. Plant Physiol. 79, 1044-1047.

Spain, J.L., 1990. Arguments for diagnoses based on unaltered wall structures. Mycotaxon 38, 71-76.

Türkan, I., Demiral, T., 2009. Recent developments in understanding salinity tolerance. Environ. Exp. Bot. 67, 2-9.

Uehlein, N., Fileschi, K., Eckert, M., Bienert, G.P., Bertl, A., Kaldenhoff, R., 2007. Arbuscular mycorrhizal symbiosis and plant aquaporin expression. Phytochemistry 68, 122-129.

Uehleln, N., Lovisolo, C., Siefritz, F., Kaldenhoff, R., 2003. The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. Nature 425, 734-737.

Urano, K., Kurihara, Y., Seki, M., Shinozaki, K., 2010. 'Omics' analyses of regulatory networks in plant abiotic stress responses. Curr. Opin. Plant Biol. 13, 132-138.

Vinocur, B., Altman, A., 2005. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr. Opin. Biotechnol. 16, 123-132.

Vivas, A., Barea, J.M., Biró, B., Azcón, R., 2006. Effectiveness of autochthonous bacterium and mycorrhizal fungus on *Trifolium* growth, symbiotic development and soil enzymatic activities in Zn contaminated soil. J. Appl. Microbiol. 100, 587-598.

Warren, G.F., 1998. Spectacular increases in crop yields in the United States in the twentieth century. Weed Technol. 12, 752-760.

White, I., Knight, J.H., Zegelin, S.J., Topp, G.C., 1994. Comments to 'considerations on the use of time-domain reflectometry (TDR) for masuring soil water content' by WR Whalley. J. Soil Sci. 45, 503-508.

Yang, J., Kloepper, J.W., Ryu, C.-M., 2009. Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci. 14, 1-4.

Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., Shinozaki, K., 1997. Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol 38, 1095-1102.

CONCLUSIONES GENERALES

- Las especies bacterianas aisladas de las rizosferas de arbustos autóctonos de zonas semiáridas, pertenecientes a los géneros *Bacillus* y *Enterobacter*, en condiciones *in vitro*, sometidas a altos niveles de estrés osmótico, mostraron su capacidad de tolerar el estrés y las habilidades que podrían describirse como potencial PGPR.

- La actividad de las bacterias específicas y/o residuo agrícola fermentado parece estar asociada a la protección de las plantas, para evitar así la sequía y la consiguiente alteración de componentes antioxidantes y las propiedades fisiológicas de las plantas. Podría ser un posible método para fomentar el desarrollo vegetal y la disponibilidad de nutrientes, y por consiguiente la tolerancia a soportar esas condiciones tan extremas de deficiencia de agua.

- *Lavandula dentata* demostró una mayor capacidad de soportar el estrés por sequía cuando se inoculó con *Bacillus thuringiensis*. Dicha tolerancia bacteriana a la sequía se evaluó como supervivencia y producción de prolina y ácido indolacético (AIA), mostrando el potencial de esta bacteria para ayudar a las plantas a crecer en condiciones de estrés hídrico. La inoculación de *B. thuringiensis* en plantas de *L. dentata* puede ser utilizado en los programas de revegetación de ecosistemas semiáridos.

- Cambios en la composición rizosférica (bacteriana y fúngica), y como esta responde a los distintos tipos de cobertura vegetal autóctona en condiciones ambientales de carácter semiárido. La inoculación de *B. thuringiensis* fomenta la diversidad microbiana.

- El consorcio y/o mezcla de hongos MA autóctonos con *B. thuringiensis*, demostró su potencial para la protección de las plantas contra la sequía y ayudar a las plantas a prosperar en ecosistemas semiáridos.

- Los patrones de uso de las diferentes fuentes de C y N de las comunidades bacterianas rizosféricas, se vieron alterados por el tipo de especie de hongo MA. No fue modificada por la co-inoculación con *B. thuringiensis*.

- El uso de microorganismos beneficiosos autóctonos de un área mediterránea degradada, protege no sólo las plantas nativas contra la sequía, sino también una planta agronómicamente importante, como el maíz. Destacando que *B. thuringiensis* interviene en la nutrición vegetal, y los hongos MA mejoran los procesos homeostáticos y de tolerancia, y participando en la regulación de las acuaporinas de la planta.