UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Application of Plant Growth Promoting Rhizobacteria (PGPR) in the Revegetation of SemiaridAreas

Aplicación de Rizobacterias Promotoras del Crecimiento Vegetal (RPCV) en la Reforestación de Zonas Semiáridas

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2015

MINISTERIO
DE ECONOMÍA
Y COMPETITIVIDAD

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

CENTRO DE EDAFOLOGÍA Y BIOLOGÍA
APLICADA DEL SEGURA (CEBAS)

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La presentación de la Tesis Doctoral titulada "**Application of plant growth promoting rhizobacteria (PGPR) in the revegetation of semiarid areas. Aplicación de rizobacterias promotoras del crecimiento vegetal (RPCV) en la reforestación de zonas semiáridas**", realizada por **Dª Carmen María Mengual Navarro-Soto**, bajo nuestra inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 3 de marzo de 2015.

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Dª. María Asunción Morte Gómez, Doctora en Biología y Profesora Titular del Departamento de Biología Vegetal de la Universidad de Murcia, ratifica como tutora:

Que la Tesis Doctoral titulada "**Application of plant growth promoting rhizobacteria (PGPR) in the revegetation of semiarid areas. Aplicación de rizobacterias promotoras del crecimiento vegetal (RPCV) en la reforestación de zonas semiáridas**" ha sido realizada por **Dª. Carmen María Mengual Navarro-Soto**, bajo la dirección y supervisión de D. Antonio Roldán Garrigós y D. Mauricio Iván Antonio Schoebitz Cid, dando mi conformidad para que sea presentada ante la Comisión Académica de Doctorado.

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Agradecimientos

Esta Tesis es el resultado del trabajo y esfuerzo en una etapa de mi vida que ha resultado ser de las más intensas y satisfactorias que he tenido. Sin embargo, no habría sido posible llevar a cabo este proyecto sin la ayuda de muchas personas que me han apoyado de una u otra manera.

En primer lugar, me gustaría agradecer a mi director de Tesis, Antonio Roldán Garrigós, la confianza depositada en mí para la realización de este trabajo. Su apoyo tanto profesional como personal ha sido clave en mi desarrollo como científico a lo largo de estos años. Brillante como científico y como persona, me ha demostrado, aún a pesar de los malos momentos, que siempre me ha respaldado, ayudando a que me levantara cuando pensaba que había tocado fondo.

A mi codirector, Mauricio Schoebitz, que llegó en un momento en el que me encontraba perdida. Me mostró el campo de la microbiología con paciencia y tras su marcha, dejó el camino marcado, camino que me ha permitido crecer en este área. Sus consejos y sus confidencias han hecho que este recorrido haya sido más amable, a pesar de la distancia.

A Fuensanta Caravaca Ballester quisiera decirle en unas líneas algo que, seguramente, no pueda expresarse ni en cientos de páginas. Su dedicación, su confianza y sus ánimos han hecho que pueda con todo. Siempre ha estado dispuesta a ayudarme con cualquier problema, ha comprendido cada una de mis situaciones y me ha demostrado que toda ella es Ciencia. Me ha enseñado que la investigación es algo fascinante y cuanto más se profundiza en ella, más enriquecedora es. Me ha inculcado su pasión y su capacidad de trabajo, porque si hay algo que tenga claro con respecto a mi futuro, ella es la responsable.

A Asunción Morte Gómez le debo parte de este logro porque aceptó ser mi tutora casi sin conocerme. Me ha animado, y ha creído fantástica cada cosa que he hecho, brindándome ayuda cuando aún no me había dado tiempo a pedírsela.

María del Mar Alguacil, Pilar Torres y Gisela Díaz, siempre han estado dispuestas a ayudarme en lo que fuera necesario: bibliografía, congresos… Nunca

les ha importado, siempre lo han hecho de la forma más amable, haciendo que los pequeños problemas del día a día se volviesen insignificantes.

A mis compañeros de grupo y sobre todo, amigos, Manolo, Asun, Emma y Gema les pertenece esta Tesis tanto como a mí, haciendo de estos años los mejores de mi vida. Sin ellos no estaría donde estoy ahora. Manolo siempre ha hecho que el trabajo fuese divertido, enseñándome, al mismo tiempo todo lo que debía saber en el laboratorio con rigurosidad. Ha sabido aconsejarme tanto en los temas laborales como personales, involucrándose en cualquiera de mis problemas; sin su presencia, me habría quedado atrás. Asun se ha convertido en uno de los pilares fundamentales que me mantienen en pie, por su implicación en el trabajo y en nuestra amistad. Es auténtica, trabajadora, comprensiva y jamás me ha dejado caer. Emma ha sido para mí una de esas personas que cuanto más se conocen, más se quieren. Me ha demostrado que un jamás no existe en su vida y puede con todo, levantando mi ánimo cada vez que me veía debilitarme. Nunca olvidaré las torpezas con las que hemos hecho historia en este centro. Sin ella, nada habría sido igual. A Gema tengo que agradecerle el apoyo que me ha dado desde que llegó, pareciéndole maravilloso todo lo que hacía, permitiéndome creer que me había convertido en una persona importante.

A todos los compañeros del Centro, porque de una forma u otra, siempre me han ayudado, especialmente el grupo de Patología Vegetal, que sigue considerándome un miembro más del equipo.

A mis compañeros de la cuarta planta, Elvira, José, André, Felipe, Sara, Antonio, Noelia, Pepa, Ana, Anais, Rocío, Irene y Elena, por prestarme cualquier cosa que he necesitado; porque sin ellos no podría haber hecho ni una cuarta parte del trabajo, permitiéndome patalear cuando el estrés me ha vencido. A Tania en especial, porque me ha ayudado en las cosas más simples que parecían un mundo. Siempre que la he necesitado ha estado apoyándome y eso ha sido fundamental en este último tramo. Gracias por brindarme tu amistad más sincera. Le deseo lo mejor tanto profesional como personalmente, y espero, que a pesar de la distancia que un día nos separará, siempre nos sintamos tan cerca como ahora.

Al Servicio de Apoyo a la Investigación (SAI), especialmente a José, Almudena, Inma y María del Mar, por su amabilidad y sus ganas de involucrarse de lleno en cada uno de los proyectos.

A mis amigos, especialmente Raquel y Asun con las que he contado siempre para poder desarrollarme profesionalmente y, especialmente, como madre. Por sus consejos y ánimos. Por la fortaleza que me dan.

A David por ser mi compañero en este viaje, por ayudarme, por aguantar mis nervios y mi estrés, por permitirme cumplir mi sueño sin pedir nada a cambio. Por estar ahí en lo bueno y en lo malo. Por disfrutar al verme feliz con mi trabajo. Por su generosidad y su paciencia. Se lo debo todo.

A Alejandro, mi hijo, porque cada sonrisa suya me ha hecho olvidar los enfados, frustraciones y los momentos menos agradables, convirtiendo en maravillosos cada uno de mis días, por muy mal que hubieran empezado. Por no tener en cuenta las horas que he estado fuera y disfrutar de mi cada rato que he pasado con él. Gracias por ser el motor que me da vida.

A mis padres y mi hermano, porque sin ellos no me habría convertido en quien soy ni estaría donde estoy. Porque me lo han dado todo sin pedirles nada. Porque han confiado en mí y me han apoyado en cada uno de los momentos malos que he tenido. Porque han dejado la distancia atrás cada vez que los he llamado. Por estar siempre ahí.

A mi familia, que siempre me ha dado ánimos y ha confiado en mi capacidad para madurar en la Investigación.

Al Ayuntamiento de Molina de Segura (Murcia) por las facilidades para realizar la investigación en el parque Ecológico Vicente Blanes.

Finalmente, mi agradecimiento al Ministerio de Economía y Competitividad, por otorgarme una beca FPI (ref: BES-2010-038654) y a los Proyectos Nacionales (ref: AGL 2009-12530-C02-01 y AGL 2012-39057-C02-01) gracias los cuales he podido realizar mi Tesis Doctoral.

A la memoria de mis abuelos

Juan Pedro, Carmen, Francisco y María Antonia

A mis padres

Publicaciones científico-técnicas derivadas de esta Tesis

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Resumen

Desde mediados del siglo XIX la erosión del suelo ha sido considerada como uno de los problemas medioambientales más relevantes en todo el mundo, particularmente en zonas con fuertes contrastes de precipitación dependiendo de la estación climática. Éste es el caso de las zonas localizadas en la cuenca del Mediterráneo, donde se estima que sólo un 9 o 10 % de sus áreas presentan una cubierta vegetal bien desarrollada, probablemente como consecuencia de sus peculiares condiciones ambientales consistentes en lluvias escasas e irregulares, y largos periodos estivales caracterizados por sus altas temperaturas y una intensa sequía, además de la presión antrópica que ha contribuido a la degradación de estas regiones debido a labores de cultivo extensivas y no reguladas o a actividades de deforestación.

En las regiones áridas y semiáridas del Mediterráneo español, se han llevado a cabo gran cantidad de estudios basados en el uso de especies vegetales autóctonas para la restauración de suelos degradados, reforzando el establecimiento y crecimiento de dichas plantas mediante la aplicación de enmiendas orgánicas y la inoculación de microorganismos beneficiosos, esencialmente hongos micorrícicos arbusculares. En este marco se planteó la siguiente investigación, cuyo objetivo principal fue evaluar, en áreas degradas, la eficacia de diversas cepas de rizobacterias promotoras del crecimiento vegetal junto a la aplicación de enmiendas orgánicas sobre el desarrollo de la cubierta vegetal y la calidad de las propiedades del suelo, así como verificar la efectividad como rizobacterias promotoras del crecimiento vegetal de varias cepas de actinobacterias, previamente aisladas de diferentes suelos de la Región de Murcia. Es por ello que esta Tesis está dividida en tres partes:

- Una primera parte en la que se llevaron a cabo tres experimentos de campo en el Parque Ecológico Vicente Blanes, Molina de Segura, mediante la adición de diferentes enmiendas orgánicas y la inoculación con varias cepas de rizobacterias promotoras del crecimiento vegetal a tres especies de plantas autóctonas.

- Una segunda parte consistente en un muestreo en campo en dos localidades diferentes de la Región de Murcia, con el fin de aislar y caracterizar distintas cepas de actinobacterias del suelo rizosférico de una planta autóctona de la zona.
- Una tercera parte basada en un ensayo de campo que se llevó a cabo en dos zonas diferentes de la región, el parque Ecológico Vicente Blanes y la Reserva Natural de Calblanque, utilizando *Rhamnus lycioides* como planta modelo. Como inoculantes microbianos se ensayaron las cepas de actinobacterias previamente aisladas.

Con respecto a la primera parte de la Tesis, el primer ensayo de campo se llevó a cabo con el fin de evaluar la efectividad de un inoculante microbiano consistente en dos cepas de rizobacterias promotoras del crecimiento vegetal (*Azospirillum brasilense* y *Pantoea dispersa*) inmovilizadas en cápsulas de arcilla y la adición de residuo orgánico de oliva sobre el desarrollo del *Cistus albidus* L., así como sobre la mejora de las propiedades del suelo. Al final de un periodo de 16 meses en campo, las plantas fueron recolectadas junto al suelo rizosférico, para medir los parámetros indicadores de crecimiento y las respuestas al estrés ocasionado por la falta de agua además de las propiedades fisicoquímicas, químicas y biológicas del suelo. Se observó que el tratamiento combinado consistente en la inoculación de las rizobacterias junto a la adición de la enmienda orgánica fue el tratamiento más efectivo en las estimulación del crecimiento de la raíz, cuyo peso seco aumentó un 133% con respecto a la raíz de las plantas control. La inoculación microbiana, por su parte, aumentó en un 106% el peso seco de la planta frente al control, siendo el tratamiento más efectivo a este respecto. Por su parte, el tratamiento combinado fue responsable de un incremento del contenido de las fracciones de carbono (carbono total, carbono orgánico total y carbono de la biomasa microbiana) y de las actividades enzimáticas deshidrogenasa, ureasa y proteasa en la rizosfera de *C. albidus*, a diferencia de los resultados obtenidos cuando los tratamientos se aplicaron por separado. De este modo, se observó un efecto aditivo en el tratamiento

combinado que permitió acrecentar las propiedades bioquímicas y microbiológicas del suelo.

El segundo experimento en campo estuvo basado en el estudio de la eficacia de la inoculación de *Pantoea dispersa* y *Azospirillum brasilense* inmovilizadas en arcilla y la adición de residuo orgánico de oliva sobre el crecimiento de *Pinus halepensis* Mill. y sus respuestas al estrés, así como sobre las propiedades físico-químicas, químicas y biológicas del suelo. Tras veintiocho meses de plantación, las plantas y el suelo rizosférico fueron recolectadas para realizar los pertinentes análisis. Así pues, los resultados mostraron que la inoculación microbiana fue el tratamiento más eficaz para promover la absorción de nutrientes y el crecimiento vegetal, aumentando el peso de las plantas tratadas un 48% respecto a las no tratadas. Además, las plantas inoculadas mostraron una mejor respuesta al estrés, observándose los valores más bajos en cuanto a la prolina acumulada en las hojas y daño oxidativo a lípidos, viéndose aumentados los correspondientes al potencial hídrico. A nivel de suelo, se observó una clara mejoría en el contenido de carbohidratos totales, carbono de la biomasa microbiana y nutrientes disponibles tras el tratamiento basado en la inoculación de las rizobacterias y el tratamiento combinado rizobacterias + enmienda orgánica. En este caso, la eficacia de la inoculación microbiana sobre el desarrollo de la planta y sobre las propiedades del suelo, haría de éste un tratamiento preferente en programas destinados a revegetación, conllevando un abaratamiento del coste de estas prácticas al poder prescindir del refuerzo aportado por el residuo orgánico.

El tercer y último ensayo de esta primera parte de la Tesis, también en campo, se focalizó en la determinación de los efectos de tratamientos combinados basados en la inoculación con varias cepas de rizobacterias libres (*Bacillus megaterium*, *Enterobacter* sp., *Bacillus thuringiensis* y *Bacillus* sp.) y la aplicación de residuo orgánico de remolacha azucarera compostado en las propiedades físico-químicas, químicas y biológicas del suelo, así como en el establecimiento de *Lavandula dentata* L. como cubierta vegetal. Pasado un año desde la plantación, los ejemplares de *L. dentata* fueron recolectados junto al suelo rizosférico con el fin de llevar a cabo los

diferentes análisis en planta y suelo. Los resultados mostraron que la inoculación con *Bacillus* sp. y la combinación *B. megaterium* + enmienda fueron los tratamientos responsables de los mayores índices de crecimiento observados en las plantas, aumentando el peso seco de *L. dentata* 5 veces respecto al peso de los ejemplares control. Por su parte, el peso seco de la raíz mostró los datos más elevados tras la aplicación del tratamiento combinado *Enterobacter* sp.+ enmienda. Con respecto al contenido de nutrientes en hoja, todos los tratamientos evaluados, excepto la combinación *B. thuringiensis* + enmienda, mediaron un incremento respecto a las plantas no tratadas. Considerando las propiedades del suelo rizosférico, tras la adición de la enmienda orgánica se observó un aumento en la concentración de fósforo disponible (29% respecto al control), mientras que el nitrógeno total se vio incrementado con el tratamiento combinado *Enterobacter* sp.+ enmienda en un 46%. Además, la aplicación de este último tratamiento también mejoró de manera significativa las propiedades físico-químicas y biológicas del suelo. Por tanto, la selección de las rizobacterias efectivas y la combinación de su inoculación junto con la aplicación de la enmienda orgánica podría considerarse el punto crucial que del que dependería la eficacia de estas herramientas biotecnológicas en la revegetación y rehabilitación de suelos degradados en ambientes mediterráneos.

La segunda parte de la Tesis está basada en metodologías de muestreo, aislamiento y caracterización de cepas bacterianas. Para ello, se llevaron a cabo dos muestreos en dos localidades diferentes de la Región de Murcia, Parque Ecológico Vicente Blanes, en Molina de Segura y Reserva Natural de Calblanque. En ambas zonas, se recolectaron muestras de suelo rizosférico de tres ejemplares del arbusto autóctono, *Rhamnus lycioides* L. Las muestras fueron trasladadas al laboratorio donde se llevaron a cabo técnicas de aislamiento diseñadas específicamente para actinobacterias, y diferentes análisis que permitieron caracterizarlas e identificarlas. Tras el aislamiento y la purificación, se obtuvieron un total de doce cepas, seis procedentes del Parque Ecológico Vicente Blanes y seis procedentes de la Reserva Natural de Calblanque. Estas cepas fueron observadas tanto macroscópica como microscópicamente lo que llevó a identificarlas como actinobacterias, debido a su

morfología y a la correspondiente tinción positiva de Gram, con la que se observaron bacterias teñidas en violeta que presentaban acúmulos de hifas. Así pues, se comprobó el peculiar desarrollo miceliar de estas bacterias, despejando cualquier duda sobre su clasificación. Posteriormente, se realizaron diferentes análisis de laboratorio para caracterizarlas con respecto a sus habilidades como bacterias fijadoras de nitrógeno y solubilizadoras de fósforo, además de su capacidad para excretar sideróforos, capacidades que las convertirían en rizobacterias promotoras del crecimiento vegetal. De las doce cepas, cuatro de ellas, dos aisladas del suelo del Parque Ecológico Vicente Blanes, y dos provenientes de la Reserva Natural de Calblanque, mostraron los mejores resultados en estos ensayos, observándose, además que sus comportamientos eran similares. De estas cuatro cepas se extrajo el ADN y mediante técnicas moleculares, éste fue amplificado, purificado y secuenciado, revelando que dichas actinobacterias pertenecían al género *Streptomyces*. Las secuencias obtenidas fueron depositadas en el banco de genes, GenBank, con sus correspondientes números de accesión. Tras su identificación molecular, se les realizó una prueba a las cuatro cepas seleccionadas con el fin de verificar su capacidad de inhibir el crecimiento de cuatro conocidos hongos patógenos para las plantas (*Phytophthora parasitica*, *Pythium ultimum*, *Botrytis cinerea* y *Fusarium* sp.), observándose una clara actividad antagonista frente a estos patógenos. Por tanto, las técnicas de aislamiento caracterizaron e identificación se consideraron las adecuadas, puesto que obtuvimos cuatro cepas de actinobacterias pertenecientes al género *Streptomyces* capaces de fijar nitrógeno, solubilizar fósforo, excretar sideróforos e inhibir el crecimiento de hogos patógenos de vegetales, reuniendo así las condiciones para ser consideradas rizobacterias promotoras del crecimiento vegetal bajo condiciones de laboratorio.

Para concluir el trabajo, se realizó un ensayo de campo, correspondiente a la tercera y última parte de la Tesis, en dos localidades diferentes, en este caso el Parque Ecológico Vicente Blanes y la Reserva Natural de Calblanque con el objeto de evaluar la efectividad de las cepas de actinobacterias, previamente aisladas e inmovilizadas en un medio compuesto por alginato y almidón de patata, como

promotoras del crecimiento vegetal, y a su vez comprobar la incidencia relativa del origen de la cepa y la zona de plantación en la revegetación de zonas semiáridas, utilizando *R. lycioides* como planta modelo. Un año después de la plantación, los ejemplares de *R. lycioides* fueron recolectados junto al suelo rizosférico y transportados al laboratorio para realizar los análisis de crecimiento, contenido de nutrientes en hoja y respuesta al estrés de las plantas, así como para determinar posibles diferencias en las propiedades físico-químicas, químicas y biológicas del suelo. Los resultados mostraron que en Calblanque, la inoculación con cepas nativas fue más efectiva que la inoculación con cepas alóctonas, en lo que al crecimiento de la planta se refiere, incrementando el peso seco de la planta sobre un 48% y 28%, respectivamente con respecto a las plantas control. Esto fue debido, principalmente a la absorción de nitrógeno, fósforo y potasio y a una mayor tolerancia al estrés por falta de agua. En el Parque Ecológico Vicente Blanes, por el contrario, la eficacia en la promoción del crecimiento vegetal no se vio influenciada por el origen de la cepa. En esta localidad y a nivel de suelo, el mayor incremento en las actividades enzimáticas ureasa, proteasa y deshidrogenasa así como en el carbono de la biomasa microbiana (200%, 28%, 29% y 30% respectivamente) mediado por la inoculación de las actinobacterias fue independiente del origen de las cepas ensayadas. Por tanto, se debería considerar el origen de la cepa y la fertilidad biológica del suelo sujeto a plantación como factores fundamentales para la selección de cepas de actinobacteria destinadas a uso en revegetación en ambientes semiáridos con especies arbustivas.

Summary

Since, at least, the second half of 19h century, the erosion has been considered as one of the most relevant environmental problems worldwide, particularly in areas with resilient precipitation contrast depending of the annual season. This is the case of Mediterranean regions where it is estimated that only 9 or 10 % of their areas are, currently, forested. It is probably due to the characteristic environmental conditions, consisting of irregular and limited rainfalls and long summers with high temperatures and severe drought periods as well as to the human actions that have contributed to the deterioration of these sites as a consequence of non-controlled cultivation labours and/ or deforestation activities.

Several research studies have been carried out in Spanish Mediterranean semiarid regions, based on the utilization of autochthonous shrubs species for the restoration of degraded soils, reinforcing the establishment and growth of the vegetal cover by the addition of organic amendments and inoculations with beneficial microorganisms, mainly with arbuscular mycorrhizal fungi. In this framework, it was strategised the investigation which main objective is to evaluate, in degraded areas, the effectiveness of diverse plat growth promoting rhizobacteria strains jointly with the addition of an organic waste on plant performance and on the soil quality properties, as well as to verify the efficacy of some actinobacteria strains as plant growth promoting rhizobacteria, previously isolated from different soils of Province of Murcia. For that, this Thesis has been divided in three parts:

- The first part consisting of three field experiment developed in Vicente Blanes Ecological Park, Molina de Segura, by the addition of different organic amendments and the inoculation with certain plant growth promoting rhizobacteria strains to three autochthonous vegetal species.
- A second part based on field samplings carried out in two different sites of Province of Murcia, with the purpose of isolating and characterising different actinobacteria strains from the rhizosphere soil of an autochthonous plant species of these areas.

- The third part, composed by a field, assay was developed in two different sites of Province of Murcia, Vicente Blanes Ecological Park and Calblanque Nature Reserve, by the use of *Rhamnus lycioides* as target plant. As microbial inoculant, the actinobacteria strains previously isolated and characterised were utilized.

Regarding the first part of the Thesis, the first field experiment was carried out with the purpose of assessing the effectiveness of an immobilised microbial inoculant (*Azospirillum brasilense* and *Pantoea dispersa*) and the addition of organic olive residue (alperujo), for plant growth promotion of *Cistus albidus* L. and enhancement of soil properties. Sixteen months after planting, the plants were harvested jointly the rhizosphere soil to evaluate the parameters that indicate the plant growth and its responses to stress due to scarcity of water, as well as the physico-chemical, chemical and biological properties of soil. The microbial inoculant and organic residue combined treatment was the most effective for stimulating the roots dry weight of *C. albidus* (by 133% with respect to control plants) and microbial inoculant was the most effective treatment for increasing the shoot dry weight of plants (by 106% with respect to non-treated plants). Total C, total organic C and microbial biomass C content and enzyme activities (dehydrogenase, urease and protease) of the rhizosphere of *C. albidus* were increased by microbial inoculant and organic residue combined treatment, but not by the microbial inoculation and organic residue applied independently. The combined treatment, involving microbial inoculant and addition of the organic residue, had an additive effect improving the biochemical and microbiological quality of the soil.

The second field experiment was developed to assess the influence of the inoculation with a mixture of two immobilised strains of rhizobacteria (*Azospirillum brasilense* and *Pantoea dispersa*) and the addition of organic olive residue (alperujo) on the growth of *Pinus halepensis* Mill. and plant stress parameters, as well as on soil physicochemical, chemical and biological properties. Twenty-eight months after planting, the plants were collected jointly the rhizosphere soil to develop the corresponding analyses. The results showed that the microbial inoculation was the

most-effective treatment regarding stimulation of seedling growth (by 48% with respect to the control) and nutrient uptake. The inoculated plants had the lowest proline accumulation, less oxidative damage to lipids and higher shoot water potential. At level soil, the microbial inoculation and combined treatment enhanced enzyme activities, total carbohydrates, microbial biomass C and nutrients on the rhizosphere of *P. halepensis*. The effectiveness of the microbial inoculant with respect to promotion of plant growth and the lower cost of implementation of this restoration biotechnology support its preferential use in re-afforestation tasks with *P. halepensis* in semiarid environments.

The third and last assay of this first part of the Thesis, also developed under field conditions, was focused to assess the combined effects of the inoculation with free native rhizobacteria (*Bacillus megaterium*, *Enterobacter* sp, *Bacillus thuringiensis* and *Bacillus* sp.) and the addition of composted sugar beet (SB) residue on soil properties and *Lavandula dentata* L. establishment. One year after planting, the plants and their rhizosphere soil were harvested to realize the different analyses on plants and soil. The data demonstrated that *Bacillus* sp. and *B. megaterium* + SB were the most effective treatments for increasing shoot dry biomass (by 5-fold with respect to control) and *Enterobacter* sp.+ SB was the most effective treatment for increasing dry root biomass. All the treatments evaluated significantly increased the foliar nutrient content (nitrogen, phosphorus and potassium) compared to control values (except *B. thuringiensis* + SB). The organic amendment had significantly increased available phosphorus content in rhizosphere soil by 29% respect to the control. *Enterobacter* sp. combined with sugar beet residue improved total N content in soil (by 46% respect to the control) as well as microbiological and biochemical properties. The selection of the most efficient rhizobacteria strains and their combined effect with organic residue seems to be a critical point that drives the effectiveness of using these biotechnological tools for the revegetation and rehabilitation of degraded soils under semiarid conditions.

The second part of the Thesis is based on methodologies focused to filed sampling, isolation and characterisation of bacteria strains. For that, two samplings

were carried out in two different sites of Province of Murcia, Vicente Blanes Ecological Park and Calblanque Nature Reserve. In both sites, rhizosphere soil samples of three specimens of autochthonous shrub *Rhamnus lycioides* L. were collected and transported to the laboratory. There, techniques specifically designed to isolated actinobacteria and different analyses to characterise and identify them were carried out. After the isolation and purification of actinobacteria, a total of twelve strains were obtained, six coming from Vicente Blanes Ecological Park and six belonging to Calblanque Nature Reserve. These strains were observed macroscopically and microscopically and were identified as actinobacteria, due to their morphology and to the corresponding Gram's stain that make possible to observe violet dyed bacteria that showed hyphal accumulations. So, it was confirmed the peculiar mycelial development, clearing their classification up. Subsequently, distinct analysis under laboratory conditions were carried out to characterise them as nitrogen fixing and phosphorus solubilising bacteria, besides their capacity to excrete siderophores, abilities that should make them plant growth promoting rhizobacteria under laboratory conditions. Among the twelve strains, four of them, two coming from Vicente Blanes Ecological Park and two belonging to Calblanque Nature Reserve, showed the best results in these laboratory assays, being observed similar behaviours between them. DNA was extracted from these four isolations and by the use of molecular techniques, it was amplified, purified and sequenced, being disclosed that these actinobacteria belonged to *Streptomyces* genus. The sequences were deposited on GenBank with their corresponding accession numbers. After the molecular identification, a final analysis was developed to verify the capacity of these four strains to inhibit the growth of four known plants pathogenic fungi (*Phytophthora parasitica*, *Pythium ultimum*, *Botrytis cinerea* and *Fusarium* sp.), being demonstrated an obvious antagonist activity with these pathogens. Therefore, the isolation, characterisation and identification techniques were considered as adequate due to it was obtained four actinobacteria strains belonging to genus *Streptomyces*, capable of fixing nitrogen, solubilising phosphates, excreting siderophores and inhibiting the plant pathogenic fungi growth. These actinobacteria

met the required abilities to be considered plant growth promoting rhizobacteria under laboratory conditions.

To complete the investigation, it was carried out a filed assay, corresponding to the third and last part of this Thesis. The field experiment was developed in two distinct sites of Province of Murcia, Vicente Blanes Ecological Park and Calblanque Nature Reserve, with the purpose to evaluate the efficacy of actinobacteria strains as plant growth promoting rhizobacteria and to assess the relative incidence of the strain origin and plantation area on the revegetation of semiarid zones. For that, *Rhamnus lycioides* was selected as target plant and the microbial inocula were bioencapsulated on alginate-potato starch by the inverse gelation technique. One year after planting, the plants were collected jointly their rhizosphere soil to analyse the parameters that indicated differences on plant performance as well as the physico-chemical, chemical and biological properties of soil. In this way, the results demonstrated that at Calblanque Nature Reserve, the inoculation with native strains was more effective than that with allochthonous strains, with respect to increasing shoot dry weight (about 48% and 28%, respectively, compared to non-treated plants), primarily due to improvements in nitrogen, phosphorus and potassium uptake and plant drought tolerance. However, at Rellano, the efficacy of plant growth promotion was not influenced by the strain origin. The highest increases in the urease, protease, and dehydrogenase activities and in microbial biomass C in response to inoculation with actinobacteria occurred at Vicente Blanes Ecological Park (about 200%, 28%, 29%, and 30%, respectively, compared to the respective controls), regardless of the origin of the strain assayed. Strain origin and the biological fertility of the plantation site should be considered in the selection of strains of actinobacteria for use in the revegetation with shrub species in semiarid environments.

Chapter I

Introduction

1. History and contemporary situation

Since at least the second half of the 19h century, erosion has been recognised as one of the most significant environmental problems worldwide, particularly in areas having seasonally contrasted climate (García-Ruiz et al., 2013). According to Hafidi et al. (2013), this is the case for the Mediterranean region, where it has been estimated that only 9-10% of the Mediterranean areas are currently forested, establishing the causes into the framework of the effects of the peculiar environmental conditions based on irregularity of rainfall distribution and long dry and hot summer associated, in part, to man-mediated deterioration activities (overgrazing, non-regulated cultivation techniques, deforestation, etc.), leading to short- and mid-term unsustainability (García-Ruiz et al., 2013). In addition, the development of a highly specialized flora and the emergence of a large number of endemic species make a return to natural conditions difficult following perturbation (Alados et al., 2011).

Analyses of precipitation and temperature confirm that Mediterranean climate is unique worldwide, being the only climate in which summer is both dry (frequently, extremely dry) and very hot, in order that the characteristics of Mediterranean region directly condition the composition and structure of plant cover. (García-Ruizet al., 2013). These consequences must be applied to microorganisms living in soil (Curiel-Yuste et. al, 2014), often acting as indicators of soil quality. In fact, it is well known that water scarcity and high temperatures strongly limits the physiological performance of microbes and the diffusion of nutrients in the soil pore space (Sardans et al., 2008). Nevertheless, other environmental factors such as soil type, nutrient availability or competitive interactions (Gibson et al, 2014) may also influence the relative composition of herbaceous and woody plants within a community. The understanding of the mechanisms behind the establishment of plant communities is a significant area of research in plant ecology. The data reported previously should allow perceiving plant-soil systems as a complex structure that goes further than the simple

interaction of vegetal covering and soil as a physical system, because soil can be understood as an ecosystem. In this fact, the recovery of soil characteristics is the first step leading to reduction of desertification processes and increasing soil cover by plantation is a successful way to preserve soil (De Gato et al., 2009).

1.1. Soil as ecosystem

Traditionally, soil has been considered as part of the different ecosystems; although it possesses all the basic elements that allow characterise it as an entire ecosystem unit: a physical area (biotope), a biotic community which organisms live together in the biotope (biocenosis) and a complex system of relations between them (Pardo, 2013) (Figure 1).

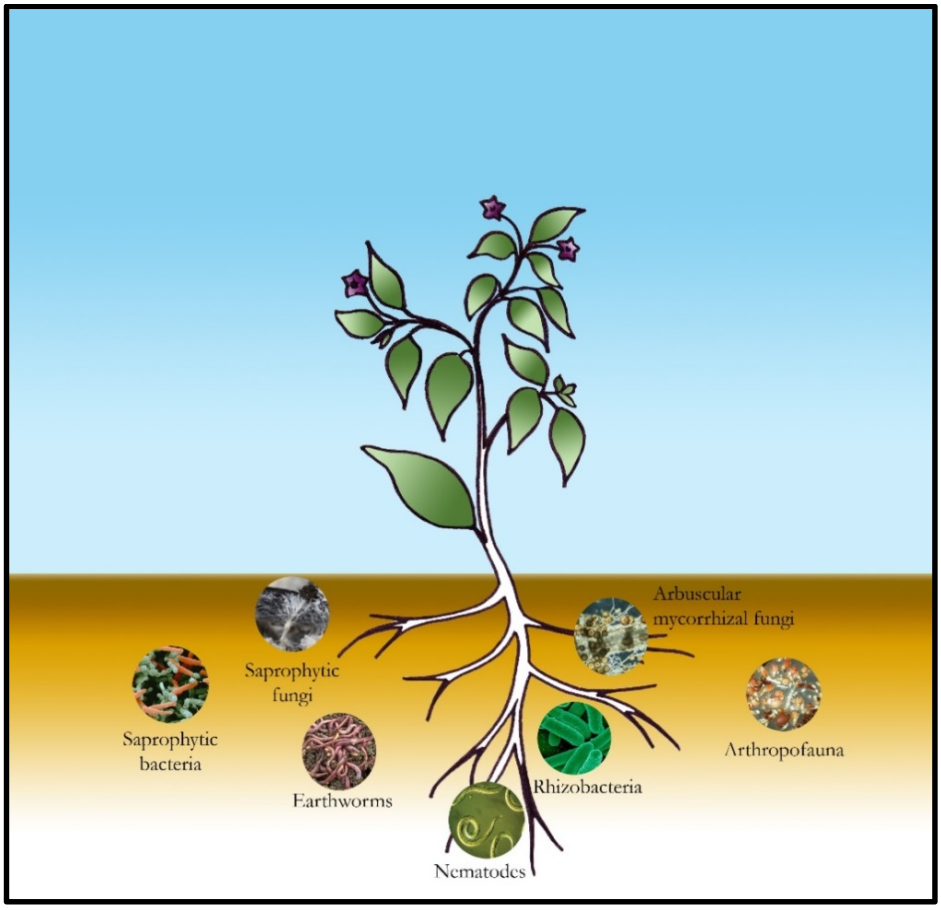


Figure 1. Soil biocenosis (plant-soil drawing modified from Pardo, 2013).

The biological community in soil is composed by a high amount of organisms belonging to all known groups (CCE, 2010) and they have a quite relevant role in the ecosystem because their activity and interrelations determines the soil utility (Breure, 2004; Pardo, 2013). Based in the concept biocenosis, ecological communities can take in various forms:

- Zoocenosis, for the faunal community.
- Phytocenosis, for the flora community.
- Microbiocenosis, for the microbial community.

1.2. Legislation

The Forest Acts of 8th June 1957 and its Regulation, approved by Royal Decree Law 485/1962 of 22 February (BOE, 1962) constituted the basic forest policy and in spite of it was a largely serviceable law, its antiquity and pre-constitutional character made evident the necessity of adequate it to current requests. In Act 5/1977 (BOE, 1977) this legislation suffered slight modifications on Development of Forestry Production of 4 January, but it was decentralized in the mid-eighties through the approval and implementation of the Royal Decrees on Transfer.

By virtue of the Royal Decrees on Transfers, the national government, at least partially, retained some management powers in, for example, forest hydrology and measures to combat desertification; co-ordination of forest fire fighting, management of the List of Basic Materials for producing forest reproduction material and the network of Forest Genetic Enhancement Centres, management of the network of indicators of the effects of atmospheric pollution on forests, co-ordination of measures to maintain and restore biological balances, co-ordination to maintain the administrative public records of the Catalogue of Public Utility Forests, the National Forest Inventory, the Forest Map and the national inventories of erosion zones and specially protected areas (BOE, 2003).

Inclusion of forest policy in the Ministry of Environment since 1996 has left competencies in some fields with the Ministry of Agriculture, Fisheries and Food.

These fields have been: statistics forest harvesting, plant health, reforestation of agricultural land and, in part, all the activities included in rural development, as well as management of the powers of public spending related with the European Union such as application of the Community of EAGGF (European Agricultural Guidance and Guarantee Fund) funds. There is also a lack of definition as to which agricultural or forest policies are applicable, as in the case of *dehesas (*grazing forests), woody crops, forest crops and others, in which the Autonomous Communities have not yet managed to define the policies that are applicable, thereby giving rise to dysfunctions from the point of view of those administered.

The currently forestry policy based on Forest Acts of 28th April 2006 (BOE, 2006), affirms and ratifies the notion of Public Utility Forests and maintains full valid the Ministerial Order 31th May 1966 (BOE, 1966), containing specify regulations to bring up to date the rosters of Public Utility Forests, correcting the failures that could be observed and introducing all the incidents happened since 1901 with the objective of perfecting their identification as figure and registration.

The Catalogue of Public Utility Forests, developed at Province of Murcia was approved by Royal Decree Law of 31th October 1975, suffering subsequent modifications that brought up to date its content. By Royal Decree Law 2102/1984 of 10th October about Transfer of Functions and National Services to Province of Murcia (BOE, 1984), the Catalogue of Public Utility Forests was assumed at autonomous level.

As established in Law 42/2007 of 13th December, Spain drafted a Strategic Plan for Natural Heritage and Biodiversity (BOE, 2007; 2011) and classified the protected nature reserves with ecological and scenic value on different ranks according to the values to protect and the objectives and good managements to accomplish. In Murcia Region, Autonomic Law 4/1992 (BORM, 1992), in its third additional order, lays down as protected some natural areas with the following ranking: Regional Park, Natural Reserve, Protected Landscape and Areas without assigned legal entity. They are detailed in Table 1.

Table 1. Ranking of protected natural areas in Murcia Region.

Regional Park	Natural Reserve	Protected Landscape	Areas without legal entity
▪ Cabo Cope-Puntas de Calnegre ▪ Carrascoy y El Valle ▪ Salinas y Arenales de San pedro del Pinatar	▪ Sotos y Bosques de Rivera de Cañaverosa ▪ Calblanque, Monte de Las Cenizas y Peña del Águila	▪ Barrancos de Gebas ▪ Cuatro Calas ▪ Espacios abiertos e Islas del Mar menor ▪ Humedal del Ajauque y Rambla Salada ▪ Sierra de las Moreras	▪ Cañón de Almádenes ▪ Islas e islotes del litoral Mediterráneo ▪ Sierra de la Muela, Cabo Tiñoso y Roldán ▪ Cabezo Gordo ▪ Saladares del Guadalentín ▪ Sierra de Salinas

Ministry of Agriculture Food and Environment is carrying out some intervention plans on favour of fight against desertification, since 1981 by LUCDEME Project (Lucha Contra la Desertificación en el Mediterráneo) (BOE, 1981) (Figure 2).

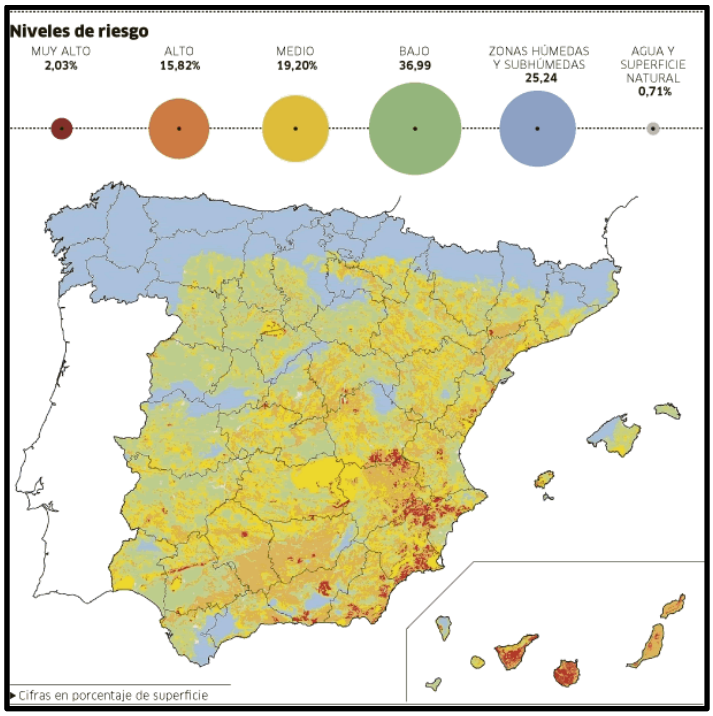


Figure 2. Spain map showing areas that are threatened by desertification. Red areas show very high risk, orange areas show high risk and yellow areas show medium risk (obtained from https://recursosccss.wordpress.com/about/geografia-de-espana/).

These project resources were spent on basic investigation and on projects based on the development of environmental methodologies under the coordination of Universities and Research Centers at Valencia, Almería, Málaga and Murcia.

With the approval of Spain, from United Nations Convention to Combat Desertification (UNCDD) (1996) it was developed the National Action Programme to Combat Desertification "Plan Nacional Contra la Desertificación" (PAND). Order ARM/2444/2008 of 12th August 2008, the last updated of PAND was approved in compliance of UNCDD (BOE, 2008). It describes as principal objectives:

1. The prevention and reduction of lands degradation.
2. Restoration of partially degraded soils.
3. Reclamation of desertified lands to contribute to sustainable development in arid, semiarid and dry sub-humid zones in Spanish territory.

2. Research studies and intervention techniques

When considering the develop of revegetation programs with the objective of reclaiming degraded areas, it is necessary to use technological restoration methods that can improve both soil quality and the ability of the seedlings to resist semiarid environmental conditions (Caravaca et al., 2005a), being the use of native plant species the most appropriate strategy to make a satisfactory recovery (Alguacil et al., 2003; Caravaca et al., 2005a). The introduction of native plant species has been increasingly advocated to achieve the sustainable recovery of degraded lands. Such species are presumed to be better suited to the local environmental conditions and better to establish balanced biotic interactions within the new community, as well as with the surrounding community (Oliveira et al., 2014) stimulating, at the same time, natural ecological succession (Pardo et al., 2014). However, not only the selection of an adequate plant can guarantee the successful in restoration tasks, but the compendium of different disciplines such as botany, physiology, microbiology,

chemistry, pedology, etc., could contribute to the upgrade of degraded lands. For this purpose, it is necessary to know some terms related to soil condition and plant performance.

2.1. Soil quality indicators

Doran and Parkin (1994) defined soil quality as "the capacity of the soil to function within ecosystems boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health". They cautioned that in selecting indicators, it is important to ensure that:

1. They correlate well with natural processes in the ecosystem.
2. They integrate soil physical, chemical and biological properties and processes, and serve as basic inputs required for estimation of soil properties or functions which are more difficult to measure directly.
3. They are relatively easy to use under field conditions.
4. They are sensitive to changes in management and climate.
5. They are components of existing soil databases wherever possible.

The indicators may directly monitoring the soil, or the outcomes influenced by the soil, such as increased biomass, improved water efficiency, and better aeration (Sharma et al., 2015). In Table 2, there are recorded physical, chemical and biological properties that were proposed as key indicator of quality soil in function of the spatial scale (Karlen et al., 2001).

Table 2. Potential physical, chemical and biological indicators of soil quality, measurable at various scales of assessment (Karlen et al., 2001).

Biological	Chemical	Physical
	Point scale indicators	
Microbial biomass	pH	Aggregate stability
Potential N mineralisation	Organic C and N	Aggregate size distribution
Particulate organic matter	Extractable macronutrients	Bulk density
Respiration	Electrical conductivity	Porosity
Earthworms	Micronutrient concentrations	Penetration resistance
Microbial communities	Heavy metals	Water- filled pore space
Soil enzymes	CEC and cation ratios	Profile depth
Fatty acid profiles	Caesium- 137 distribution	Crust formation and strength
Mycorrhiza populations	Xenobiotic loadings	Infiltration
	Field-, farm-, or watershed-scale indicators	
Crop yield	Soil organic matter changes	Topsoil thickness and
Weed infestations	Nutrient loading or mining	Compaction or ease of tillage
Disease pressure	Heavy metal accumulation	Ponding (infiltration)
Nutrient deficiencies	Changes in salinity	Rill and gully erosion
Growth characteristics	Leaching of runoff losses	Surface residue cover
	Regional-, national-, or international-scale indicators	
Productivity (yield stability)	Acidification	Desertification
Species richness, diversity	Salinization	Loss of vegetative cover
Keystone species and ecosystems engineers	Water quality changes	Wind and water erosion
Biomass, density, and abundance	Air quality changes (dust and chemical transport)	Siltation of rivers and lakes

2.2. Plants selection for reclamation of degraded areas

Several studies as published by and Castro et al. (2004), Navarro-Cerrillo et al. (2005) and Padilla and Pugnaire (2007) recorded that shrubs are nurse species for tree seedling, particularly in Mediterranean areas. Several studies reported that certain shrubs act facilitating the survival and growth of other neighbouring plant species by creating a better environmental habitat with low stresses from high

radiation and temperature as well as from soil nutrient and moisture deficiencies, referred to as "the nurse-plants syndrome (Hafidi et al., 2013). This ecological facilitation between plant species provides the patchy distribution of vegetation commonly observed in Mediterranean basin, especially in degraded ecosystems.

2.3. Plant performance indicators

When considering restoration programs to cover degrades areas, it is quite relevant to improve soil quality as well as plants performance. Plant performance includes their growth, shoot and roots weight, height and basal stem diameter. However, plant condition also must be determined by evaluating certain parameters than cannot be observed at first sight and must be analysed by techniques or specialized instruments, such as shoot nutrients contents and biochemical or physiological responses as a consequence of the plant subjection to biotic or abiotic stresses.

2.3.1. Nutrients uptake

In soils where nutrients are deficient, plants are known to show different responses to different specific nutrient deficiencies and the responses can vary between species. The most common changes are inhibition of primary root growth (often associated with P deficiency), increase in lateral root growth and density (often associated with N, P, Fe, and S deficiency) and increase in root hair growth and density (often associated with P and Fe deficiency) (Morgan and Connolly, 2013).

In order to maintain nutrient homeostasis, plants must regulate nutrient uptake and must respond to changes in the soil as well as within the plant. Thus, plant species develop various strategies for mobilisation and uptake of nutrients as well as chelation, transport between the various cells and organs of the plant and storage to achieve whole-plant nutrient homeostasis.

Here, a few examples are briefly cited as strategies used by plants to acquire nutrients from the soil (Courty et al., 2015; Morgan and Connolly, 2013):

1. Direct uptake from the soil.
2. Mycorrhizal symbiosis, being arbuscular mycorrhizal fungi (AMF) the most ancient and widely extended mycorrhizal relation.
3. Rhizobia symbiosis.

2.3.2. Abiotic stress parameters

Lambers et al. (1998) defined stress as "an environmental factor that reduces the rate of some physiological process below the maximum rate that the plant could otherwise sustain". Stresses can be generated by biotic and/or abiotic processes, resumed in Table 3.

Table 3. Different types of stress determined on plants.

Abiotic stresses		**Biotic stresses**
Physical	**Chemical**	
Hydric stress	Salinity	Herbivory
Excessive water	Heavy metals	Allelopathy
High-low temperatures	Atmospheric contamination	Interspecific competition
UV radiation	Toxins	Pathogens

The immediate response of the plant to stress is a reduction in performance that, generally, occurs over a time of scale from seconds to days. Plants compensate for the detrimental effects trough many mechanisms that are dependent of the nature of the stress and the physiological processes that are affected. If a plant is going to be successful in a stressful environment, then there must be some degree of stress resistance that differs widely among species. The processes range from avoidance to stress tolerance.

Following the initial stress response, acclimation can occurs like a morphological and physiological adjustment by individual plants to compensate for the decline in performance, usually within days to weeks. When acclimation requires

genetic changes in population it is necessary to define the term adaptation as an evolutionary response that requires many generations to occur (Figure 3).

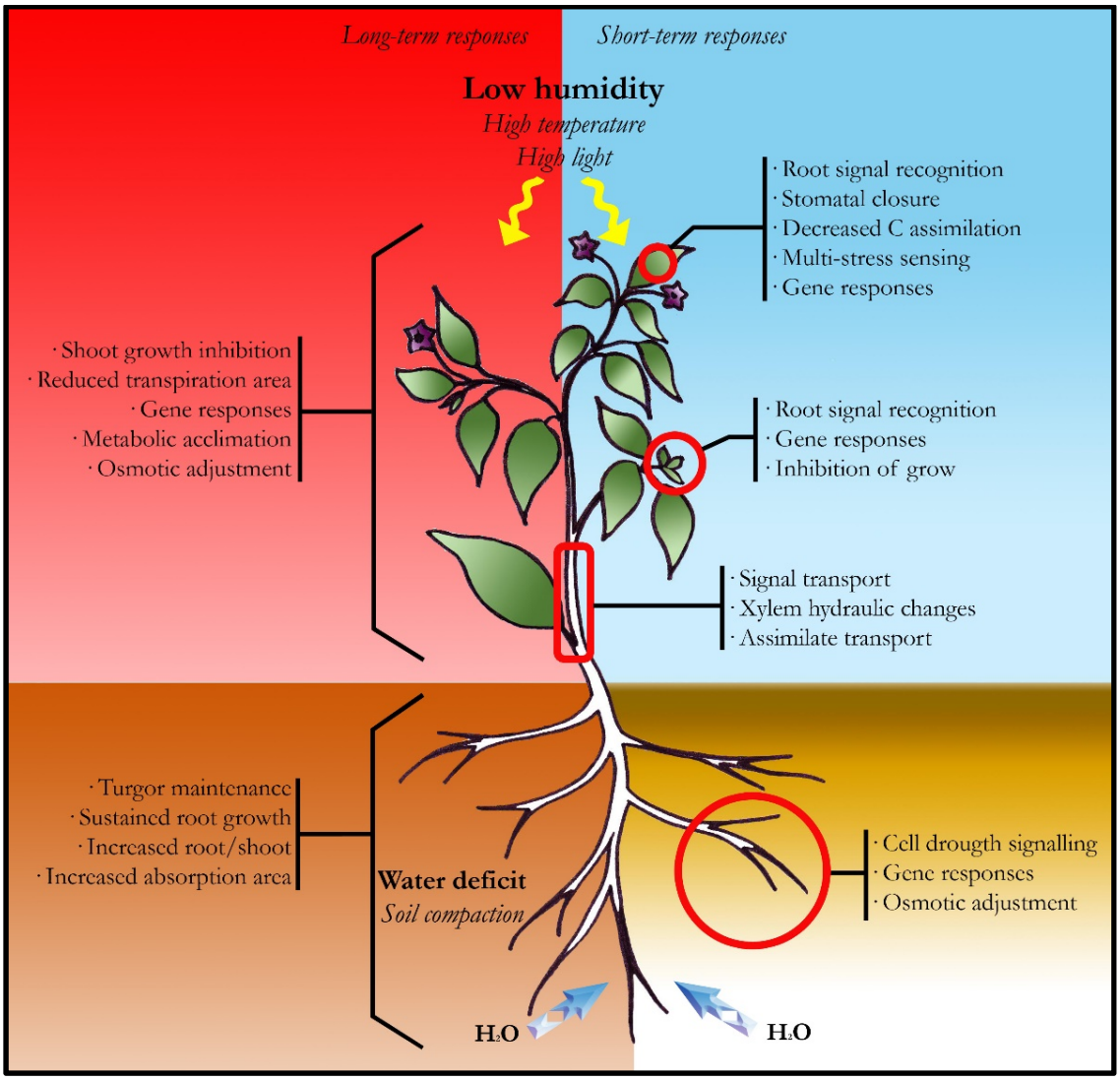


Figure 3. Whole-plant responses to drought stress. Left, long-term or acclimation responses; right short-term responses (plant-soil drawing modified from Chaves et al., 2003; Pardo, 2013).

Henceforth, the text is referred to hydric and high temperature abiotic stresses, due to their relevance in semiarid areas. On a global basis, soil and/or atmospheric water deficits, in conjunction with coincident high temperature and radiation, poses the most environmental constraints to plant survival (Chaves et al., 2003). It implies morphological, physiological, biochemical and molecular changes

on plants as response to water deficit (Kohler, 2008), affecting negatively plant performance.

Among the variables that can be measured to establish the level of stress showed by plant species, there are some considered relevant because they are indicators of their biochemical response. Besides, they have been used in Mediterranean semiarid soils (Armada et. al, 2014; Medina et al., 2010, Caravaca et al., 2005c):

- Oxidative damage: Under water stress conditions, when the use of absorbed light in either photosynthesis of photorespiration or the thermal dissipation are not enough to cope with excess energy, the production of highly reactive molecules is exacerbated (Smirnoff, 1998). These molecules, generated within the chloroplast, can cause oxidative damage to the photosynthetic apparatus (Smirnoff, 1998). It consists of a state of damage caused by reactive oxygen species (ROS): hydrogen peroxide (H_2O_2), the superoxide (O_2^-) and hydroxyl (OH) radicals and the singlet ($^1O_2$). Antioxidant molecules such as superoxide dismutases (SODs), catalases (CATs) and the enzymes and metabolites of the ascorbate-glutathione cycle can scavenge ROS (Foyer and Mullineaux, 1994).

- Proline accumulation: A crucial process in plant adaptation to water deficit is the osmotic adjustment, because it sustains tissue metabolic activity and enables regrowth upon rewetting, but varies greatly among genotypes (Morgan, 1984). Osmotic adjustment is normally a slow process and is triggered above a certain threshold of cell water deficit. Among the osmotic compounds synthetised, the amino acid proline is one of the most studied compatible solute (Hamilton and Heckathorn, 2001). Accumulation of high proline contents in cells has been associated with prevention of protein denaturation, preservation of enzyme and structure and activity (Samuel et al., 2000), and protection of membranes from damage by ROS produced under water deficit and high-light conditions (Hamilton and Heckathorn, 2001).

- Nitrate reductase: Hydric stress also takes part in the modulation of different enzymes such as nitrate reductase (Sachs et al., 1996; Mozhgan-Farzami et al., 2012). Nitrate reductase (NC 1.6.6.1) is the first enzyme in the nitrate assimilation pathway and under drought conditions its activity diminishes in plants due to a lower nitrate uptake by the roots (Caravaca et al., 2005c). Ruíz-Lozano and Azcón (1996) proposed nitrate reductase as a stress index since it is highly sensitive to the metabolic and physiological status of the plant and it was clearly demonstrated due to its increase under hydric stress conditions after plants inoculation with AM fungi (Caravaca et al., 2005c).

Other interesting parameter that could provide information about plant performance in the restoration of degraded sites is the loss of turgidity, a physiological process occurring in plants subjected to water deficiency. To determine that, it is necessary to measure certain water potentials as leaf water potential, stem water potential and leaf osmotic potentials. The difference between osmotic and water potentials results in leaf turgor potential, indicator of turgidity in plants (Cruz et al, 2012).

2.4. Use of microbial inocula in the reclamation of Mediterranean areas

Arbuscular mycorrhizal fungi as a tool for reclamation of degraded Mediterranean sites have been used over years. Thus, mycorrhizal symbiosis seems to be a key ecological factor on the functioning of ecosystems in semiarid Mediterranean regions (Requena et al., 1999). So, Alguacil et al. (2003, 2009a, 2011a, 2011b), Barea et al. (2011), Caravaca (2002a, 2003a, 2003b, 2005a), Estrada et al. (2013) and López-García et al. (2014), reported the importance, effectiveness and the functional role of arbuscular mycorrhizal fungi in semiarid Mediterranean areas. AM fungi are obligate symbiotic microorganisms which form mutualistic associations with the roots of most land plants. They may help plant to thrive in semiarid conditions (Caravaca et al., 2005a) by increasing the supply of nutrients to the plant (Kafkas and Ortas, 2009) and improving soil structure in eroded soils (Caravaca et al., 2002a) by enhancing aggregate stability on soil and by the

production of glomalin (Wright and Anderson, 2000) a glycoprotein that acts to bind soil mineral particles together.

Therefore, it is obvious the efficacy of the inoculation with AM fungi to favour the establishment of a vegetal cover in semiarid areas. Some studies have demonstrate that bacteria are often successful inoculants in revegetation tasks (Armada et al., 2014; Bashan et al. 2009, 2012 b; Ortiz et al., 2014) but their role in semiarid Mediterranean areas, specially under field conditions is a pending matter. Plant roots host a large variety of bacteria, many of them cooperating with the plant and enhancing plant nutrition, stress tolerance or health (Vacheron et al., 2013). They often are labelled Plant Growth Promoting Rhizobacteria (PGPR). Direct effects on plants may involve enhanced availability of nutrients, stimulation of root system development via production of phytohormones and other signals, or interference with synthesis of ethylene on plants and/ or induced systemic resistance. Indirect beneficial effects of PGPR on plants entail competition or antagonisms against phytoparasites (Bruto et al., 2014).

2.5. Use of organic amendments in the reclamation of Mediterranean areas

Several studies about the application of organic amendments as "alperujo" (olive mill residue) (Kohler et al., 2008), urban refuse (Alguacil et al., 2009), sugar beet residue (Caravaca et al., 2005a) and leftover material from wastewater treatment (Trejo et al., 2012) have reported a beneficial effect on semiarid and arid soils, increasing the proliferation and development of natural populations of soil microorganisms and improving soil properties. Besides, composting procedures can also help to reduce the amount of waste that is being directed into landfills. This means a reduction of concentrated, toxic leachates and methane gas that is being released into the atmosphere, which equates to a decrease in overall pollution. Composting also cuts down on the usage of chemical fertilizers, which are harmful to our water supply what enhances it high ecological valour.

2.6. Bioencapsulation as a tool to improve inocula performance

Colonisation of plant roots by direct inoculation of free PGPR cells into the soil is not easy because it is susceptible to environmental variations, such as soil conditions, fluctuation of pH and temperature, humidity, protozoa predation, and salt stress (Wu et al. 2012). This unpredictability of the PGPR inoculation success on plants is mainly due to the quality of formulations of inoculants containing an effective bacterial strain and determines the success or failure of a biological agent. Cell survival can be improved by immobilizing them into biodegradable carriers (Schoebitz et al., 2013, Figure 4).

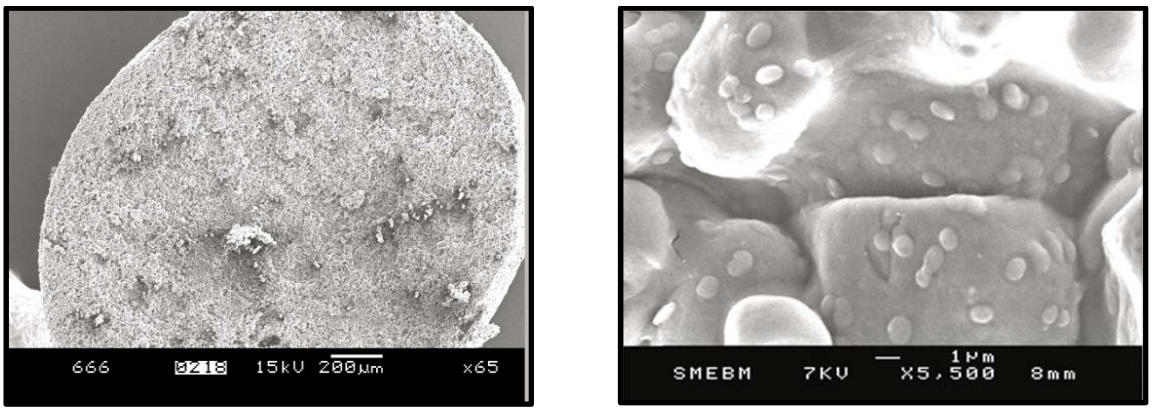


Figure 4. Scanning electron microscope pictures of alginate-starch beads: u at left, half of a biocapsule showing alginate-starch composition; at right, *Raoultella terrigena* immobilised on and adhered to the surface of starch granules (Schoebitz et al., 2012).

The principle of rhizobacteria bioencapsulation is to protect the microorganisms introduced into the soil and to ensure a gradual and prolonged release (Bashan 1998; Kim et al. 2012, Schoebitz et al, 2013). Due to climate conditions of semiarid areas and the benefits that bioencapsulation provides to microbial inocula, it can be assumed that this technology could be effective to improve the efficacy of rhizobacteria to reclaim degraded areas.

Chapter II

Objectives

In Mediterranean semiarid zones of Southeast of Spain, limited and irregular rainfalls and a long and dry summer periods have contributed, drastically, to the acceleration of soil degradation processes (Alguacil et al., 2011b). Environmental changes as a consequence of loss of natural vegetal communities are, often, accompanied by the physical and chemical soil properties degeneration and by a loss or reduction of microbial activity (Alguacil et al., 2011b). It is a corroborate fact that the proper functioning and stability of terrestrial ecosystems depends, to a large extent, of the diversity and composition of their vegetal cover. However, the ecological mechanisms that adjust and maintain the peculiar diversity of vegetal species in an ecosystem throughout the time are only known in a fragmentary way. Nowadays, it is permissible to think that the soil microbiota diversity and activity constitute the basis of one of the mechanisms that influences on soil preservation, on the development and maintenance of the vegetal cover and, consequently, on the ecosystem stability and functioning (Jenkins and Jain, 2010).

In consequence, it is necessary the understanding of different factors that regulate microbial activity associated to soil fertility and vegetal nutrition. Several studies have focused their purposes on the hunt and manipulation of microorganisms that are able to improve the roots development, solubilise mineral phosphorus non assimilable by the plant, fix atmospheric nitrogen, excrete siderophores, etc., and different activities that, often, are developed by interaction with other microorganisms (Franco-Correa, 2008). In short, to investigate the capacity of rhizosphere microorganisms acting as plant growth promoting microorganisms (PGPM).

The actions developed by microorganism in the rhizosphere, and that provide benefit in soil-plant systems can be summarized as follows (Franco-Correa, 2008):

1. Stimulation of seeds germination and roots development.
2. Enhancement of nutrients supply/availability.
3. Improvement of soil structure by stable aggregate formations as consequence of microbial development.

4. Plant protection against biotic and abiotic stresses by microbial activity.

At this point, it is important to define the term "rhizobacteria" that refers to the capacity of bacteria living in the rhizosphere of colonizing the soil-roots interfaces, where maintain a rate of development that allows them to be effective (Barea at al., 2005; Kloepper et al., 1991). Several studies have demonstrated the capacity of rhizobacteria to act as Plant Growth Promoting Rhizobacteria (PGPR). Among them, it can be found different species belonging to genera: *Agrobacterium, Alcaligenes, Arthrobacter, Enterobacter, Erwinia, Flavobacterium, Hafnia, Klebsiella, Serratia, Xanthomonas, Azotobacter, Azospirillum, Clostridium, Pseudomonas, Acetobacter, Burkholderia* and *Bacillus* (Kloepper et al., 1989; Bashan and Levanony, 1990; Okon and Lavandera-González, 1994; Tang, 1994; Martins et al., 2015; Peix et al., 2015) and some species have been tested to promote plant growth in arid or semiarid conditions (Bashan et al., 1999, 2009, 2012b; Valdenegro et al., 2001; Domínguez-Núñez et al., 2012). Actinobacteria are known as soil beneficial bacteria due to their ability to promote diverse functions over the ecosystem such as the improvement of soil structure and excretion of bioactive products with antagonism activity against pathogenic microorganisms, being the principal antibiotic producers. Particularly, it has been described capacities that can catalogue them as PGPR, although it is necessary to direct investigations to demonstrate this fact, due to these abilities have been observed separately in different species (Hamdali et al., 2008a). Besides, studies conducted to verify their field applications are scarce, being limited to assays in agricultural ecosystems (Jog et al., 2014) so that it is assumable that they could have a potential role on revegetation programmes.

The main objective in this Thesis is to evaluate, in degraded areas, the effectiveness of diverse plat growth promoting rhizobacteria strains jointly the addition of an organic waste on plant performance and on the quality of soil properties, as well as to verify the efficacy of some actinobacteria strains as plant growth promoting rhizobacteria, previously isolated from different soils of Province of Murcia.

To go into detail about this main objective, the following specific objectives have been proposed:

1. To select couplings PGPR-plant could be effective in the revegetation of semiarid areas
2. To evaluate the beneficial effect on the reclamation of degraded areas resulting from the addition of organic amendments to soil, verifying the improvement level achieved in plant-soil system after the inoculation of rhizobacteria and the addition of an organic amendment, separately and in combination.
3. To determine the presence of actinobacteria as a component of the bacterial community in the rhizosphere of *Rhamnus lycioides*, a target plant species capable of grow naturally in degraded Mediterranean areas, and the subsequent characterisation and determination under laboratory conditions.
4. To select different strains of actinobacteria that have met the abilities to be considered plant growth promoting rhizobacteria under laboratory conditions, and to test their efficacy under field conditions, as well as to verify the importance of their native or non-native character in the effectiveness of reclaiming areas subjected to deterioration processes.

Basing on the described purposes, this Thesis can be divided in three different parts:

- The first part consists of three field assays developed to evaluate the efficacy of the microbial inoculation and organic amendment addition to improve the performance of a target plant and the quality of soil properties at Vicente Blanes Ecological Park (Table 1).

Table 1. First part of the Thesis details.

Chapter	Target plant	Microbial inoculants	Organic residue	Objective
IV	*Cistus Albidus*	*Azospirillum brasilense- Pantoea dispersa*	Olive mill	1, 2
V	*Pinus halepensis*	*Azospirillum brasilense- Pantoea dispersa*	Olive mill	1,2
VI	*Lavandula dentata*	*Bacillus megaterium* *Enterobacter* sp. *Bacillus thuringiensis* *Bacillus* sp.	Sugar beet	1,2

- Second part is carried on, mainly, under laboratory conditions. The development of this assay tries to tackle, in a factory way, the isolation, characterisation and identification of actinobacteria that become part from the rhizobacteria group of a representative plant species of semiarid Mediterranean areas (Table 2).

Table 2. Second part of the Thesis details.

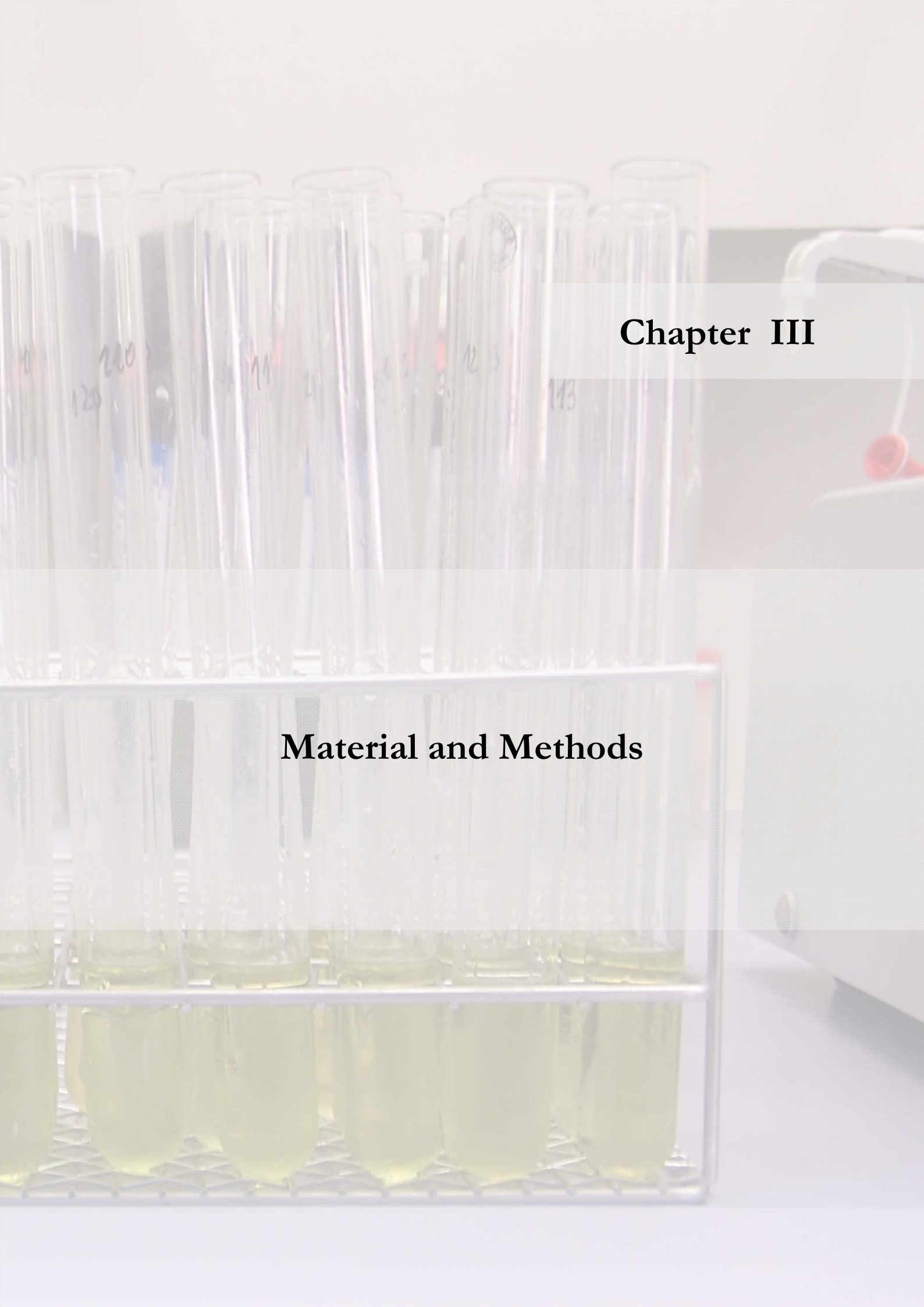
Chapter	Target plant	Bacteria subjected to isolation	Isolation areas	Objective
VII	*Rhamnus lycioides*	Actinobacteria	Vicente Blanes Ecological Park Calblanque Nature Reserve	3

- The third part of this Thesis is developed by a field assay with the purpose of verify the effectiveness of the inoculation of actinobacteria previously isolated and identified on the performance of a target plant and on the quality of two different soils properties (Vicente Blanes Ecological Park and Calblanque Nature Reserve) as well as study the importance of native or non-native character in their efficacy (Table 3).

Table 3. Third part of the third part of the Thesis details.

Chapter	**Target plant**	**Microbial inoculants**	**Experimental areas**	**Objective**
VIII	*Rhamnus lycioides*	Actinobacteria	Vicente Blanes Ecological Park Calblanque Nature Reserve	4


10 5
106
10 7
10 8



Chapter III

Material and Methods

1. Experimental areas

1.1. Location

The plantations in experimental fields were carried out in two different semiarid Mediterranean areas: Vicente Blanes Ecological Park in Rellano, Molina de Segura (coordinates 38°12' N, 1°13' W) and Calblanque Nature Reserve (coordinates 37º36' N, 0º45' W), both in the Province of Murcia, Spain.

Figure 1. Aerial and panoramic views of Vicente Blanes Ecological Park.

Figure 2. Aerial and panoramic views of Calblanque Nature Reserve.

1.2. Description

1.2.1. Climate

- Vicente Blanes Ecological Park has a potential evapotranspiration (ETP) that reaches 1000 mm and an annual average rainfall less than 250 mm. The mean annual temperature is 17.5°C.
- Calblanque Nature Reserve shows a mean annual temperature of 18.7 ºC and no frost period. The annual rainfall is around 270 mm and the ETP reaches out to 1000 mm per year.

1.2.2. Vegetal covering

- Vicente Blanes Ecological Park supports vegetation dominated by *Piptatherum miliaceum* (L.) Cosson, and some shrubs of *Thymus vulgaris* L., *Pistacia lentiscus* L., *Rhamnus lycioides* L., *Cistus clusii* Dunal and *Rosmarinus officinalis* L.
- Calblanque Nature Reserve shows flora composed by several shrubs species such as *Tetraclinis articulata* (Vahl) Mast., *Rhamnus lycioides* L., *Maytenus senegalensis* (Lam.) Loes., *Periploca angustifolia* Labill., and *Calicotome intermedia* C. Presl in Abh., and some tree species like *Pinus halepensis* Mill. and *Chamaerops humilis* L. the unique autochthonous palm in Spain.

1.2.3. Soil

- Vicente Blanes Ecological Park: The soil is classified as a Typic Torriorthent (SSS, 2010). The Typic subgroup is fixed on the driest Torriorthents. Many of these soils are shallow to weakly cemented rock. Some are moderately deep or deep to hard rock. Rellano soil is very little developed with low organic matter content due to it is gently sloping soil that are mostly on fans or piedmont slopes where the sediments are recent and have little organic carbon. It facilitates the degradation of soil structure.

- Calblanque Nature Reserve: The soil is a Lithic Torriorthent (SSS, 2010). It has a lithic contact that is within 50 cm of the surface and commonly is at a depth of less than 25 cm. It has a low moisture-storage capacity, and occurs mostly in association with soils of other orders or subgroups that have more moisture available to plants.

The methodology used to determinate the physico-chemical and microbiological characteristics of both soils is described in the subsection 6 "Analytical determination of soil physico-chemical, chemical and biological properties". The characterisation is detailed in Table 1.

Table 1. Soil physico-chemical, chemical and biological characteristics of Rellano and Calblanque soils.

Properties	Rellano	Calblanque
pH (H_2O)	8.5 ± 0.0[a]	7.74 ± 0.0
Electrical conductivity (1:5, μS cm^{-1})	176 ± 3	250 ± 3
Total C (g kg^{-1})	98.5 ±1.5	41.4 ± 1.3
Total organic C (g kg^{-1})	18.3 ± 5.3	23.7 ± 4.8
Water soluble C (mg kg^{-1})	76 ± 3	328 ± 3
Water soluble carbohydrates (mg kg^{-1})	11 ± 1	75 ± 2
Microbial biomass C (mg kg^{-1})	627 ± 31	1229 ± 49
Total N (g kg^{-1})	1.6 ± 0.0	2.0 ± 0.0
Available P (mg kg^{-1})	5 ± 0	8 ± 0
Extractable K (mg kg^{-1})	350 ±3	356 ± 5
Aggregate stability (%)	43.0 ± 1.0	71.4 ± 2.1

Mean ± standard error (n= 5).

2. Biological material used to develop the assays

2.1. Plant material

2.1.1. Cistus albidus

It is a representative autochthonous shrub from semiarid shrublands in southeast Spain, well adapted to water stress conditions and, therefore, frequently used in the revegetation of semiarid disturbed lands (Alguacil et al. 2011a) (Figure 3).

Figure 3. *Cistus albidus.*

2.1.2. Lavandula dentata L.

It is a small shrub that reaches a height of 30 cm, widely well adapted to water stress conditions and, therefore, could be potentially used in the restoration of Mediterranean disturbed lands (Ouahmane et al., 2006a) (Figure 4).

Figure 4. *Lavandula dentata.*

2.1.3. Pinus halepensis Mill.

It is a tree species, which can reach a height of 10 m, widely distributed in the Mediterranean areas. It supports water stress conditions and high temperatures what allows its use for revegetation assays of degraded (Díaz and Roldán, 2000; Maestre and Cortina, 2004) and arid soils (Oliveras et al., 2003) (Figure 5).

Figure 5. *Pinus halepensis.*

2.1.1. *Rhamnus lycioides* L.

It is a perennial shrub which can reach a height up to 3 m, naturally occurring in the Western Mediterranean Basin (Gulías and Traveset, 2012). It is representative from semiarid areas in southeast Spain, well adapted to water stress conditions and high temperatures, used frequently in the revegetation of semiarid disturbed lands (Caravaca et al., 2003a; 2005b, Alguacil et al., 2011b) (Figure 6).

Figure 6. *Rhamnus lycioides.*

2.2. Microbial inoculants

2.2.1. Azospirillum brasilense and Pantoea dispersa

- *Azospirillum brasilense* Tarrand, Krieg & Döbereiner, 1978 is a rhizobacteria capable of promoting the growth of plants and thus it is considered a "Plant Growth Promoting Rhizobacteria" (PGPR). Being one of the well-studied PGPR, this is a Gram-negative bacterium and belongs to the alpha subclass of Proteobacteria, alpha-Proteobacteria, to the group IV of this subclass, order Rhodospirillales and to the family *Rhodospirillaceae*. It is a diazotrophic microorganism, meaning these bacteria are able to fix atmospheric nitrogen gas into more useful forms such as ammonia (Flores et al., 2010). The phenotypic differentiation of *A. brasilense* from other diazotrophic members of group IV is based upon the size and shape of the bacterial cell, and the mode of nitrogen fixation. Within its family *Azospirillum* can be distinguished from other members by lack of phototrophy, that is the inability to use light energy to synthesise sugars and other organic molecules from carbon dioxide, the incapacity of forming root and stem hypertrophies, and G+C content. *A. brasilense* can be distinguished from other *Azosprillum* species

based upon the ability to utilise ribose and mannose (Okon and Itzigsohn, 1995) and its diazotrophic character (Flores et al, 2010).

- *Pantoea dispersa* Gavini, Mergaert, Beij, Mielcarek, Izard, Kersters, De Ley, 1989 is a genus of Gram-negative bacteria that belongs to the gamma subclass of Proteobacteria, gamma-Proteobacteria, to the order Enterobacteriales and to the family *Enterobacteriaceae*, recently separated from the *Enterobacter* genus. *Pantoea* bacteria are able to ferment lactose, are motile, and form mucoid colonies (Donnemberg, 2009).

Azospirillum has been shown to be more successful when it is co-inoculated with other microorganisms such as phosphate-solubilising bacteria (Bashan et al., 2004). To this end, *A. brasilense* was co-applied with *Pantoea dispersa* whose beneficial effect on plant development arises from its capacity to solubilise phosphorus compounds and help to control pathogenic organism (Son et al., 2006). The mixture of the two PGPR *A. brasilense* and *P. dispersa* was immobilised on clay pellets, being the cells concentrations of both rhizobacteria 10^9 CFU g^{-1}. This microbial inoculant was developed by Probelte, S.A., Murcia, and these strains were deposited in the Spanish Type Culture Collection (CECT) with the numbers CECT-5801 (*P. dispersa*) and CECT-5802 (*A. brasilense*).

The described mixture was used to develop the assays described in Chapters IV and V.

*2.2.2. Bacillu*s species and *Enterobacte*r sp.

- *Bacillus* species are rod-shaped, endospore-forming aerobic or facultative anaerobic, Gram-positive bacteria that are ubiquitous in nature. They belong to the group Firmicutes, order Bacillales and family *Bacillaceae*. The majority species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment. The genus includes (Turnbull, 1996):

- Thermophilic bacteria that thrive at relatively high temperatures, between 41 and 122 °C.
- Psychrophilic bacteria, able to grow and reproduce in cold temperatures, ranging from −20°C to 10°C.
- Acidophilic bacteria that thrive under highly acidic conditions, usually at pH 2 or below.
- Alkaliphilic bacteria capable of survival in alkaline, pH roughly 8.5-11, environments, growing optimally around a pH of 10.
- Halotolerant species, adapted to conditions of high salinity.

- *Bacillus megaterium* de Bary, 1884 is a rod-like, Gram-positive, mainly aerobic spore forming bacterium found in widely diverse habitats, growing (De Vos et al., 2009; Vary et al., 2007) at temperatures from 3 °C to 45 °C, with the optimum around 30 °C. It has been recognised as an endophytic, living within a plant for at least part of its life without causing apparent disease (Carroll, 1986) and as a potential agent for the biocontrol of plant diseases. Besides, nitrogen fixation has been demonstrated in some strains of *B. megaterium* (De Vos et al., 2009) what convert it in bacteria capable of promoting plant growth.

- *Bacillus thuringiensis* Berliner, is a soil-dwelling bacterium, Gram-positive, facultative anaerobic and spore forming which shows catalase activity. It is consider as ubiquitous bacterium due to also occurs naturally in the gut of caterpillars of various types of moths and butterflies, as well on leaf surfaces, aquatic environments, animal faeces, insect-rich environments, and flour mills and grain-storage facilities (Madigan et al, 2005)

- *Enterobacter* sp. is a genus of common Gram-negative, facultative anaerobic, rod-shaped and non-spore-forming Proteobacteria. They become part of the subclass gamma- proteobacteria, order Enterobacteriales and family *Enterobacteriaceae*. The genus *Enterobacter* is a member of the coliform group of bacteria, and although many of them are pathogenic, some species are

decomposer and can be found living on the soil organic matter. Many species possess flagella and thus are motile. Features such as motility, as well as certain biochemical properties, including the ability to synthesize an enzyme known as ornithine decarboxylase, are used to distinguish *Enterobacter* from the very similar and closely related *Klebsiella* bacteria. They ferment lactose with gas production (Russo and Johnson, 2008; Cabral, 2010; Wen-Si et al., 2014).

The plant growth promoting rhizobacteria *Bacillus* sp., *B. megaterium*, *B. thuringiensis* and *Enterobacter* sp., were used to develop the assay described in Chapter VI. They were isolated at Rellano experimental area and cultivated in Estación Experimental del Zaidín (EEZ). The rhizobacteria were grown in YEP (yeast extract peptone) broth (described in the section 8 "Media composition") for 2 days at room temperature on a Heidolph Unimax 1010 shaker. The bacterial culture was centrifuged at 2287 g for 5 m at 2ºC, and the sediment was resuspended in sterilized tap water (Alguacil et al., 2009b). The cells concentration of the bacterial suspension was 10^{10} CFU ml^{-1}.

2.2.3. Actinobacteria

- *Streptomyces* sp. *Streptomyces* species are characterised as Gram-positive aerobic bacteria of complex form. This genus of filamentous bacteria belongs to the order Actinomycetales and the family *Streptomycetaceae* that includes more than 500 species occurring in soil and water (Kämpfer, 2006). The genus *Streptomyces* produce well-developed vegetative hyphae (between 0.5-2.0 μm in diameter) with branches. They form a complex substrate mycelium that aids in scavenging organic compounds from their substrates (Chater, K., 1984). Although the mycelia and the aerial hyphae that arise from them are immotile, mobility is achieved by dispersion of spores. Many species are important in the decomposition of organic matter in soil, contributing in part to the earthy odour of soil as result from production of a volatile metabolite named geosmina, and decaying leaves to the fertility of soil. Certain species

are noted for the production of broad-spectrum antibiotics, chemicals that the bacteria naturally produce to kill or inhibit the growth of other microorganisms (Anderson and Wellington, 2001).

Streptomyces species were isolated from Vicente Blanes Ecological Park and Calblanque Nature Reserve experimental areas. They were grown on YEP broth, described in section 8 "Media composition", during 15 days at 28ºC and 180 rpm on a Heidolph Unimax 1010 shaker, reaching a cells concentration of 1.2 10^8 CFU g^{-1}. Once the strains were identified by molecular methodology, the strains were deposited in the GenBank with the accession numbers: RE1: LN610452; RE2: LN610454; CA1: LN610453 and CA2: LN610455. Actinobacteria were used to carry out the assays described in Chapter VII and VIII. Isolation protocol and molecular techniques are described in section 7 "Isolation, characterisation and encapsulation of actinobacteria."

2.2.4. Plant pathogenic fungi

- *Fusarium* sp. *Fusarium* is a large genus of filamentous fungi, part of a group often referred to as hyphomycetes, widely distributed in soil and associated with plants. Most species are harmless saprobes, and are relatively abundant members of the soil microbial community. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes (Summerell et al., 2010).
- *Phytophthora parasitica* Tucker. This fungal species belong to *Phytophthora* genus, part of a group labelled oomycetes, morphologically similar to but phylogenetically distant from true fungi. Most species in the genus *Phytophthora* are devastating plant pathogens, causing damages to both agricultural production and natural ecosystems. Thus, *P. parasitica* is a soilborne pathogen with a wide range of host plants (Meng et al, 2014).
- *Pythium ultimum* Fravel is a ubiquitous oomycete plant pathogen responsible for a variety of diseases on a broad range of crop and ornamental species.

Analysis of the *P. ultimum* genome sequence suggests that not all oomycete plant pathogens contain a similar 'toolkit' for survival and pathogenesis. This specie has a distinct effector repertoire compared to *Phytophthora* spp. It is an opportunistic pathogen of young seedlings and plant roots with little or no cuticle or heavily suberized tissue, consistent with lack of cutinase encoding genes. It is a poor competitor against secondary invaders of damaged plant tissues and soil organisms with better saprobic ability (Lévesque et al., 2010).

- *Botrytis cinerea* Pers.:Fr. is considered the second most important fungal plant pathogen (Dean et al., 2012). It belongs to order Loetiomycetes and is broad host range and ability to cause disease both pre- and postharvest lead to large economic effects (both in terms of yield loss and cost of control). *B. cinerea* is a necrotrophic pathogen, meaning it kills plant tissue prior to feeding, and uses a range of toxic molecules (Williamson et al., 2007) as well as the plant's own defence mechanisms (Govrin et al., 2006) to destroy host cells.

These pathogenic fungi were grown in nutritive agar dishes at 28ºC and were used to evaluate the ability of actinobacteria to inhibit fungal development.

3. Organic residues

3.1. Olive mill residue "alperujo"

The amendment consists on the organic fraction extracted with KOH from composted alperujo. The raw material was collected from an olive-mill located in Granada, Spain and mixed with fresh cow bedding as bulking agent for composting (Cegarra et al., 2006). The composting process was based on the Rutgers Strategy (Finstein et al., 1985) combined with mechanical turning and forced aeration. The extract was obtained by mechanical shaking during 24 h (12 h at 25 °C and 12 h at 70 °C) of the composted AL with 0.1 M KOH (1:20, w/v). The suspension was centrifuged at 14644 g for 20 m. After centrifugation the supernatant was freed of particulate matter. This amendment was used to develop the assay described in

Chapters IV and V. The analytical characteristics of alperujo residue are shown in Table 2.

Table 2. Chemical characteristics of the dissolved organic matter of composted alperujo.

Dry weight (g l^{-1})	25.0
pH	12.01
EC (mS cm^{-1})	9.78
Organic matter (g l^{-1})	17.4
Extractable C (g l^{-1})	8.6
Humic acid C (g l^{-1})	6.5
Fulvic acid (g l^{-1})	2.1
Carbohydrates (g l^{-1})	1.03
Phenols (g l^{-1})	1.58
Total N (mg l^{-1})	320
Na (mg l^{-1})	186
K (g l^{-1})	4.44
P (mg l^{-1})	62
Ca (mg l^{-1})	136
Mg (mg l^{-1})	28.9
Fe (mg l^{-1})	3.4
Cu (mg l^{-1})	0.2
Mn (mg l^{-1})	0.3
Zn (mg l^{-1})	1.2

Mean ± standard error (n= 5).

3.2. Sugar beet residue

This solid residue was dried in a 60ºC oven and then ground in an electrical grinder to 2-mm fragments. The sugar beet residue is a lignocellulosic material composed by cellulose, hemicellulose, lignin, total carbon and total nitrogen

(Caravaca et al 2005a). It was used to carry on the assay detailed in Chapter VI. The characterisation of the organic residue is detailed in Table 3.

Table 3. Sugar beet residue characterisation and composition.

Compounds	%
Cellulose	29
Hemicellulose	23
Lignin	5
Total C	55
Total N	2

4. Design and experimental factors

The design and experimental factors are exposed in each particular case in the pertinent chapter.

5. Analytical determinations on plants

5.1. Plant growth

Fresh shoot and root weights were written down. Dry weights were determined after drying vegetal material in a 70 ºC oven during 24 h. Basal stem diameter and plant height also were recorded.

5.2. Nutrients uptake

Nutrients uptake was determined after milling season of shoot dry biomass.

- Shoot nitrogen and total carbon values were established dry combustion using a LECO Tru-Spec CN analyser (Leco Corp., St. Joseph, MI, USA) after milling season of shoot dry biomass.

- Shoot phosphorus and potassium were determined by ICP/OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL).

5.3. Biochemical plant conditions

5.3.1. Nitrate reductase activity

Nitrate reductase activity was assayed in vivo by measuring NO_2^- production in tissue that had been vacuum-infiltrated with buffered NO_3^- solutions (Downs et al., 1993).

- Reagents:
  - 0.05 M Tris-HCl buffer, pH 7.8
  - 0.25 M KNO_3
  - 1% Sulphanilamide in 1 M HCl
  - 0.01% N-(1-naphthyl)-ethylenediamine hydrochloride
- Procedure:

  The leaves or needles (in each particular case) were collected in the morning over 11:00 h solar time and cut into 5-mm sections. Approximately 300 mg of leaf or needle punches were placed in tubes containing 2 ml of an incubation medium consisting of 0.05 M Tris-HCl, pH 7.8 and 0.25 M KNO3. The tubes were sealed and kept in the dark at 30ºC for 1 h. The nitrite released into the medium was determined after incubation by treating 1 ml aliquots with 1 ml of 1% sulphanilamide in 1 M HCl and 1 mL of 0.01% N-1-napthyl-ethylenediamine hydrochloride. After 15 m, the optical density was measured spectrophotometrically at 540 nm.

5.3.2. Lipid peroxidation

Lipid peroxidation was determined by the content of malondialdehyde (MDA), a product of lipid peroxidation (Zhao et al., 1994) by the method of Minotti and Aust (1987).

- Reagents:
  - 100 mM Potassium phosphate buffer
  - 15 % (w/v) Trichloroacetic acid (TCA)
  - 0.375 % (w/v) 2-thiobarbituric acid (TBA)
  - 0.1% (w/v) butyl hydroxytoluene
  - 0.25 N HCl
- Procedure:

  Lipid peroxides were extracted by grinding 0.5 g of fresh leaves or needles in an ice-cold mortar and 6 ml of 100 mM potassium phosphate buffer (pH 7). Homogenates were filtered through one Miracloth layer and centrifuged at 15 000 g for 20 m. The chromogen was formed by mixing 200 ml of supernatants with 1 ml of a reaction mixture containing 15% TCA, 0.375% TBA, 0.1% butyl hydroxytoluene, and 0.25 N HCl, and by incubating the mixture at 100 °C for 30 m. After cooling it was centrifuged at 800 g for 5 m and the absorbance of the supernatant was recorded at 535 nm. The calibration curve was carried out with different concentrations of MDA.

5.3.3. Proline accumulation

Proline accumulation was evaluated from 1 g of fresh leaves or needles by the method described by Paquin and Lechasseur (1979).

- Reagents:
  - Chloroform
  - Methanol
  - 0.88% (w/v) NaCl
  - Ninhydrin reagent
  - Glacial acetic acid
  - Toluene

- Preparation of ninhydrin reagent:

  1.25 g of ninhydrin were weighted and put into 30 ml of glacial acid acetic and 20 ml of 6M phosphoric acid shaking to dissolve the mixture. It can be kept out at 4ºC a maximum of 24 h.

- Procedure:

  The plant sample was homogenised in a cool mortar containing liquid nitrogen and added to a 50 ml polystyrene tube with 3.75 ml of methanol. After vortexing twice during 1 m, 7.5 ml of chloroform was put in the tube and the content was vortexing again for 1 m. Right after, 3.75 ml of 0.88% NaCl were added and the mixture was shaked and centrifuged at 5000 rpm and 0ºC during 10 m. The methanolic phase was used for the quantification of proline according Bates et al (1973). In a test glass tube 1 ml of the obtained extract was introduced, and then, 1 ml of ninhydrin reagent and glacial acetic acid were added. The mixture was heated at 100ºC for 1 h. The reaction was stopped by cooling on ice. The proline was extracted with 2 ml of toluene by vortexing during 20 s and the absorbance was measured at 530 nm. A standard curve of L-proline (PRO) was used for calibration and it was realized from a 1mM proline solution with the following concentrations: 0, 25, 50, 100, 200 and 300 μM proline.

5.4. Water potentials

5.4.1. Leaf water potential

Leaf water potential was measured in two fully developed needles per tree of each replicate in a pressure chamber (model 600-EXP, PMS Instrument Co., Albany, USA) (Mellisho et al., 2012).

5.4.2. Stem water potential

Stem water potential was measured at midday (12 h solar time), in a similar number and type of needles as used for leaf water potential, enclosing needles in a

small black plastic bag covered with aluminium foil for at least 2 h before measurements in the pressure chamber (Cruz et al, 2012) (Figure 7).

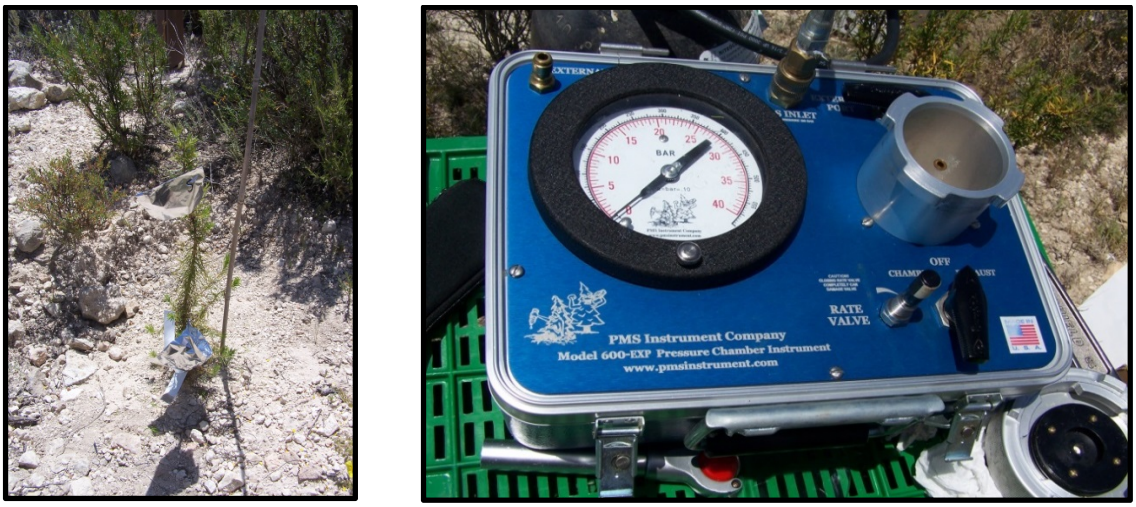


Figure 7. Stem water potential measuring by a pressure chamber.

5.4.1. Leaf osmotic potentials

Leaf osmotic potentials were determined in the same needles used for leaf water potentials. Needles were frozen in liquid nitrogen and the osmotic potential was measured after thawing the samples and expressing sap, using a vapor pressure osmometer (Wescor 5600, Logan, USA). Leaf turgor potential was derived as the difference between osmotic and water potentials (Cruz et al, 2012).

6. Analytical determinations of soil physico-chemical, chemical and biological properties

After plant growth period, some plants per treatment including root systems and rhizosphere soil were harvested, and introduced in polyethylene bags for transport to the laboratory. Rhizosphere soil samples were separated into two subsamples before physico-chemical, chemical and biological analyses: one subsample sieved to < 2 mm and other subsample sieved between 4 and 0.25 mm.

6.1. Physico-chemical analyses

6.1.1. pH

It was obtained a 1:10 (w/v) aqueous solution by mechanical shaking during 2 h. Right after, the pH measurement was carried out by using a pH-Meter Crison PH25.

6.1.2. Electrical conductivity

The extract obtained previously for pH measurement was centrifuged at 7500 rpm during 10 m and the supernatant was utilized to obtain the electrical conductivity with a conductimeter Crison, 522.

6.1.3. *Aggregate stability*

The percentage of stable aggregates was determined by the method described in Lax et al. (1994). 4 g aliquot of soil sieved between 0.25 and 4 mm was placed on a small 0.250 mm sieve and wetted by spraying with water. After 15 m the soil was subjected to an artificial rainfall of 150 ml with energy of 270 J m^{-2}. The remaining soil on the sieve was dried at 105 °C and weighted (P1). The soil was then soaked in distilled water and, after 2 h, passed through the same 0.250 mm sieve with the assistance of a spatula to break the remaining aggregates. The residue remaining on the sieve, made up of plant debris and sand particles, was dried at 105 °C and weighted (P2). The mass of stable aggregates as a percentage of the total aggregates was calculated by (P1 − P2) × 100/ (4 − P2).

6.2. Chemical analyses

6.2.1. Total carbohydrates

Total carbohydrates were determined by the method of Brink et al. (1960).

- Reagents:
  - 72% (v/v) sulphuric acid
  - 0.2% Anthrone reagent in 96% sulphuric acid
  - Glucose solution (25 mg l^{-1})

- Procedure:

  0.25 g of soil samples were introduced into glass test tubes. 1 ml of sulphuric acid 72% and 20 ml of distilled water were added. Right after, the content of test tubes was heated on a boiling water bath (Figure 8) at 104ºC during 5 h. The tubes were cooled and the content was filtered (Whatman Paper number 40). Distilled water was added to reach up 50 ml. 1 ml of this extract was introduced in clean glass test tubes and 4 ml of the anthrone was put in. The content of test tubes was vortexed for 10 s and heated on a water bath at 80ºC during 10 m. To get the total carbohydrates values, the mixture contained in the tubes was cooled and the absorbances of the samples were measured at 630 nm.

6.2.2. Water soluble carbohydrates

The same extract obtained to determine electrical conductivity was used to establish water-soluble carbohydrates data according to Brink et al. (1960) (Figure 8).

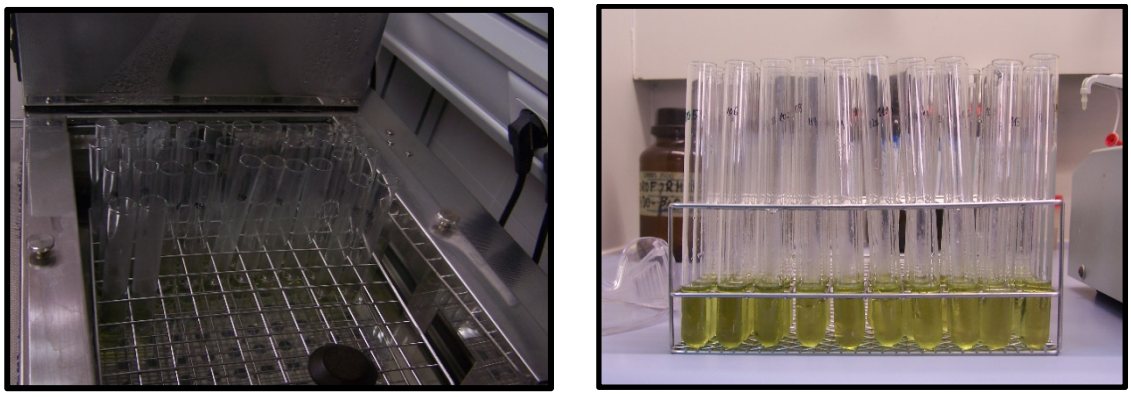


Figure 8. Water bath and test tubes prepared to the measurement of absorbances.

- Reagents:
  - Anthrone reagent (0.2% in sulphuric acid 96%)
- Procedure:

  1 ml of the extracts was introduced in glass test tubes and 4 ml of anthrone reagent were added. They were vortexing during 10 s and heated on a water

bath at 80ºC for 10 m. Once the content of the tubes got cold, the absorbances were measured at 630 nm.

6.2.3. Total carbon, total organic carbon and total nitrogen

They were measured by dry combustion using a LECO Tru-Spec CN analyser (Leco Corp., St. Joseph, MI, USA). For this purpose, soil samples were grinded with an agate mortar.

6.2.4. Water soluble carbon

Water soluble carbon was determined using the same extract obtained for electrical conductivity by using an automatic carbon analyser for liquid samples (Shimadzu TOC-5050A).

6.2.5. Available phosphorus

Available phosphorus was extracted with 0.5M $NaHCO_3$ and determined by ICP/OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL).

- Reagents:
  - 0.5 M $NaHCO_3$
- Procedure:

  5 g of soil sample sieved to 2mm was weighted and introduced in 250 ml containers. Then, the extract was obtained by adding 100 ml of extractor 0.5 M $NaHCO_3$ solution and mechanical shaking for 30 m. Right after, the extract was filtered.

6.2.6. Extractable potassium.

Extractable potassium was extracted with ammonium acetate and established by ICP/OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL).

- Reagents:
  - 1N Ammonium acetate

- Procedure:

  1 g of soil sample sieved to 2 mm was weighted and introduced in 50 ml polystyrene tubes. Then, the extract was obtained by adding 20 ml of extractor 1 N ammonium acetate solution and mechanical shaking for 30 m. Right after, the extract was centrifuged at 2500 rpm during 5 m and filtered.

6.3. Biological properties

6.3.1. Microbial biomass carbon

- Reagents:
  - Glucose
  - 0.2% (w/v) KOH
- Procedure:

  Microbial biomass C was assayed after glucose was mixed into the soil (at 60% of its field capacity) at a rate of 0.5% (w/w) and monitoring the CO_2 production for 24 h, using the μ-Trac 4200 system (SY-LAB, GmbH P.O. Box 47, A-3002 Neupurkersdorf, Austria). This system is based on the variation of electrical impedance of a KOH 0.2% water solution (Fernández et al., 2004).

6.3.2. Soil respiration

- Reagents:
  - 0.2% (w/v) KOH
- Procedure:

  Respiration rates were calculated in the linear phase of the respiration curves. Basal soil respiration was assessed with the same system described for microbial biomass C but in absence of glucose.

6.3.3. Glomalin related soil protein (GPRS)

The glomalin-related soil protein was extracted from soil samples (sieved between 0.250 and 4 mm and to < 2 mm) according to Wright and Anderson (2000).

- Reagents:
  - 20 mM sodium citrate, pH 7.0
  - Bradford reagent (BioRed)
- Procedure:

  250 mg of soil were mixed with 2 ml of sodium citrate, and then autoclaved at 121ºC for 30 m. The supernatant was removed and two additional sequential 1-h extractions were performed. All supernatants from a sample were combined, the volume was measured, an aliquot was centrifuged at 10000 × g for 15 m to remove soil particles and Bradford-reactive total protein was measured.

6.3.4. Dehydrogenase activity

Dehydrogenase activity was determined according to García et al. (1997). In presence on INT acting as electron acceptor and in absence of any buffer, it is formed the INTF salt.

- Reagents:
  - 0.4% (w/v) INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride)
  - N,N- Dimethylformamide
  - Methanol
  - INTF (Iodonitrotetrazolium formazan)
- Procedure:

  1 g of soil at 60% of its field capacity was exposed to 0.2 ml of 0.4% INT in distilled water for 20 h at 22°C in darkness. At the same time, controls not containing INT but distilled water were prepared. After the incubation period, the INTF formed was extracted with 10 ml of methanol by shaking

vigorously for 1 m and filtering through a Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm. The curve patron was developed using different concentrations of INTF.

6.3.5. Urease activity

Urease activity was calculated by the method described by Nannipieri et al. (1980) as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al., 1980).

- Reagents:
  - 6.4 % (w/v) urea
  - 0.1 M phosphate buffer, pH 7.0
  - 0.3 M sodium citrate
  - 1% (w/v) sodium nitroprusside
  - A reagent: 15.62 g of sodium salicylate were dissolved into 4 ml of sodium nitroprusside. Then, distilled water was added to reach up 200 ml.
  - B reagent: 0.5 g sodium dichloroisocyanurate and 4 g of NaOH were dissolved into 100 ml of distilled water.
- Procedure:

  Aliquots of 2 ml of 0.1 M phosphate buffer and 0.5 ml of 6.4 % were added to 0.5 g of sample followed by incubation for 90 m at 30 °C. Right after, distilled water was added to get 10 ml of total volume. The samples were centrifuged at 3400 rpm during 8 m and 1 ml of supernatants were added to 5.8 ml of distilled water, 0.8 ml of sodium citrate, 1.6 ml of A reagent and 8.0 ml of B reagent, reaching to 10 ml. Samples were conserved in darkness at room temperature for 45 m and the absorbances were measured spectrophotometrically at 660 nm. Controls were prepared in the previously described way, but in absence of urea.

6.3.6. Protease activity (BAA)

Determination of N-α-benzoyl-L-argininamide (BAA) hydrolysing protease activities was realized as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al., 1980).

- Reagents:
  - 0.3 M N-α-benzoyl-L-argininamide (BAA)
  - 0.1 M phosphate buffer, pH 7.0
  - 0.3 M sodium citrate
  - 1% (w/v) sodium nitroprusside
  - A reagent: 15.62 g of sodium salicylate were dissolved into 4 ml of sodium nitroprusside. Then, distilled water was added to reach up 200 ml.
  - B reagent: 0.5 g sodium dichloroisocyanurate and 4 g of NaOH were dissolved into 100 ml of distilled water.
- Procedure:

  This activity was determined in 0.1 M phosphate buffer at pH 7 with 0.03 M BAA as substrate. Aliquots of 2 ml of buffer and 0.5 ml of substrate were added to 0.5 g of sample followed by incubation for 90 m at 39ºC. The rest of the protocol is equal to that described for urease activity.

6.3.7. Alkaline phosphatase activity

Alkaline phosphatase activity was evaluated according to Tabatabai and Bremmer (1969) based on the spectrophotometrically determination of released *p*-nitrophenol after incubation of soil samples at 37ºC with a buffered solution (pH 11) obtained from unnatural substrate *p*-nitrophenyl phosphate disodium (Naseby and Lynch, 1997).

- Reagents:
  - MUB stock solution: 12.2 g of tris-hydroxymethyl-aminomethane (THAM), 11.6 g of maleic acid, 14 g of citric acid and 6.28 g of boric

acid were dissolved in distilled water and 488 ml of 1 M NaOH were added. Right after, distilled water was added to reach 1000 ml. The solution was conserved at 4ºC.

- MUB pH 11: from the stock solution Modified Universal Buffer (MUB) pH 11 was obtained. For that, 0.1 M NaOH was added to 200 ml of stock solution until obtain a solution with pH 11.
- 0.025 M *p*-nitrophenyl phosphate disodium
- *p*-nitrophenol (1000 μg ml^{-1}): since this solution a curve patron was obtained by reducing the *p*-nitrophenol concentration until 250 μg ml^{-1}.
- 0.5 M $CaCl_2$
- 0.5 M NaOH

- Procedure:

  2 ml of 0.5 M MUB buffer at pH 11 and 0.5 ml of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 m. The reaction was stopped by cooling at 0°C for 10 m. Then 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5M NaOH were added and the mixture centrifuged at 4000 rev min^{-1} for 5 min. The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm. Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

6.3.8. β-glucosidase activity

β-glucosidase activity was established by the colorimetric method described by Tabatabai (1982), based on the determination of *p*-nitrophenol obtained after the incubation of soil samples with artificial substrate *p*-nitrophenyl-β-D-glucopyranoside (PNG) at 37ºC and pH 6.5 and the subsequent extraction of *p*-nitrophenol after filtration once $CaCl_2$ and THAM buffer pH 12 were added.

- Reagents:
  - 0.025 M *p*-nitrophenyl-β-D-glucopyranoside (PNG)
  - *p*-nitrophenol (1000 μg ml^{-1}): since this solution a curve patron was obtained by reducing the p-nitrophenol concentration until 250 μg ml^{-1}.

- 0.5 M $CaCl_2$
- 0.5 M NaOH
- Stock solution: The same solution that the used for alkaline phosphatase activity.
- MUB pH 6.5: from the stock solution Modified Universal Buffer (MUB) pH 6.5 was obtained. For that, HCl was added to 200 ml of stock solution until obtain a solution with pH 6.5.
- Tris-hydroxymethyl aminomethano (TAMH)

▪ Procedure:

For this assay, 2 ml of 0.1 M MUB buffer at pH 6.5 and 0.5 ml of substrate were added to 0.5 g of sample and incubated at 37°C for 90 m. The reaction was stopped with TAMH. The amount of PNP was determined by spectrophotometry at 398 nm (Tabatabai and Bremmer, 1969). Controls were prepared in the identical way that samples but in absence of substrate.

7. Isolation, characterisation and encapsulation of actinobacteria

7.1. Isolation and culturing of actinobacteria

For actinomycete isolation, 9 ml of saline solution at 0.9% (w/v) were placed into polystyrene tubes and 1 g of soil from each sample was added. Then, these samples were homogenised by vortexing and heated at 50ºC for 10 s to promote growth of strains on synthetic culture media (Franco-Correa et al., 2010). After the heat treatments, the samples were diluted eight times in saline solution. Aliquots of each dilution (0.1 ml) were spread on oatmeal-agar medium, in Petri dishes. This medium was supplemented with penicillin (25mg ml^{-1}), nistatyn (0.1%), and cicloheximide (50 mg ml^{-1}) to inhibit the growth of other bacteria and fungi (Franco-Correa et al., 2010). The spread dishes were incubated at 28ºC for 15 days.

Isolated strains were purified on oat-meal agar medium (Franco-Correa et al., 2010) and placed into flaks which contained 50 ml of liquid Yeast Extract Peptone

(YEP) medium (the composition of this medium is detailed in section 8 "Media composition") The flaks were placed on an orbital agitator at 180 rpm during 15 days at 28ºC.

7.2. Macroscopic and microscopic identification

Visual observation of both morphological and microscopic characteristics using Gram staining and light microscopy was performed. Morphological characters were observed on oat meal agar and were used for classification and differentiation considering the aerial mass colour and substrate mycelium (Bergey, 2000).

7.3. Growth in N-free medium and acetylene reduction

The fixation of nitrogen was studied by streaking actinomycetes in a solid nitrogen-free medium (NFB) (Döbereiner and Day, 1976) described in section 8 "Media composition". Cultures were examined for growth characteristics after 15 days of incubation at 28ºC.

A CFU of each actinobacteria strain that showed growth in solid NFB medium was inoculated in an Erlenmeyer flask containing 30 ml of NFB broth, and was shaked during 30 days at 160 rpm at 28 ºC. Ethylene was measured by reduction of acetylene to ethylene activity (ARA) according to Hardy et al. (1968). 2 ml NFB broth containing actinobacteria in a concentration of 10^8 CFU were placed into vials of 10 ml, containing 5 ml of semi-solid NFB medium (composition is described in section 8 "Media composition") and were incubated during 10 days in the vessels at room temperature (22 º) (Correa, 2008). After the inoculation time, 0.5 ml of air was replaced with acetylene in each vial, and then, they were incubated during 3 h. At the end of the incubation time, 0.5 ml of the contents in each vessel was injected in a Hewlett-Packard gas chromatograph model 5890, equipped with a flame ionization detector and stainless steel column (3 m × 3.25 mm i.d.) containing activated 80/100 mesh alumina. The quantification of ethylene was carried out by interpolation of the area obtained in a curve pattern prepared with pure acetylene. The reduction activity of acetylene was determined in nm ml^{-1} h of ethylene.

7.4. Siderophore production

The qualitative evaluation of siderophore production was performed using a commercial kit, SideroTec AssayTM (Emergen Bio) following the manufacturer's recommendations.

7.5. Phosphate solubilising capacity

Actinomycete strains were cultivated on solid minimal medium based on the Pikovskaya (PVK) medium (Pikovskaya, 1948), described in section 8. It was supplemented with bromocresol purple as a pH indicator for determining the implication of organic acid production in this activity (Franco-Correa et al., 2010). Before autoclaving, the media pH was adjusted to 7.5. Holes of 5 mm of diameter were made in plates with 30 ml of the medium, and 30 µl of sample consisting on 10 ml of a bacterial suspension in saline solution at 0.9% with a concentration ~10^8 CFU ml^{-1}, were put in the holes. The halos due to the phosphate solubilisation were measured at 15th day of the plate incubation at 28ºC. Solubilisation index (SI) was calculated using the following formula (Premono et al., 1996): SI = (colony diameter + halozone diameter) / colony diameter.

Once this test was assayed, the strains that recorded the greatest values to phosphate solubilisation were selected to their molecular identification and to evaluate their biocontrol capacity.

7.6. Molecular identification

For each actinobacteria strain, total DNA was extracted from a CFU using Chelex 100 Resin following the manufacturer's recommendations (BIO-RAD). The actinomycete samples were placed into a 2 ml screw-crap propylene tube with 0.4 of Chelex solution (20%), homogenized by vortexing and heated, first at 56ºC for 30 m and after at 95ºC for 10'. After centrifuged at 14000 rpm, the supernatants (DNA extracts) were stored a -20ºC.

PCR amplification of the partial ribosomal small subunit (SSU) DNA fragments was performed using the universal primer pair fD1 and rp2 (Weisburg et

al., 1991). PCR was made in a final volume of 25 µl using the "ready to go" PCR beads (Amersham Pharmacia Biotech) and 0.5 µM of each primer (PCR conditions: 95ºC for 3 m, then 30 cycles of 30 s denaturation at 94ºC, 30 s primer annealing at 55ºC and 1 m extension at 72ºC, followed by a final extension period of 7 m at 72ºC). DNA extracts were stored at -20ºC. All the PCR reactions were run on a Perkin Elmer Cetus DNA Thermal Cycler.

The PCR products were purified using a Gel extraction Kit (Qiagen) (Alguacil et al., 2012). The sequencing was done by the section of Molecular Biology of the SAI (Campus de Espinardo- Murcia, Spain) using the universal primers fD1 and rp2. Sequence editing was done using the program FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTn) sequence similarity search tool (Altschul et al., 1997).

7.7. Antagonism of actinobacteria strains against pathogenic fungi

This assay was carried out in mixed cultures of the selected strains of actinomycetes and four pathogenic fungi *Pythium ultimum*, *Fusarium* sp., *Botritis cinerea* and *Phytophthora parasitica*. For that, Petri dishes with nutritive agar medium were used according to method reported by (Cafaro and Currie, 2005). In this case, the results of the radial growth of the pathogenic fungi in mixed cultures were compared with the fungal control cultures after 15 days of incubation at 28ºC. The level of inhibition was determined by the differences between the diameters of the radial fungal growth of a control culture ($\gamma 0$) and the radial fungal growth of paired-cultures (γ) in the direction of actinobacteria as indicated by this equation; $\Delta\gamma = \gamma 0 - \gamma$ (Yuan and Crawford, 1995). The ratings of inhibition levels were established from those of Prapagdee et al. (2008) as follows: $\Delta\gamma \geq 20$ mm = + + +; $\Delta\gamma \geq 10$ - 19 mm = + +; $\Delta\gamma \geq 5$ - 9 mm= +; $\Delta\gamma < 5$ mm (No antifungal activity) = -.

7.8. Encapsulation

Actinobacteria were immobilised on pellets composed by sodium alginate and starch from potato, by a technique denominated Inverse Gelation (Figure 10) (Madene et al., 2006), based in the precipitation of alginate in calcium chloride, reaching a cells concentration of 1.2 10^8 CFU g^{-1}. For that, it was designed a formulation that allowed the pellets to remain into soil in perfect conditions until they were easily decomposed, avoiding the desiccation and loss of microbial material.

Figure 9. Encapsulation by Inverse Gelation technique, using alginate, potato starch and the actinobacteria strains.

- Reagents:
  - 3% alginate
  - 2% starch from potato
  - 15 g l^{-1} $CaCl_2$
  - Saline solution at 9%
- Procedure:

  All the material used for the bioencapsulation process was previously sterilised during 30 m at 121ºC and the process was carried out under aseptic conditions. The matrix solution was prepared by mixing alginate and starch with the strains growth in YEP broth and the matriceswere then stirred for 30 min for homogenisation. Righ after, the matrix mixture containing cells was transferred to a syringe (10 ml) and dropped into sterile calcium chloride. Gelling of alginate-starch beads was completed after 30 min in contact with the calcium solution. The collected beads were placed

in 50 ml pasltic container with saline solution (0.9%) at 4ºC until their use. To develop in vitro assays, which objective is to characterise the microorganisms, the biological material must be washed with saline solution and any rest of culture medium must be eliminated. In our case, the growth medium was used to encapsulate the actinobacteria with the purpose of providing an energy source that favour the bacterial survival on the pellets, after the inoculation in plants subjected to grow in arird soils. It is recommended not to prepare the pellets if their use is not expected in a short-time period (Figure 10).

Figure 10. Alginate biocapsules containing *Streptomyces* sp.

8. Media composition

8.1. Yeast Extract Peptone (YEP) broth medium

Yeast Extract Peptone (YEP) is a nutritive medium that facilitate the growth and culture of bacterial and fungal microorganisms. The pH must be adjusted to 7. Its composition is (per litre):

- 10 g yeast extract
- 10 g peptone
- 5 g NaCl

8.2. Pikovskaya solid medium

This medium is quite relevant to evaluate the capacity of microorganisms to solubilise phosphate after the addition of a pigment which colour are modified due to a variation of pH as consequence of the phosphate solubilisation. To evaluate this ability on microorganism is advisable to use different phospahtes. The pH must be adjusted to 7. It composition per litre is as follows:

- 10 g glucose
- 5g $Ca_3(PO_4)_2$ that can be substituted for or $FePO_4$ or $AlPO_4$
- 0.5 g $(NH_4)_2SO_4$
- 0.2 g NaCl
- 0.1 g $MgSO_4 \bullet 7H_2O$
- 0.2 g KCl
- 0.5 g yeast extract
- 0.002 g $MnSO_4 \bullet 7H_2O$
- 0.002 g $FeSO_4 \bullet 7H_2O$
- 15 g agar

8.3. Nitrogen free medium (NFB)

This medium is particularly designed to test the ability of microorganisms to fix atmospheric nitrogen. It is observed when they are capable to growth in a medium without nitrogen, an elemental nutrient, as consequence of the reduction of N_2 to NH_4^+. The composition per litre to get a solid medium is:

- 5 g DL-malic acid
- 4 g KOH
- 0.5 g K_2HPO_4
- 0.2 g $MgSO_4 \bullet 7H_2O$
- 0.02 g $CaCl_2$
- 0.1 g NaCl
- 10 mg $FeSO_4 \bullet 7H2O$

- 2 mg $NaMoO_4 \cdot 2H_2O$
- 10 mg $MnSO4 \cdot H_2O$
- 0.5% alcoholic solution (or dissolved in 0.2 N KOH) bromothymol blue 2 ml
- 15 g agar

To get a semisolid medium the amount of agar can be reduced to 5 g and to obtain a broth agar must be eliminated of the composition.

Chapter IV

Combined effects of clay immobilised *Azospirillum brasilense* anda *Pantoea dispersa* and olive mill residue on plant performance and soil properties in the revegetation of a semiarid area

1. Introduction

Establishment of native plant species is a widely used practice for reclaiming degraded lands and constitutes the most effective strategy in semiarid Mediterranean areas (Alguacil et al., 2003) and other arid ecosystems (de-Bashan et al., 2012). Shrub species such as *Cistus albidus* L. associated with other small woody plants are indigenous of the plant communities in these semiarid ecosystems. In semiarid Mediterranean areas, the establishment of shrubs is made difficult by low soil fertility and the severe climate, characterised by low precipitation and frequent drought periods. Therefore, it is necessary to apply methods that improve soil quality and the ability of the seedlings to resist semiarid environmental conditions (Caravaca et al., 2005a).

The term rhizosphere was coined by Hiltner (1904) and describes the volume of soil affected by plant roots (Hartmann et al., 2008). Soil microbial populations undergo a proliferation, process known as rhizosphere effect, induced by the roots, due to the excretion of organic compounds in several forms (Benizri, 2002). Among all the components of soil microbiota, rhizobacteria are of great interest. They are free-living bacteria often labelled as plant growth-promoting rhizobacteria (PGPR), which can colonise the surface or intercellular spaces of the host plant roots, frequently improving root system establishment. In this regard PGPR has a potential role in establishment of plant species in arid environmental conditions (Puente et al., 2004), where these mechanisms lead to plant growth promotion of diverse nature such as non-symbiotic nitrogen fixation, phosphate solubilisation and production of various phytohormones improving root growth, water absorption and nutrients uptake (Bashan et al., 2004). Nevertheless, colonisation around plants roots by direct inoculation of free PGPR cells into soil is not easy because this process is very susceptible to environmental variations such as UV radiation, temperature fluctuation, protozoa depredation and salt stress (Wu et al., 2012). This unpredictability of the PGPR inoculation success on plants is mainly due to the

quality in the inoculants formulations containing an effective bacterial strain and determines the success or failure of a plant growth promotion.

Immobilisation of microbial inoculants has been used to improve their effectiveness by supplying nutrients, protection from desiccation and slow cells release (Bashan, 1998; Kim et al., 2012). The success of using microbial inoculants introduced into soil requires the survival of adequate numbers of bacteria reaching suitable habitats where they can stay alive (Heijnen and Van Veen, 1991). The principle of immobilisation of rhizobacteria is to protect the microorganisms (Schoebitz et al., 2012; 2013) and to ensure a gradual and prolonged release into the soil (Bashan et al., 2002; Wu et al., 2011). The use of clay in inoculant formulation can increase cells survival in the soil, due to the formation of a protective clay envelope around rhizobacteria which modifies the rates of water flow into and out of the cells during drying and rewetting (Cassidy et al., 1996).

Several studies about the application of organic amendments, for example, alperujo (organic olive residue) (Kohler et al., 2008), urban refuse (Alguacil et al., 2009a), sugar beet residue (Caravaca et al., 2005a) and leftover material from wastewater treatment (Trejo et al., 2012) have reported a beneficial effect on semiarid and arid soils, increasing the proliferation and development of natural populations of soil microorganisms and improving soil properties. In addition, it is known that immobilised rhizobia and rhizobacteria play a very important role in growth promotion of plants in crop systems (Vassileva et al., 1999; Albareda et al., 2008; Wu et al., 2012). Recent studies have also reported that the inoculation with rhizobacteria and organic residue addition can improve the revegetation with cacti and leguminous trees in the Sonoran Desert (Bashan et al., 2009, 2012b). However, to the best of our knowledge, no studies have been reported on the effects of the inoculation of clay immobilised rhizobacteria and organic olive residues evaluated to facilitate the revegetation in a degraded Mediterranean area. We hypothesise that the combined effect of clay immobilised rhizobacteria and organic addition can improve both the plant performance and soil properties in the revegetation of a degraded area. The objectives of this study were to assess the effect of these management

techniques on *C. albidus* growth and nutrient uptake and to evaluate the influence of the rhizosphere effect on the physicochemical and biological soil properties.

2. Material and methods

2.1. Study site

The experimental area was located in Vicente Blanes Ecological Park in Molina de Segura, Province of Murcia, Spain. The characterisation of the experimental zone is detailed in Chapter III "Matherial and Methods", section 1: Experimental areas, subsections 1.2 and 1.3.

2.2. Plants

The target plant species used in our experiment was *Cistus albidus* L. that is described in Chapter III "Material and Methods", section 2: Biological material used to develop the assays, subsection 2.1. The initial biomass characteristics of *C. albidus* plants used were 2.11 g (shoot dry weight); 1.23 g (root dry weight); 2.80 mm (basal stem diameter) and 31 cm (height of plants). The initial foliar concentrations were: total C, 438.08; N, 11.87; P, 0.78 and K, 7.23 (values are expressed in mg g plant^{-1}).

2.3. Microbial inoculants and organic residue

The microbial inoculant used for replanting was a mixture of two PGPR species *Azospirillum brasilense* and *Pantoea dispersa* immobilised on clay pellets. Bacteria and type of inoculum description are recorded in Chapter III "Material and Methods", section 2: Biological material used to develop the assays, subsection 2.2. The amendment used was composted "alperujo" and it characterisation is showed in Chapter III "Material and Methods", section 3: Organic residues.

2.4. Experimental design

A complete randomised factorial assay was established with two factors and five-fold replication in a split plot design. The first factor was the inoculation of *C. albidus* seedlings with microbial inoculant (*A. brasilense* and *P. dispersa*) and the second

was the addition of olive residue into the soil. The experimental design was performed as follows: treatment 1, *C. albidus* without rhizobacteria treatment and soil without organic residue addition (Control); treatment 2, *C. albidus* inoculated with microbial inoculant and soil without organic residue addition (MI); treatment 3, *C. albidus* without microbial inoculant treatment and soil with organic residue addition (OR) and treatment 4, *C. albidus* inoculated with microbial inoculant and soil with organic olive residue addition (MI + OR) (Figure 1) .

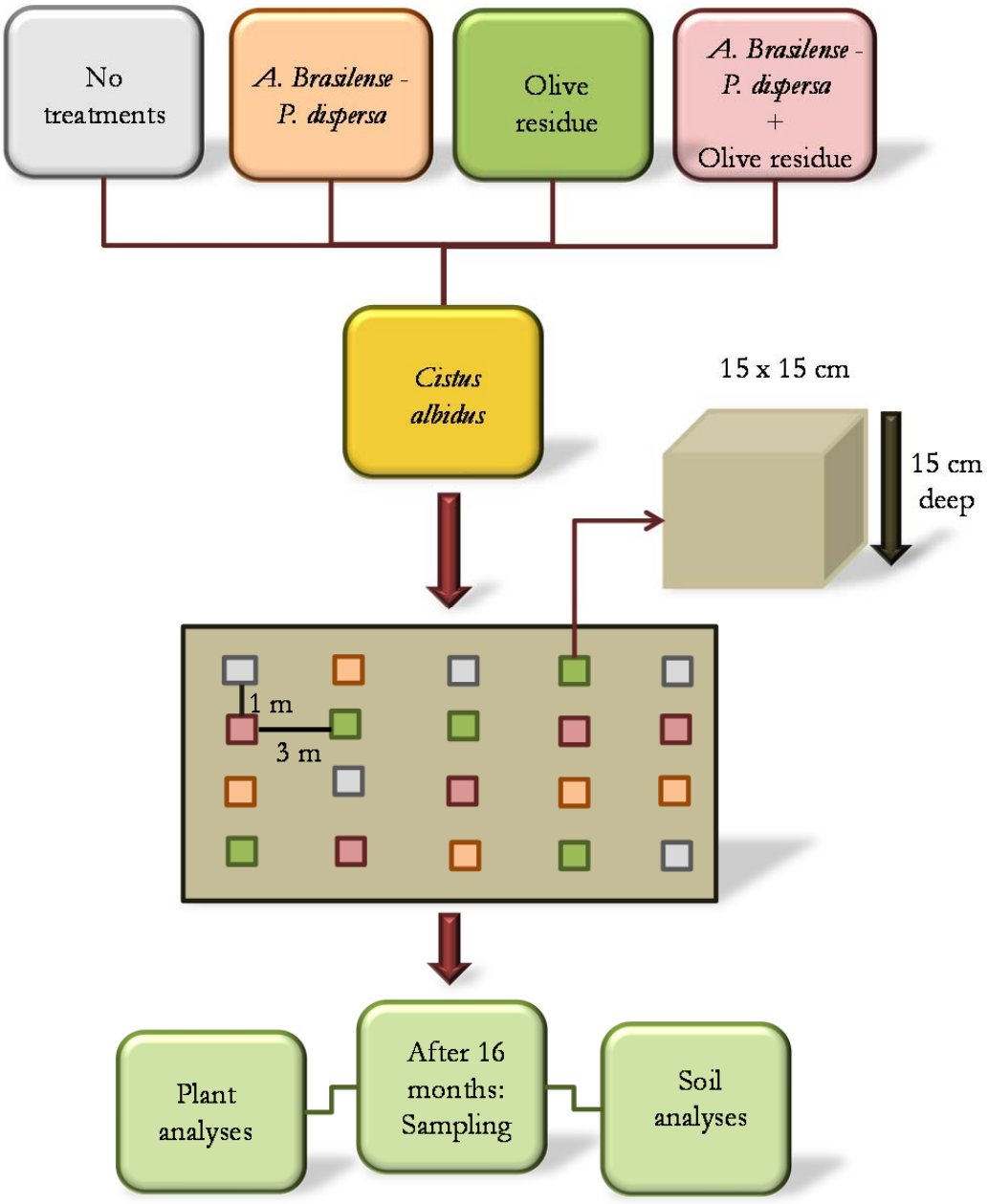


Figure 1. Diagram showing experimental design.

Planting holes 15 x 15 cm wide and 15 cm deep were dug manually. In early February 2011, microbial inoculant was applied at a rate of 30 g per plant. The same amount of sterilised inoculant was applied to the non-inoculated plants. Organic olive residue was added to the holes (0±15 cm depth) corresponding with a rate of 2% by weight (186 g of organic olive residue per plant). Microbial inoculant and organic olive residue were manually mixed into 2 kg of soil in plastic bags an introduced in the plantation holes. The seedlings were planted at least 1 m apart between holes, with 3 m between treatment levels in five 40 m² replicated plots. (Figure1, Figure 2).

Figure 2. Plantation of *Cistus albidus* in Vicente Blanes Ecological Park.

2.5. Sampling procedures

Sixteen months after planting, soil samples of each treatment and replicate were collected. Twenty rhizosphere soil samples were collected at 0–15 cm depth from planting holes. The same number of bulk soil samples was simultaneously taken from outside the canopy of the seedlings (at a distance of 20 cm from the planting holes). The sampling was carried out in early June 2012. At the same time, five plants per treatment were harvested.

2.6. Plant analyses

To evaluate the response to rhizobacteria inoculation and organic residues application the following growth parameters were evaluated: dry weights of shoots

and roots, basal stem diameter and plant height were recorded before chemical analyses. The foliar concentrations of phosphorus (P) and potassium (K) were determined by ICP-OES spectrometry while shoot total carbon (C) and nitrogen (N) were determined by dry combustion. Proline accumulation was determined by the method described by Paquin and Lechasseur (1979) and was estimated according to Bates et al. (1973). Plant analyses are detailed in Chapter III "Material and Methods", section 5: Analytical determinations on plants.

2.7. Soil physico-chemical, chemical and biological analyses

Soil pH and electrical conductivity were measured in a 1:10 (w/v) aqueous solution. Total N, total carbon C and total organic C (TOC) were determined by dry combustion using a LECO Tru-Spec CN analyser (Leco Corp., St. Joseph, MI, USA). Available P and extractable K were determined by ICP-OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL). Water-soluble carbohydrates and total carbohydrates were determined by the method of Brink et al. (1960). Soil respiration was calculated as the amount of CO_2 emitted during a 24 h incubation period. Soil microbial biomass C was evaluated by the Substrate Induced Respiration (SIR) method and the transformation of the amount of CO_2 emitted to microbial biomass C was done with the equation developed by Anderson and Domsch (1978). Soil respiration and soil microbial biomass C were determined with an automatic analyser (μ-TRAC 4200, SY-LAB). Glomalin related soil protein (GRSP) was determined in the easily extractable glomalin form according to Wright and Anderson (2000).

Dehydrogenase activity was determined according to García et al. (1997). Urease and N-a-benzoyl-L-arginine amide (BAA) hydrolysing protease activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al. 1980). Alkaline phosphatase activity was determined based on the production of *p*-nitrophenol (PNP) using *p*-nitrophenyl phosphate disodium (PNPP) as substrate according to Tabatabai and Bremmer 1969. β-glucosidase was determined using *p*-nitrophenyl-β -D-glucopyranoside (PNG) as substrate to obtain *p*-nitrophenol

(PNP) which amount was established by spectrophotometry (Tabatabai and Bremmer 1969).

Soil analysis methodologies are detailed in Chapter III "Material and Methods", section 6: Analytical determination of soil physico-chemical, chemical and biological properties.

2.8. Statistical analyses

The effects on plants and soil of organic residue and microbial inoculation and their interactions were analysed by a two-way ANOVA and post-hoc mean separation was performed by Duncan's multiple range test at $P \leq 0.05$.

3. Results

3.1. Effects of the inoculation with microbial inoculant and organic residue on growth of *C. albidus*

The ANOVA revealed that none of the experimental factors tested significantly affected the basal stem diameter and plant height (Table 1).

Table 1. Two-way ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic amendment (OA) and their interaction on the measured variables are shown. NS: Not significance.

Factors and interaction	Shoot	Root	Basal stem diameter	Height
MI	<0.001	0.001	NS	NS
OR	NS	0.021	NS	NS
MI x OR	0.001	NS	NS	NS

The inoculation with PGPR strains promoted an increase in shoots dry weight higher than 100% and the MI + OR treatment increased the roots dry weight and the root/shoot ratio compared with control plants (133% and 51% respectively) (Figure 3).

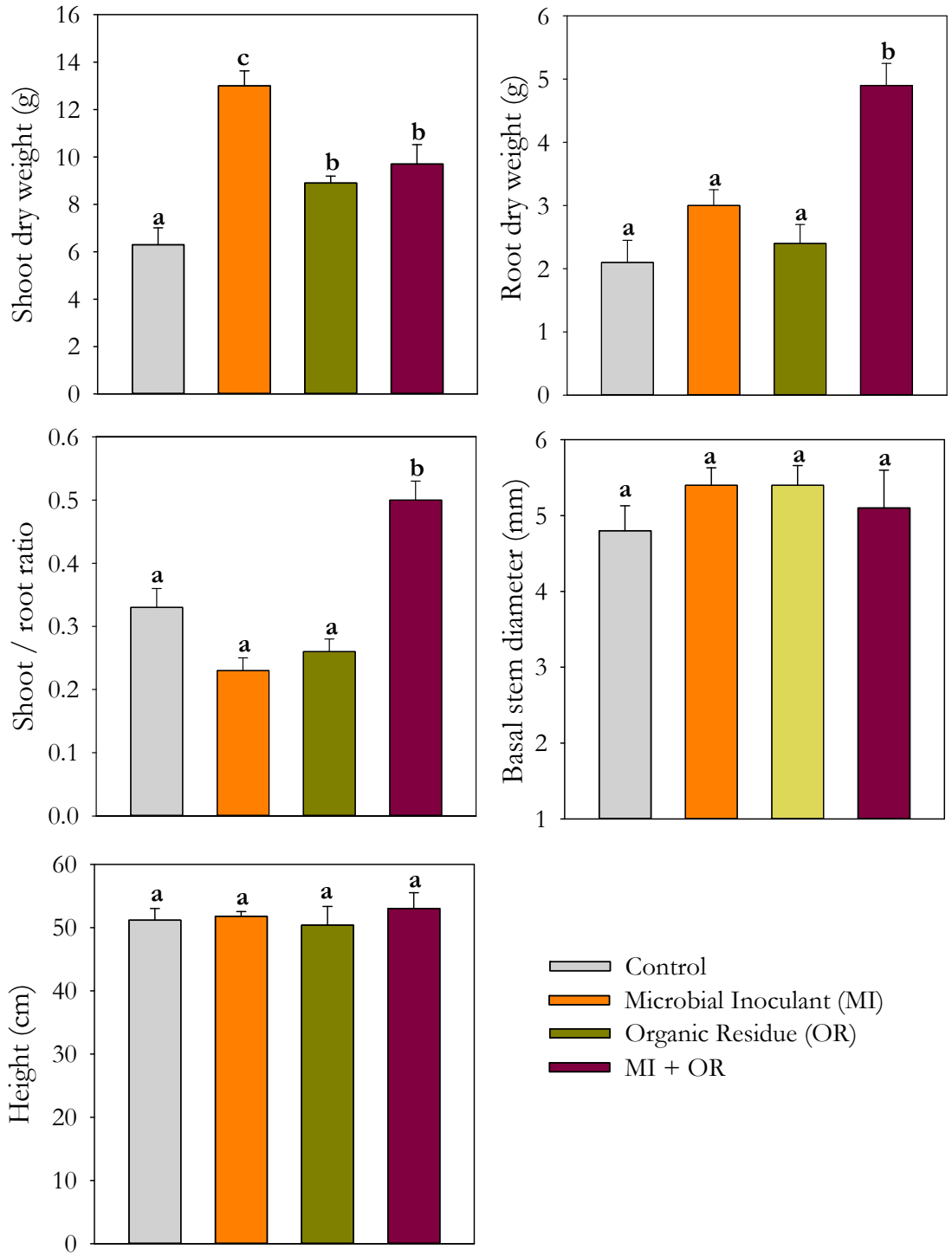


Figure 3. *C. albidus* growth parameters. Values are mean of five replicates. Significant differences according to the Duncan's test at $P < 0.05$ levels were indicated by different letters.

3.2. Nutrients uptake

Experimental treatments did not significantly affect the total C and N shoot content. However, the total P concentration in the plants was significantly improved by the addition of the organic amendment (around 100%) and microbial inoculation (68%) (Figure 4).

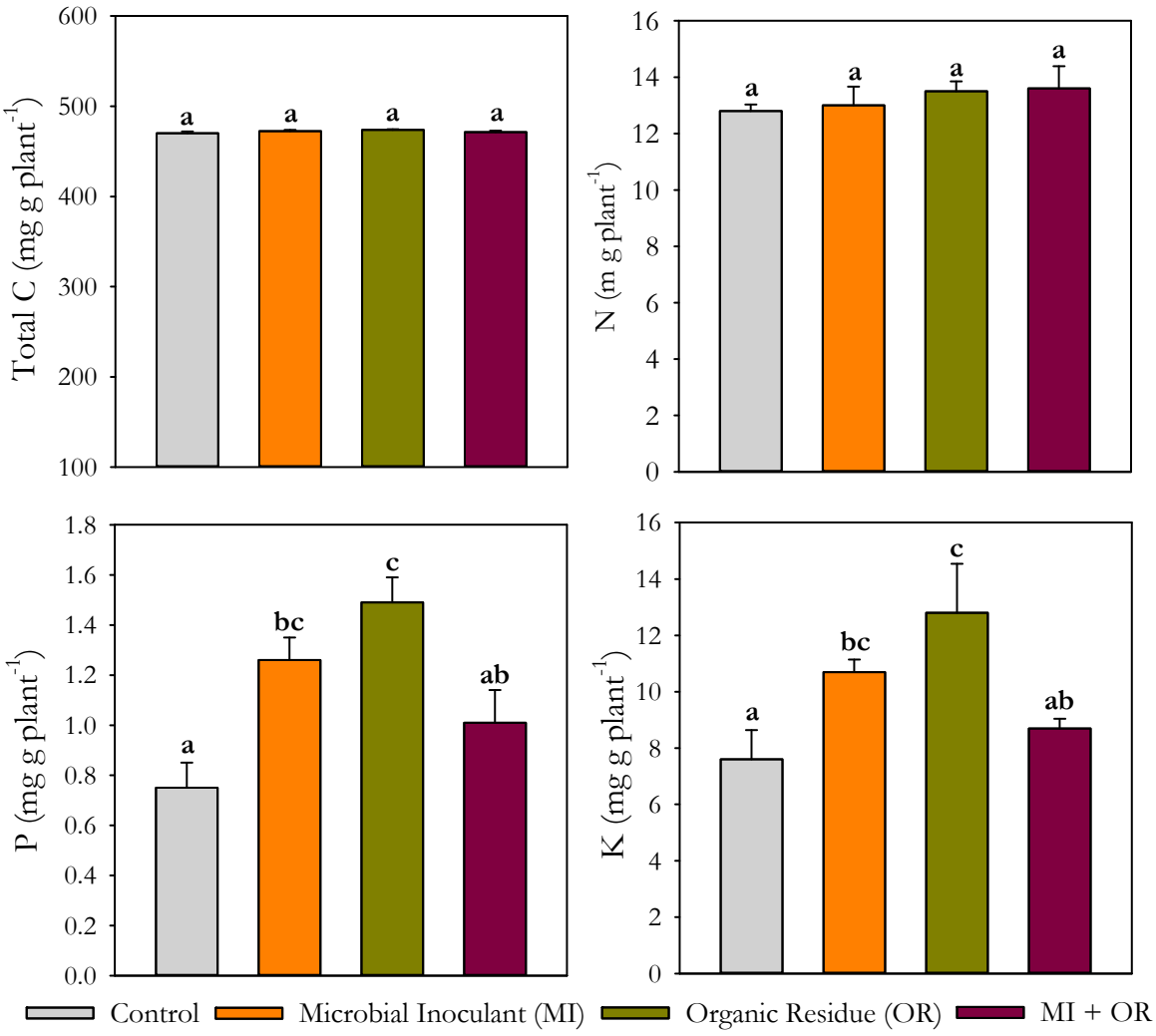


Figure 4. Nutrient contents in shoot of *C. albidus* seedlings. Values are mean of five replicates. Significant differences according to the Duncan's test at $P < 0.05$ levels were indicated by different letters.

The combination of these two factors increased P content only by 34 %. The total K concentration in shoots was increased significantly by the microbial inoculant (40%) and the addition of the organic residue also showed a significant

increase (68% greater than control plants) (Figure 4). ANOVA showed that for K and P plant uptake, a negative interaction between the two factors considered was recorded (Table 2).

Table 2. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic residue amendment (OR) and their interaction on the measured variables are shown. NS: Not significance.

Factors and interaction	Total C	N	P	K
MI	NS	NS	NS	NS
OR	NS	NS	0.033	NS
MI x OR	NS	NS	<0.001	0.003

3.3. Plant stress parameter

The comparative effects of microbial inoculant, organic residue and MI + OR treatment regarding accumulation of proline, demonstrated that this osmoregulatory compound was accumulated to a greater extent in control plants, microbial inoculant and organic residue treatment (Figure 5).

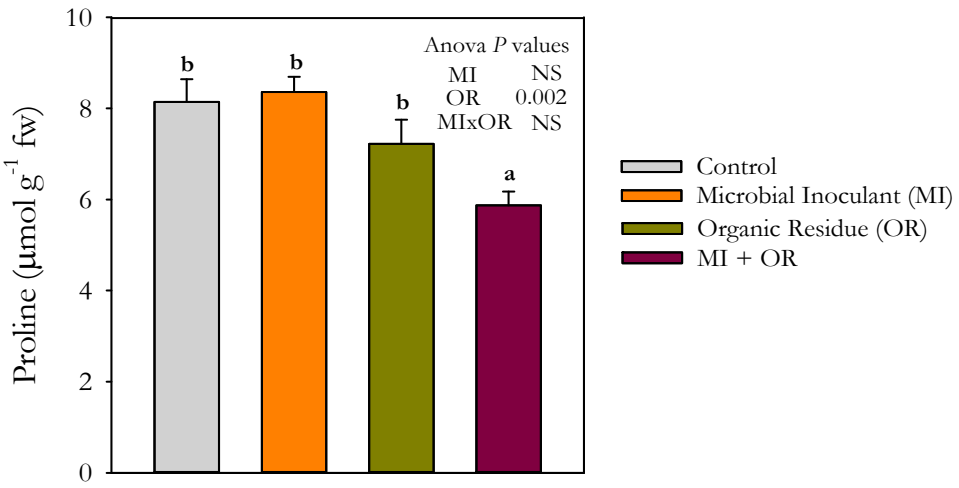


Figure 5. Proline contents in leaves of *C. albidus*. Values are mean of five replicates. Significant differences are showed according to the Duncan's test at $P < 0.05$ levels. Significance of the effects of the factors and their interaction on the measured variable is also shown. NS: not significance.

3.4. Soil physico-chemical, chemical and biological analyses

Microbial inoculant and organic residue treatments significantly increased pH values in the rhizosphere soil. The microbial inoculant and MI + OR treatments also significantly increased the electrical conductivity in the rhizosphere soil, but only te combined treatment caused an increment in bulk soil of *C. albidus* (Table 3).

Table 3. Changes observed on rhizosphere and bulk soil physico-chemical and chemical properties after microbial inoculation and organic residue addition.

Treatments	Soil type	pH (H_2O)	EC ($\mu S\ cm^{-1}$)	Total N ($g\ kg^{-1}$)	P available ($mg\ kg^{-1}$)	K extractable ($mg\ kg^{-1}$)
Control	Rs	8.4 ± 0.0 ab	309 ± 6 a	2.1 ± 0.1 cd	13 ± 2 bc	310 ± 15 a
	Bs	8.3 ± 0.0 a	405 ± 17 c	2.2 ±0.1 cd	10 ± 1 ab	292 ± 17 a
MI	Rs	8.5 ± 0.0 c	346 ± 14 b	1.1 ± 0.1 a	18 ± 2 cde	618 ± 29 c
	Bs	8.4 ± 0.01ab	330 ± 8 ab	1.7 ± 0.1 b	8 ± 1 a	294 ± 14 a
OR	Rs	8.5 ± 0.0 c	342 ± 19 ab	2.3 ± 0.1 d	19 ± 1 de	662 ± 15 c
	Bs	8.4 ± 0.0 ab	358 ± 10 b	2.1 ± 0.0 cd	12 ± 2 b	469 ± 13 b
MI + OR	Rs	8.4 ± 0.0 ab	435 ± 16 c	2.4 ± 0.1 d	20 ± 2 e	840 ± 34 d
	Bs	8.4 ± 0.0 ab	560 ± 11 d	1.9 ± 0.1 c	14 ± 2 bcd	466 ± 18 b
ANOVA *P* values						
MI		NS	<0.001	<0.001	NS	<0.001
OR		NS	<0.001	<0.001	<0.001	<0.001
SP		0.001	<0.001	NS	<0.001	<0.001
MI x OR		0.008	<0.001	<0.001	NS	<0.001
MI x SP		NS	NS	NS	NS	<0.001
OR x SP		NS	NS	<0.001	NS	NS
MI x OR x SP		NS	<0.001	0.001	0.041	0.001

Rs: Rhizosphere soil; Bs: Bulk soil; EC: Electrical conductivity. Values are means of five replicates. Mean ± standard error. For each species, values in columns followed by the same letter do not differ significantly ($P < 0.05$) as determined by Duncan's test. Significance of effects of microbial inoculant (MI), organic residue amendment (OR) soil position (SP) and their interactions on the measured variables are also shown. NS: Not significance.

Total N significantly decreased in both rhizosphere and bulk soil soil after microbial inoculation respect to the control (Table 3). Regarding the factorial analysis the organic residue addition, soil sampling position and MI x OR x SP interactions were significant on available P (Table 3). Thus, the addition of the amendment and MI + OR treatment significantly increased the available P in rhizosphere soil. The available P content levels in rhizosphere soil mediated by the microbial inoculant, organic amendment and their combination were about 38, 46 and 54 % higher than in controls. Extractable K content in rhizosphere soil showed a statistically significant difference (<0.05) twofold increase for the microbial inoculant and organic residue and approximately threefold increase for the MI + OR treatment. In bulk, soil this variable significantly increased with the organic amendment and MI + OR treatments (Table 3). In fact, the factorial analysis showed that the microbial inoculation, the addition of the amendment, soil sampling position and their interactions (except OR x SP) were significantly affected the extractable K content (Table 3).

The MI + OR treatment significantly increased the total C in the rhizosphere soil and the total organic C in both rhizosphere and bulk soil. However, after microbial inoculation, a significant decrease was found for total C in rhizosphere soil and for total organic C in both rhizosphere and bulk soil (Table 4). The ANOVA elucidated that among all the interactions analysed, microbial inoculant, organic amendment, soil sampling position and their interaction were significant for total carbohydrates contents (except MI x SP and MI x OR x SP interactions) (Table 4) but none of them significantly affected water-soluble carbohydrates. In deed, not significant differences between microbial inoculant, organic residue and MI + OR treatment were found for water-soluble carbohydrates (Table 4). The organic amendment and the MI + OR combined treatment significantly increased the total carbohydrates concentration of the rhizosphere soil (Table 4) while after microbial inoculation, a significant decrease on total carbohydrates was observed in bulk soil.

Table 4. Values observed on total C and carbon fractions after microbial inoculation and organic residue addition.

Treatments	Soil type	Total C (g kg^{-1})	TOC (g kg^{-1})	Total CH (mg kg^{-1})	W-S CH (mg kg^{-1})
Control	Rs	101 ± 1.2 b	14.4 ± 0.65 cd	2025 ± 66 b	11 ± 1 a
	Bs	98 ± 0.7 ab	13.4 ± 0.37 bc	2149 ± 69 bc	11 ± 1 a
MI	Rs	94 ± 0.8 a	12.1 ± 0.63 b	2057 ± 158 b	13 ± 1 a
	Bs	95 ± 1.1 a	10.1 ± 0.44 a	1531 ± 61 a	14 ± 3 a
OR	Rs	102 ± 0.7 b	13.9 ± 0.44 c	2357 ± 129 c	10 ± 1 a
	Bs	97 ± 1.3 ab	13.3 ± 0.60 bc	2206 ± 76 bc	14 ± 3 a
MI + OR	Rs	108 ± 2.5 c	19.3 ± 0.48 e	2763 ± 75 d	12 ± 1 a
	Bs	99 ± 1.2 b	15.9 ± 0.69 d	2376 ±74 c	16 ± 3 a
ANOVA *P* values					
MI		NS	NS	<0.001	NS
OR		<0.001	<0.001	0.002	NS
SP		<0.001	<0.001	<0.001	NS
MI x OR		<0.001	<0.001	<0.001	NS
MI x SP		NS	0.029	NS	NS
OR x SP		0.003	NS	0.002	NS
MI x OR x SP		NS	NS	NS	NS

Rs: Rhizosphere soil; Bs: Bulk soil; TOC: Total organic C; Total CH: Total carbohydrates; W-S CH: water soluble carbohydrates. Mean ± standard error. Values are mean of five replicates. For each species, values in columns followed by the same letter do not differ significantly (P< 0.05) as determined by Duncan's test. ANOVA for microbial inoculant (MI), organic residue amendment (OR) soil position (SP) and their interactions, given as F values (significance level); NS: Not significance.

The application of MI + OR treatment significantly increased enzyme activities in rhizosphere soil (urease, protease and dehydrogenase), but the microbial inoculant and organic residue independently failed to do that (Table 5). The greatest increase on enzyme activities was observed in rhizosphere soil in comparison to bulk soil; in this sense the soil factor was significant for enzyme activities (except β-glucosidase; Table 5).

Table 5. Changes observed on enzymatic activities after microbial inoculation and organic residue addition.

Treatments	Soil type	Urease (μmol NH_3 g^{-1} h^{-1})	β-glucosidase (μmol PNF g^{-1} h^{-1})	Phosphatase (μmol PNF g^{-1} h^{-1})	Protease (μmol NH_3 g^{-1} h^{-1})	Dehydrogenase (μg g^{-1} INTF)
Control	Rs	0.8 ± 0.1 bc	0.37 ± 0.02 abc	2.1 ± 0.1 bc	1.9 ± 0.1 bc	102 ± 4 bc
	Bs	0.5 ± 0.1 ab	0.38 ± 0.03 abc	2.0 ± 0.1 abc	1.3 ± 0.1 a	101 ± 7 bc
MI	Rs	0.6 ± 0.1 ab	0.27 ± 0.03 a	1.7 ± 0.2 ab	1.7 ± 0.1 ab	103 ± 7 bc
	Bs	0.3 ± 0.1 a	0.29 ± 0.03 ab	1.6 ± 0.1 a	1.2 ± 0.2 a	80 ± 4 a
OR	Rs	1.0 ± 0.1 c	0.39 ± 0.02 abc	2.3 ± 0.2 c	2.2 ± 0.1 cd	110 ± 6 c
	Bs	0.5 ± 0.1 ab	0.40 ± 0.04 bc	1.9 ± 0.1 abc	1.6 ± 0.2 ab	86 ± 8 ab
MI + OR	Rs	1.4 ± 0.2 d	0.44 ± 0.07 c	2.4 ± 0.1 c	2.5 ± 0.1 d	148 ± 8 d
	Bs	0.6 ± 0.1 ab	0.36 ± 0.04 abc	1.7 ± 0.2 ab	1.6 ± 0.2 ab	106 ± 8 c
ANOVA *P* values						
MI		NS	NS	0.043	NS	NS
OR		<0.001	0.017	0.050	<0.001	0.004
SP		<0.001	NS	0.001	<0.001	<0.001
MI x OR		0.007	NS	NS	NS	<0.001
MI x SP		NS	NS	NS	NS	NS
OR x SP		0.029	NS	0.020	NS	NS
MI x OR x SP		NS	NS	NS	NS	NS

Rs: Rhizosphere soil; Bs: Bulk soil; Values are means of five replicates. Mean ± standard error. For each species, values in columns followed by the same letter do not differ significantly ($P < 0.05$) as determined by Duncan's test. ANOVA for microbial inoculant (MI), organic residue amendment (OR) soil type (ST) and their interactions, given as F values (significance level); NS: Not significance.

All factors and their interactions (except MI x OR x SP) significantly affected soil respiration, while microbial biomass C was not affected by MI x SP and glomalin related soil protein was not affected by OR x SP (Table 6).

Table 6. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic residue amendment (OR) soil position (SP) and their interactions on the measured variables, NS: Not significance.

Factors and interactions	Soil respiration	Microbial biomass C	GRPS
MI	<0.001	0.010	0.002
OR	<0.001	<0.001	<0.001
SP	<0.001	<0.001	<0.001
MI x OR	<0.001	<0.001	<0.001
MI x SP	<0.001	NS	0.001
OR x SP	0.015	<0.001	NS
MI x OR x SP	NS	<0.001	<0.001

Respect to soil respiration rates, a significant decrease mediated by the microbial inoculant treatment was found when compared to control in rhizosphere soil, whereas in bulk soil the soil respiration rates significantly decreased with the microbial inoculant and MI + OR treatments (Figure 6). Microbial biomass C and glomalin-related protein were significantly increased in rhizosphere soil in comparison with bulk soil (Figure 6). The MI + OR treatment significantly increased the microbial biomass of the rhizosphere and bulk soil. However, the microbial inoculant decreased significantly the microbial biomass in rhizosphere and bulk soil respect to control samples. The glomalin-related protein was increased significantly by the organic residue, about 80 % and MI + OR treatment showed also a significant increase (100% greater than the control rhizosphere soil) (Figure 6).

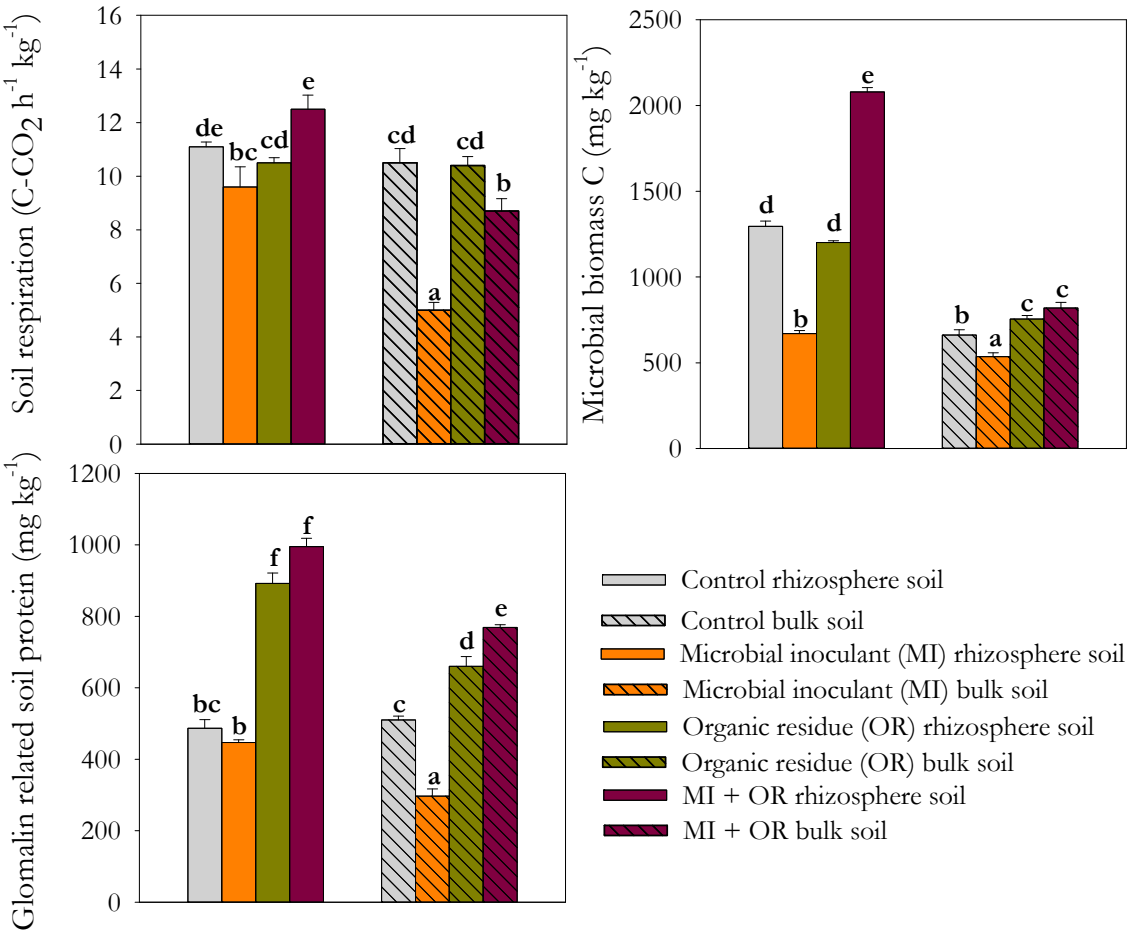


Figure 6. Soil biological properties in rhizosphere and bulk soil after microbial inoculation and organic residue addition. Values are mean of five replicates. For each species, bars entitled by the same letter do not differ significantly (P< 0.05) as determined by Duncan's test.

4. Discussion

Information about the beneficial effects of rhizobacteria inoculation on the growth of plants is well documented under greenhouse and laboratory conditions (Vessey, 2003; Bashan et al., 2004; Schoebitz et al., 2009). Nevertheless, reports of rhizobacteria inoculation and/or organic residues addition evaluated in field experiments are scarce, even more in non-agricultural systems (Bashan et al., 1999, 2009, 2012b; Carrillo-García et al., 2000). Sixteen months was considered enough period of time (one drought period and two growth periods) to ascertain the

combined effects of immobilised rhizobacteria and organic amendment on plant performance and soil properties in the revegetation of a degraded area. Plants showed different levels of response to the microbial inoculant and the addition of the organic residue. Thus, it was observed an increase in shoot biomass when microbial inoculant was applied. It is well documented that PGPR exert a beneficial effect on plant growth and development, and many different rhizobacteria have been commercialised for using in agriculture (Bashan et al., 2004; Adesemoye et al., 2009; Adesemoye and Kloepper, 2009). Assuming that root/shoot ratio could reflect the degree of microorganisms effectiveness (Tobar et al., 1999), *C. albidus* did not respond to inoculation and organic amendment applied independently. MI + OR was the treatment with the greatest response, reaching the most significant increases in root/shoot ratio. Decreased root/shoot ratio in the inoculated treatment with respect to control plants indicates low microorganisms activity in relation to root biomass production. And in this sense we can partially conclude that the combined treatment was the most effective for improving seedling performance. However, root biomass and nutrient uptake, as affected by microbial inoculant, must all be considered together

Immobilised microbial inoculants have solved many problems associated with traditional liquid inoculants finding numerous advantages such as increasing cell survival and controlling the release of rhizobacteria into the soil, as well as protecting the microorganisms against drought stress, especially in semiarid regions. Our results are in agreement with those found by other authors, who reported that the use of immobilised *A. brasilense* and *P. dispersa* had a pronounced beneficial effect on pepper biomass and N uptake in greenhouse conditions (Flores et al., 2010). Nevertheless, in our revegetation experiment with *C. albidus* seedlings, the microbial inoculants, organic residue and MI + OR did not increase the total N, but appeared effective for improving the uptake of other nutrients such as P and K, which might explain why the microbial inoculant yielded the highest shoot biomass of *C. albidus*. Higher P and K uptake may be attributable to the mobilisation of nutrients from soil because of the secretion of organic acids mediated by rhizobacteria (Basak and

Biswas, 2010) and also to the addition of the organic olive residues due to the P and K amounts incorporated with the amendment, in comparison with previous field experiment in a degraded semiarid Mediterranean area adding composted urban residue to soil, Caravaca et al. (2003b) found an increase of plant nutrient content (NPK) 18 months after the organic residue addition. Rhizobacteria are rhizosphere competent bacteria that colonise plant roots; they are able to colonise all the ecological niches found on the rhizosphere (Antoun and Kloepper, 2001) and consequently can explore a wider range for nutrients mobilisation. In this sense, total nutrient content can be taken as a representative parameter of rhizobacteria immobilised effectiveness. In addition it has been reported that rhizobacteria could help plants to compensate for deficiencies of immobile nutrients such as P and K, by the increase of root biomass due to the production of phytohormones which can mediate the extent of a greater root surface area for nutrients uptake (Bashan et al., 2004). It can be concluded that the activity of the inoculated bacteria and the fertilisers added with the olive residue can improve the nutrient uptake by the seedlings with the exception of the N.

Most plant species can accumulate proline, which plays a major role in the process of osmotic adjustment decreasing the cell osmotic potential, thus allowing higher water retention during drought (Medina et al., 2010). Our results suggest the role of the proline in the protection of plants against drought stress. Here, control plants and those grown with microbial inoculant and organic residue independently having high proline contents were also the most affected by drought. We observed that the MI + OR treatment presented lower proline content suggesting that this combined treatment was more effective to induce resistance to drought conditions in this semiarid area.

The rhizosphere effect is an area of intensive interactions between plant roots and soil. The rhizosphere effect is mainly based on the microbiota activity influenced by root growth (Berg and Smalla, 2009). Physico-chemical and biological properties of the soil samples were measured and we observed that the rhizosphere effects did not influence the bulk soil in the majority of the evaluated soil properties.

Nevertheless, we found that the combined treatment increased the electrical conductivity and organic C in bulk soil. Besides, the application of the organic residue, independently and combined, also increased extractable K, glomalin and biomass C.

In rhizosphere soil, supplemented with organic residue and microbial inoculant combined treatment, it was produced a significant increase in the amounts of total C, total organic C, total carbohydrates and glomalin, which can be used as carbon and energy sources for soil microflora. Wright and Anderson (2000) have indicated that arbuscular mycorrhizal fungi produce glomalin, a glycoprotein able to increase the hydrophobicity of soil particles and to promote soil aggregation. Total N levels in the rhizosphere were not increased by the treatments tested, even a significant decrease was observed in the microbial inoculant treatment and this may be explained through the assimilation of N by the soil microorganisms. In the treatments involving organic amendment it is assumed that the addition of the amendment mediates an input on N levels, even so the total N levels in soil were not modified. MI + OR treatment increased microbial biomass and in particular enhanced the protease and urease activity, which is involved in the N cycle and could reveal a shift in microbial populations mediated by an increase in N assimilation by the soil microorganisms.

Soil microorganisms are able to excrete organic acids and phosphatases, which increase the concentration of P in rhizosphere soil (Rodríguez et al., 2006; Vassilev et al., 2006). The application of the organic residue with microorganisms has been used for increasing soil available P and improvement of plant growth and nutrients uptake. Caravaca et al. (2005a) demonstrated the effectiveness of these biosystems for increasing the growth and nutrient uptake of *C. albidus* using an amendment with sugar beet, rock phosphate and *Aspergillus niger*. In our experiment, available P in the rhizosphere was increased by the use of both organic residue and MI+OR treatments but not by the inoculant, assuming that the rhizobacteria inoculated was able to solubilise P from the organic residue applied and not from the soil.

All the treatments tested increased K contents in rhizosphere soil with regard to the control. Microbial inoculant and organic olive residue helped plants to compensate for deficiencies of immobile nutrients such as potassium, which may be attributed to the mobilisation of K by the soil rhizobacteria and also by the input of K provided with the addition of organic residue. The inoculation of soil with immobilised rhizobacteria can be considered an effective tool for the development of biotechnological products than can be used as a partial substitute for chemical fertilisation. In that way, introduction of a microbial inoculant can improve nutrients availability for plants and thereby to increase the efficiency of the applied manures (Adesemoye and Kloepper, 2009).

In this assay, the effect of the microbial inoculant combined with organic residue on soil properties might also be attributed to a greater promotion of biological activity in the rhizosphere soil of the *C. albidus* plants. In fact, microbial biomass C, glomalin, dehydrogenase, protease and urease activities were higher in the rhizosphere soil of MI + OR treatment in comparison to control plants and these parameters have frequently been used as indicators of soil microbial activity (Caravaca et al., 2002d). Alguacil et al. (2003) also reported an increase in enzyme activities when measured in the rhizosphere soil of *C. albidus* after amendment with fermented sugar beet residue in a semiarid Mediterranean area. Enzyme activities are sufficiently sensitive to indicate changes caused by microbial inoculation (Naseby and Lynch, 1997). Furthermore, *A. brasilense* and *P. dispersa* in combination with organic amendment may release enzymes involved in the mineralisation of organic matter. Thus, a positive correlation in MI + OR treatment has been reported between enzyme activity and microbial biomass C. However, we observed a significant decrease on microbial biomass C and soil respiration when the microbial inoculant treatment was applied independently, suggesting than the introduced microbiota presented a lower biological activity in comparison to the autochthonous soil microorganisms. In general, the combined treatment was the most effective for increasing microbial activities in the soil of our experiment.

5. Conclusions

The microbial inoculation of the seedlings with *A. brasilense* and *P. dispersa* immobilised in clay increased the performance of *C. albidus*, even more when the microbial inoculation was combined with the addition of organic olive residue. When considering the improvement of soil quality, the combination of both treatments was the most effective, yielding a significant increase in C fractions, microbial biomass and enzyme activities, but microbial inoculation and organic residue applied independently failed to restore soil properties. Therefore, the application of combined treatments involving immobilised PGPR inoculation and organic amendments appear to be the most suitable tool to aid in the restoration of both plant cover and soil quality in semiarid degraded lands.

Chapter V

Advantages of inoculation with immobilised rhizobacteria versus amendment with olive mill residue in the afforestation of a semiarid area with *Pinus halepensis* Mill.

1. Introduction

In semiarid Mediterranean areas, the establishment of plant cover is difficult under the severe climate, characterised by low precipitation and frequent drought periods, particularly in soils with low microbial activity. Therefore, it is necessary to use technological restoration methods that can improve both soil quality and the ability of the seedlings to resist semiarid environmental conditions (Caravaca et al., 2005a). The establishment of native plant species is a practice widely used for reclaiming degraded lands and constitutes the most-effective strategy in semiarid areas (Alguacil et al., 2003; Mengual et al., 2014a; Schoebitz et al., 2014). *Pinus halepensis* Mill. is the prevailing tree species in semiarid areas of central-southern Spain, and it has been used in afforestation programmes for degraded soils as it is a pioneer species and one of the few tree species that can thrive in these conditions (Maestre and Cortina, 2004).

Recent studies about the reclamation of semiarid soils have shown the beneficial effects of the application of organic amendments on soil quality, with increases in the proliferation and development of natural populations of soil microflora, since the organic residues can be used by soil microorganisms, as substrates and as carbon and energy sources (Medina and Azcón, 2010), and also improve soil properties. This effect could be extended to the enhancement of soil enzyme activities, which are key factors contributing to soil-borne microorganism activity and soil fertility (Caravaca et al., 2005a). The use of organic waste materials not only increases the organic matter and fertility of soils, but also contributes to the palliation of environmental and economic inconveniences related with waste disposal (Rincón et al., 2006).

The Spanish olive-mill industry produces a huge amount of wastes that are difficult to reuse (four million tons per year). The main by-product is alperujo, which can be composted before its application to the soil in order to obtain a high-quality amendment, rich in K and partially-humified organic matter (Alburquerque et al., 2009, 2006). Such characteristics suggest that this residue could be useful for

improving soil quality and in the development of afforestation programs in semiarid and degraded areas. The beneficial short-term effects of the addition of alperujo compost in horticultural and revegetation practices have been reported (Alburquerque et al., 2006; Schoebitz et al., 2014). However, their effect on the establishment of tree seedlings under semiarid field conditions remains unknown. Additionally, the application of olive-mill waste interacts positively with soil microorganisms (Schoebitz et al., 2014). Among the components of the soil microbiota, rhizobacteria are free-living bacteria, often labelled as plant growth-promoting rhizobacteria (PGPR), which can colonise the rhizosphere and improve root system establishment (Antoun and Kloepper, 2001). In this regard, PGPR have a potential role in the establishment of plant cover in arid environmental conditions (Puente et al., 2004), where they can promote plant growth and improve both water and nutrients uptake (Bashan et al., 2004). Nevertheless, colonisation around plant roots following the direct inoculation of free PGPR cells into soil is not easy because this process is highly susceptible to environmental variations (Wu et al., 2012). This unpredictability of the success of PGPR inoculation of plants is due mainly to the quality of the inoculant formulations containing effective rhizobacterial strains, which determines the success or failure of plant growth promotion. Immobilisation of microbial inoculants has been used to enhance their effectiveness, by providing nutrients and protection from desiccation (Kim et al., 2012). The success of microbial inoculants introduced into soil requires that an adequate number of bacteria reach suitable habitats where they can survive (Heijnen and Van Veen, 1991). The aim of the immobilisation of rhizobacteria is to protect the microorganisms (Schoebitz et al., 2013) and ensure a gradual and prolonged release into the soil (Wu et al., 2011). In spite of their potential viability, the use of immobilised bacteria has never been tested in the reafforestation with tree species like *P. halepensis* in Mediterranean semiarid conditions. The aim of this work was to study the medium-term effect of olive-mill waste compost and a microbial inoculum constituted by two immobilised strains of rhizobacteria on *P. halepensis* establishment under semiarid field conditions. We hypothesised that the revegetation treatments

assayed would confer drought tolerance on the plants and/or enhance soil quality, leading to enhanced plant growth. In this respect, we measured soil physico-chemical, biochemical and microbiological variations as well as the changes in shoot nitrate reductase activity, proline accumulation, oxidative damage to lipids and plant water relations induced by these treatments.

2. Material and methods

2.1. Study site

The study area was located in Vicente Blanes Ecological Park in Molina de Segura, (southeast Spain) (Lat. 38º 12' N, Long. 1º 13' W, Elev. 392 m). The characterisation of the experimental zone is detailed in Chapter III "Matherial and Methods", section 1: Experimental areas, subsections 1.2 and 1.3.

2.2. Plants

The selected plant to carry out this study was *Pinus halepensis* Mill., detailed in Chapter III "Matherial and Methods", section 2: Biological material used to develop the assays, subsection 2.1. Seedlings were grown in Muzalé nursery (Murcia, Spain) with peat as substrate for 1 year prior to experimental procedures. At planting, *P. halepensis* was 20 cm high, with a shoot dry weight of 2.77 g and root dry weight of 1.37 g.

2.3. Microbial inoculants and organic residue

The microbial inoculant used for replanting was a mixture of two PGPR species *Azospirillum brasilense* and *Pantoea dispersa* immobilised on clay pellets. Bacteria and type of inoculum description are recorded in Chapter III "Matherial and Methods", section 2: Biological material used to develop the assays, subsection 2.2. The amendment used was composted "alperujo" and it characterisation is showed in Chapter III "Matherial and Methods", section 3: Organic residues.

2.4. Experimental design

A full-factorial assay was established with two factors and fivefold replication in a split plot design. The first factor was the inoculation or not of *P. halepensis* seedlings with microbial inoculant and the second was the addition or not of organic olive residue into the soil. In early February 2011, the seedlings were transported to the experimental field, where planting holes 15 x 15 cm wide and 15 cm deep were dug manually (Figure 1).

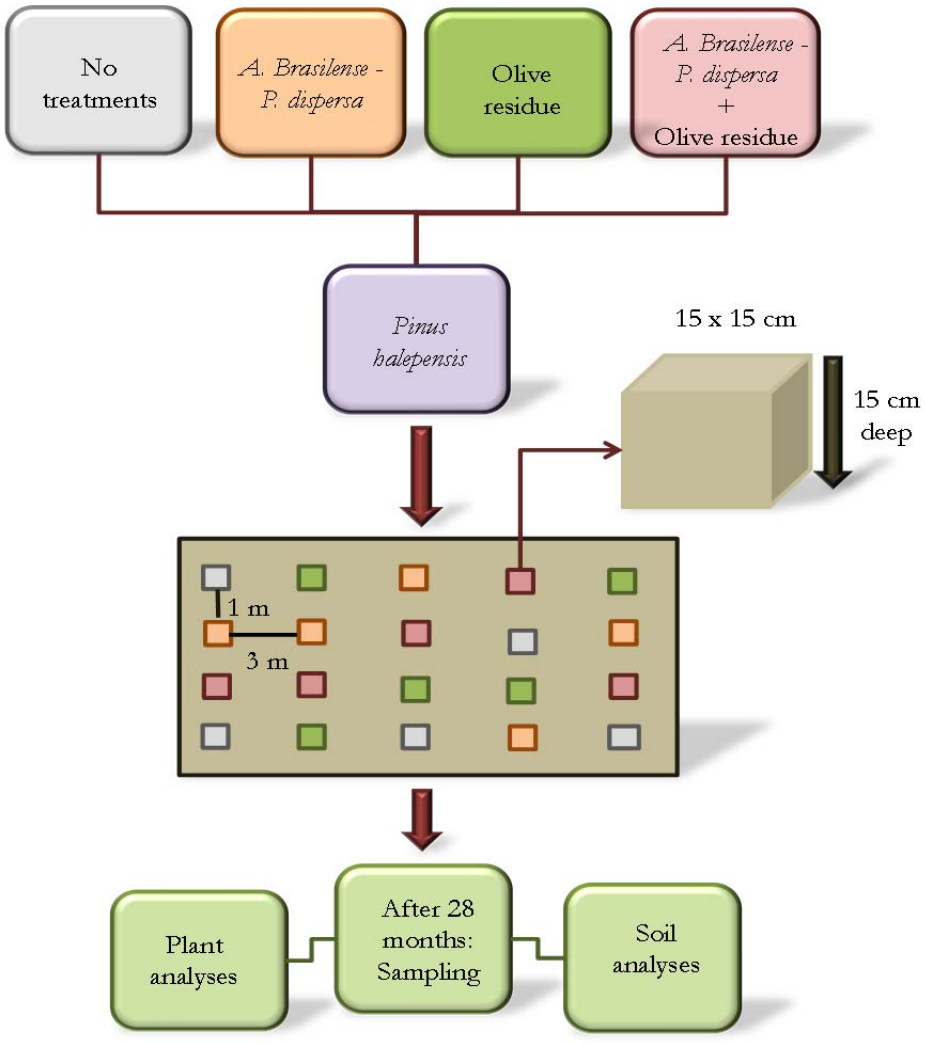


Figure 1. Diagram showing the experimental design.

There, an amount of 30 g of microbial inoculant pellets was applied per plant. The same quantity of sterilised inoculant was applied to the non-inoculated plants. Olive residue was added at a rate of 2% by weight (186 g of organic olive residue

per plant). Microbial inoculant and organic olive residue were manually mixed into 2 kg of soil in plastic bags an introduced in the plantation holes. The seedlings were planted at least 1 m apart between holes, with 3 m between treatment levels (Figure 1, Figure 2).

Figure 2. *Pinus halepensis* plantation (Vicente Blanes Ecological Park).

2.5. Sampling procedures

Twenty-eight months after planting, in early June 2013, samples were collected. Five plants per treatment including root systems and rhizosphere soil were harvested, and introduced in polyethylene bags for transport to the laboratory. Rhizosphere soil samples were separated into two subsamples before physicochemical and biochemical analyses: one subsample sieved to < 2 mm and other subsample sieved between 4 and 0.25 mm.

2.6. Plant analyses

The sampling day, before the harvest, leaf water potential was measured in two fully developed needles per tree of each replicate in a pressure chamber (Mellisho et al., 2012). Midday (12 h solar time) stem water potential was measured in a similar number and type of needles as used for leaf water potential (Cruz et al., 2012). Leaf osmotic potentials were determined in the same needles used for leaf water potentials, using a vapour pressure osmometer. Leaf turgor potential was derived as the difference between osmotic and water potentials (Cruz et al, 2012). Dry weights

of shoots and roots, basal stem diameter and plant height were recorded before chemical analyses. Shoot P and K were determined ICP/OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL) while shoot N was determined by dry combustion using a LECO Tru-Spec CN analyser (Leco Corp., St. Joseph, MI, USA). Nitrate reductase activity was assayed in vivo by measuring NO_2^- production in tissue that had been vacuum-infiltrated with buffered NO_3^- solutions (Downs et al., 1993) and proline accumulation was evaluated using the method developed by Paquin and Lechasseur (1979). Lipid peroxidation rates were determined by measuring the content of malondialdehyde (MDA) according to Minotti and Aust (1987). Plant analyses are detailed in Chapter III "Matherial and Methods", section 5: Analytical determinations on plants.

2.7. Soil physical, chemical, and biochemical analyses

Soil pH and electrical conductivity were measured in a 1:10 (w/v) aqueous solution. Total organic carbon (TOC) and total nitrogen (N) were determined by dry combustion while available P and extractable K were determined by ICP/OES spectrometry. Total carbohydrates were determined by the method of Brink et al. (1960).

Soil microbial biomass C was evaluated by the Substrate Induced Respiration (SIR) method and the transformation of the amount of CO_2 emitted to microbial biomass C was done with the equation developed by Anderson and Domsch (1978). Soil respiration and soil microbial biomass C were determined with an automatic analyser (μ-TRAC 4200, SY-LAB).

Dehydrogenase activity was determined according to García et al. (1997). Urease and N-a-benzoyl-L-arginine amide (BAA) hydrolysing protease activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al. 1980). Alkaline phosphatase activity was determined based on the production of *p*-nitrophenol (PNP) using *p*-nitrophenyl phosphate disodium (PNPP) as substrate according to Tabatabai and Bremmer 1969. β-glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG) as substrate to obtain *p*-nitrophenol

(PNP) which amount was established by spectrophotometry (Tabatabai and Bremmer 1969).

Soil analysis methodologies are detailed in Chapter III "Material and Methods", section 6: Analytical determination of soil physico-chemical, chemical and biological properties.

2.8. Statistical analyses

The effects of amendment addition, microbial inoculation and their interaction on measured variables were analysed by a two-way ANOVA and post hoc mean separation was performed by Duncan's multiple range test, calculated at $P < 0.05$.

3. Results

3.1. Effects of the inoculation with microbial inoculant and olive mill residue on growth of *P. halepensis*

The experimental factors tested, the microbial inoculation (MI) and the addition of organic residue (OR), as well as the MI x OR interaction were significant for *P. halepensis* height, while only MI had a significant effect on shoot biomass (Table 1).

Table 1. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic residue amendment (OR) and their interactions on the measured variables are shown.

Factors and interaction	Shoot (dw)	Root (dw)	Height	BSD
MI	<0.001	NS	<0.001	NS
OR	NS	NS	<0.001	NS
MI x OR	NS	NS	0.038	NS

Dw: Dry weight; BSD: Basal stem diameter; NS: Not significance.

The microbial inoculant produced a significant increase in shoot dry weight by about 48% compared to the control plants and by 4 fold with respect to the initial seedlings grown in the nursery (Figure 3). However, the shoot biomass of the plants

after the addition of olive residue and the combined treatment did not undergo significant changes respect to the control plants. Plant height was increased significantly by all the treatments evaluated. The organic residue and the MI + OR treatment did not increase root dry weight or basal stem diameter, compared with control plants (Figure 3).

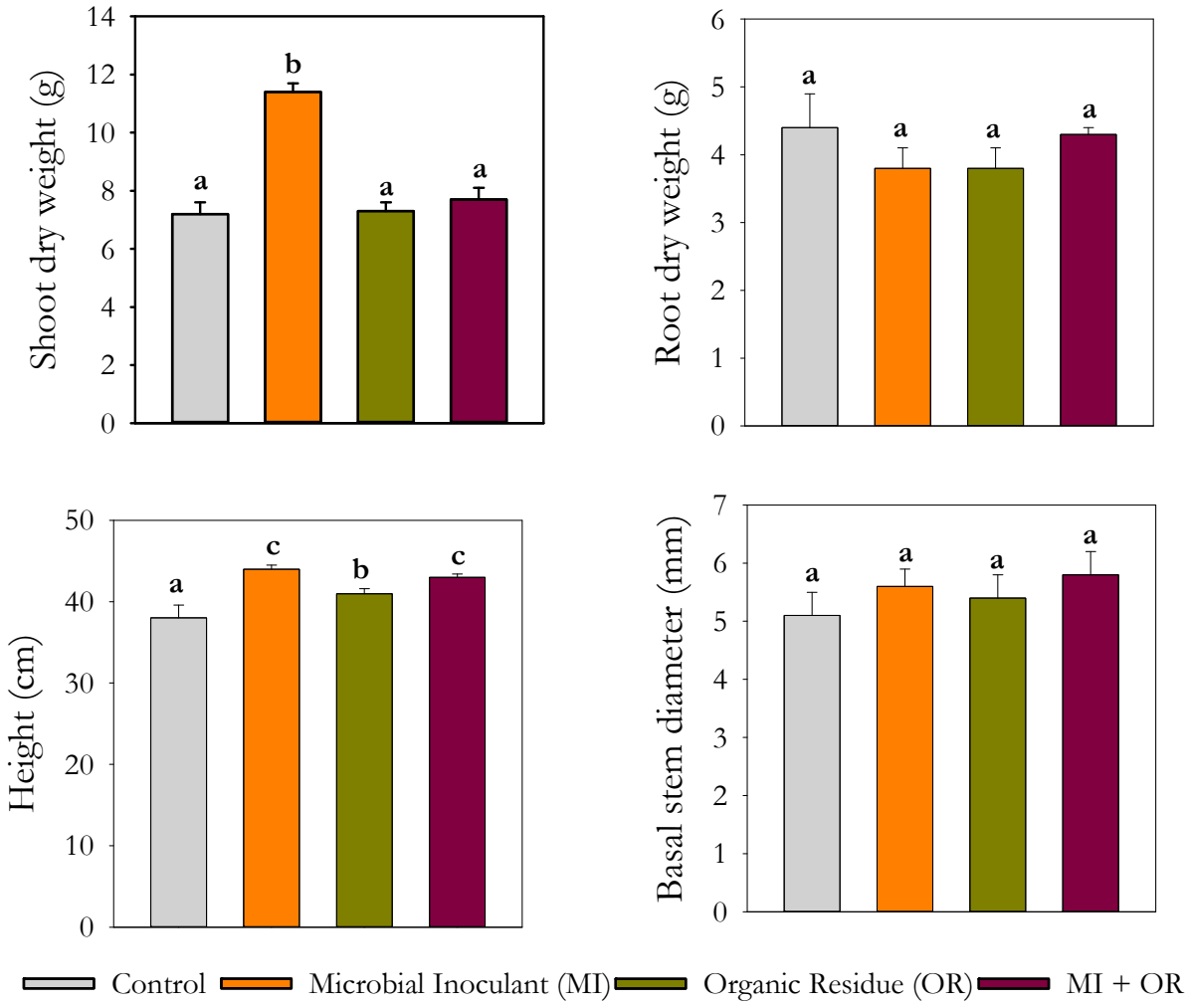


Figure 3. Growth parameters observed on *P. halepensis* seedlings in response to microbial inoculation and organic residue addition. Values are means of five replicates. For each species, bars entitled with the same letter do not differ significantly ($P < 0.05$).

3.2. Nutrients uptake

The ANOVA revealed that the microbial inoculation, organic amendment and MI x OR interaction affected significantly the N, P and K uptake (Table 2).

Table 2. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic residue amendment (OR) and their interaction on nutrients contents, NS: Not significance.

Factors and interaction	N	P	K
MI	<0.001	0.020	<0.001
OR	0.034	0.001	<0.001
MI x OR	<0.001	0.001	<0.001

The experimental treatments significantly increased the shoot tissue total N, particularly the microbial inoculant, which yielded an increase of 30% (Figure 4).

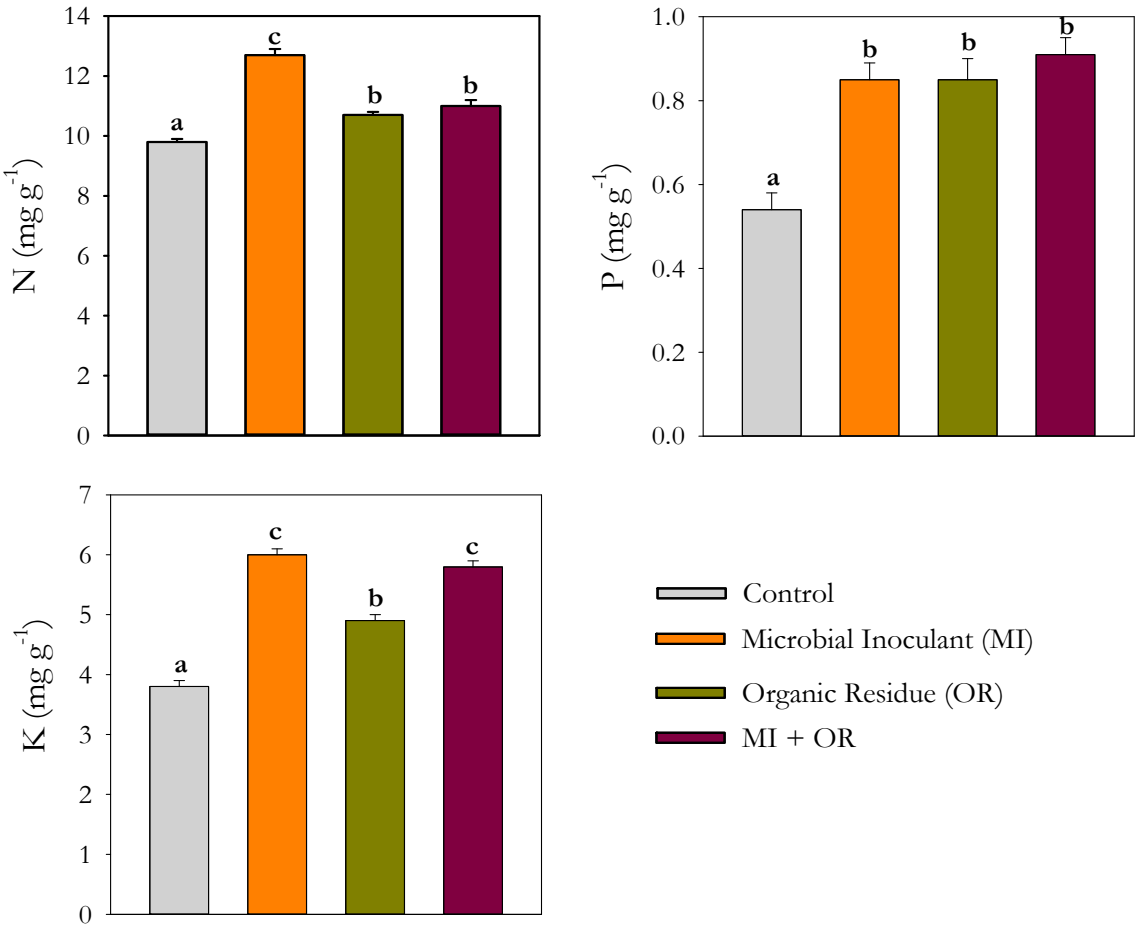


Figure 4. Nutrients content in shoot of *P. halepensis* in response to microbial inoculation and organic residue addition. Values are means of five replicates. For each species, bars entitled with the same letter do not differ significantly ($P < 0.05$).

The P concentration in the plants was improved significantly by the microbial inoculation (57%) and organic amendment (57%), as well as by their combination (69%). Regarding the total K concentration in the shoot, the highest and most-significant increases were recorded with the microbial inoculant (58% greater than control plants) and the combined treatmen (52%) while the addition of the organic residue also gave a significant increase (29% respect to the control plants) (Figure 4).

3.3. Plants stress parameters

The microbial inoculation, the organic amendment and the MI x OR interaction significantly affected the nitrate reductase activity, stem and leaf water potential and leaf turgor potential, while only MI affected the proline and oxidative damage values (Table 3).

Table 3. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic residue amendment (OR) and their interactions on plant stress parameters.

Factors and interaction	Nitrate reductase	Oxidative damage	Proline	Ψstem	Ψl	Ψp
MI	0.001	0.036	0.001	<0.001	<0.001	<0.001
OR	0.024	NS	NS	<0.001	<0.001	<0.001
MI x OR	0.001	NS	NS	<0.001	<0.001	<0.001

Ψstem: Stem water potential; Ψl: Leaf water potential; Ψp: Leaf turgor potential; NS: Not significance.

The post hoc test showed a significant decrease in proline values after the MI and the combined treatment, and a significant decrease in oxidative damage after MI. Nitrate reductase activity underwent a significant increase after the MI and the application of the combined treatment (33% and 67%, respectively), whereas a significant decrease was recorded with the addition of the organic amendment (Figure 5).

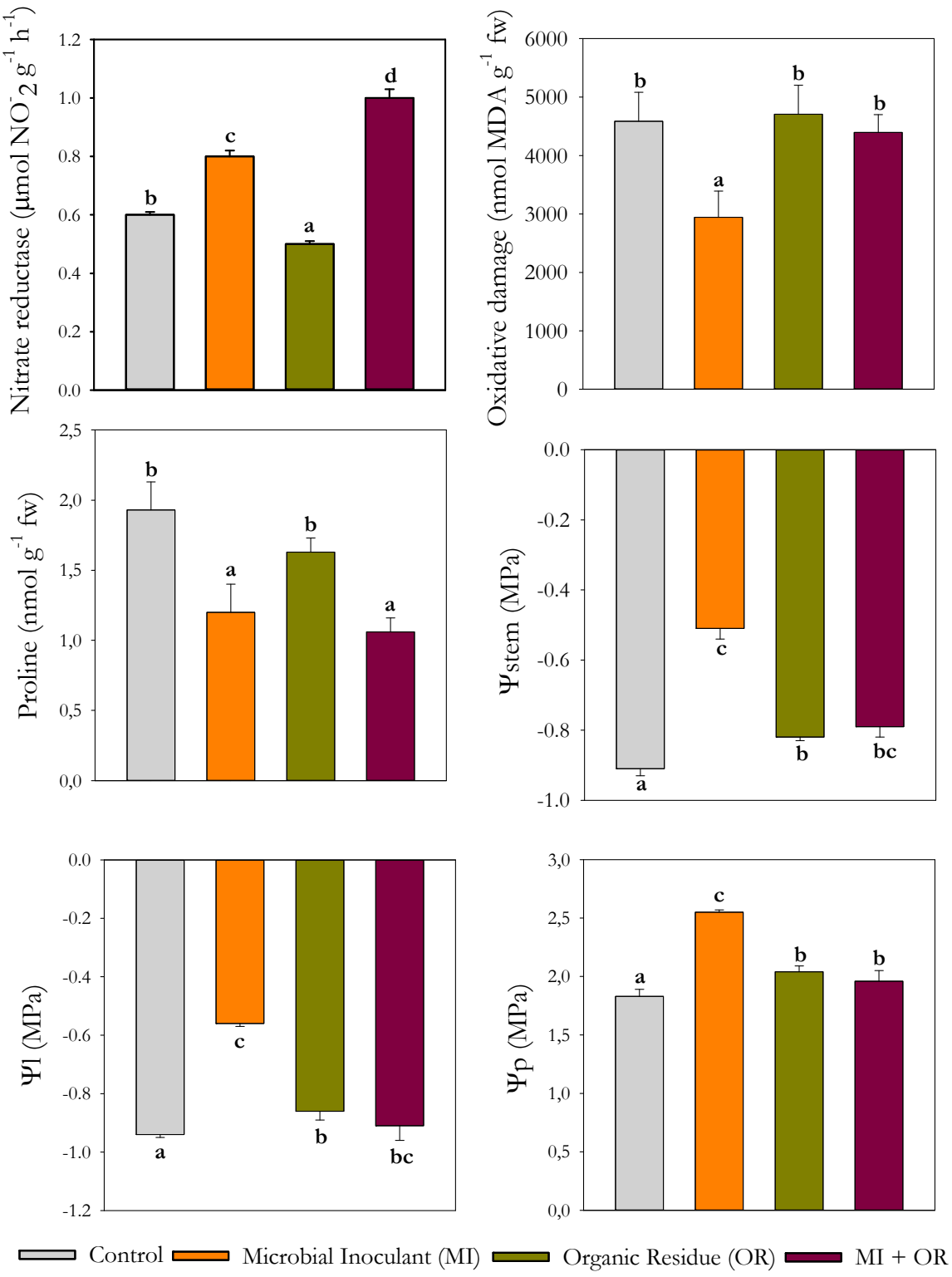


Figure 5. Plant stress parameters on *P. halepensis* shoot. Significant differences according to the Duncan's test at $P < 0.05$ are signed with different letters.

With regard to the stem water potential, leaf water potential and leaf turgor potential, all the treatments significantly improved the values relative to the control, the highest values occurring after the MI (increases of 56%, 60% and 23%, respectively) (Figure 5).

3.4. Soil physico-chemical, chemical and biological analyses

There were significant effects of the microbial inoculant, organic amendment and MI x OR interaction on the pH, electrical conductivity, available P and extractable K. Total N was significantly affected by MI and the MI x OR interaction, while a significant variation in total organic C was mediated by the organic residue and the MI x OR interaction. In many cases, the Duncan test confirmed a significant increase in the values for these treatments compared with the control (Table 4).

Table 4. Changes in pH, electrical conductivity and nutrients in rhizosphere soil of *P. halepensis* as response to immobilised rhizobacteria and organic residue addition.

Treatments	pH (H_2O)	EC ($\mu S\ cm^{-1}$)	TOC ($g\ kg^{-1}$)	Total N ($g\ kg^{-1}$)	P available ($mg\ kg^{-1}$)	K extractable ($mg\ kg^{-1}$)
Control	8.5 ± 0.0 a	149 ± 2 b	18.6 ± 0.1 a	1.4 ± 0.0 a	3 ± 0 a	237 ± 20 a
MI	8.7 ± 0.0 c	170 ± 1 d	18.8 ± 0.2 a	1.5 ± 0.1 b	7 ± 0 c	437 ± 29 b
OR	8.7 ± 0.0 c	144 ± 1 a	20.9 ± 0.3 c	1.4 ± 0.1a	15 ± 0 d	306 ± 16 a
MI + OR	8.6 ± 0.0 b	154 ± 2 c	20.3 ±0.5 b	1.7 ± 0.1 c	5 ± 0 b	730 ± 10 c
Anova *P* values						
MI	<0.001	<0.001	NS	<0.001	<0.001	<0.001
OR	0.018	<0.001	<0.001	NS	<0.001	<0.001
MI x OR	<0.001	0.002	0.041	0.033	<0.001	0.005

EC: Electrical conductivity; TOC: Total organic carbon. Values are means of five replicates. Mean ± standard error. For each species, values in columns followed by the same letter do not differ significantly ($P < 0.05$) as determined by Duncan's test. NS: Not significance.

Only for electrical conductivity a decrease was recorded after the amendment. The pH values increased with all the treatments. With regard to available P, the

greatest value was observed after the addition of the organic amendment (a 5-fold increase with respect to the control) (Table 4). The electrical conductivity, total N and extractable K underwent increases after the MI and the combined treatment, the highest values being obtained with the MI for electrical conductivity (14% increase) and with the combined treatment for total N (21%) and extractable K (3-fold higher with respect to the control) (Table 4). The total organic C values were increased by the olive residue and the combined treatment.

The ANOVA showed that the microbial inoculant, organic residue and MI x OR interaction significantly affected urease and protease enzyme activities. Microbial inoculation had a significant effect on β-glucosidase, while the addition of organic amendment and the MI x OR interaction affected dehydrogenase activity. Neither of the factors nor the MI x OR interaction had a significant effect on phosphatase (Table 5).

Table 5. Changes in enzymatic activities of rhizosphere soil of *P. halepensis* in response to immobilised rhizobacteria and organic residue addition.

Treatments	Dehydrogenase (μg g^{-1} INTF)	β-glucosidase (μmol PNF g^{-1} h^{-1})	Urease (μmol NH3 g^{-1} h-1)	Protease (μmol NH_3 g-1 h^{-1})	Phosphatase (μmol PNF g^{-1} h^{-1})
Control	100 ± 4 a	1.5 ± 0.1 a	1.1 ± 0.0 a	1.8 ± 0.1 a	5.2 ± 0.6 a
MI	94 ± 6 a	1.8 ± 0.1 b	1.3 ± 0.0 b	2.6 ± 0.0 d	5.6 ± 0.3 a
OR	114 ± 6 b	1.6 ± 0.1 a	1.3 ± 0.0 b	2.4 ± 0.0 c	5.3 ± 0.4 a
MI + OR	136 ± 4 c	1.9 ± 0.1 c	1.4 ± 0.0 c	2.1 ± 0.0 b	5.9 ± 0.2 a
Anova *P* values					
MI	NS	<0.001	<0.001	<0.001	NS
OR	<0.001	NS	<0.001	0.028	NS
MI x OR	0.017	NS	0.030	<0.001	NS

Mean ± standard error. Significant difference according to the Duncan test at $P < 0.05$ levels were indicated by different letters. Significance of effects of microbial inoculant, organic amendment and their interaction on the measured variables is also shown. NS: not significance.

Thus, the post hoc test recorded that phosphatase did not undergo any change with respect to the control (Table 5). The activity of β-glucosidase showed an increase with MI and MI + OR treatments; the highest value was achieved with the combined treatment (a 26% increase, with respect to the control). Urease activity increased with all the treatments, especially the combined treatment (by 27%, with respect to the control). Protease activity also was improved by all the treatments, the MI yielding an increase of 44%. Dehydrogenase activity was improved by the application of the olive residue and the combined treatment, obtaining its maximum value with the latter (a rise of 36% relative to the control) (Table 5).

The MI, the organic residue and the MI x OR interaction significantly affected the total carbohydrates, microbial biomass and soil respiration (Table 6).

Table 6. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic residue amendment (OR) and their interaction on the measured variables are shown.

Factors and interaction	Soil respiration	Microbial biomass C	Total carbohydrates
MI	<0.001	<0.001	0.015
OR	<0.001	0.049	0.002
MI x OR	0.0031	<0.001	<0.001

The Duncan test confirmed increases in the values of all these parameters for all the treatments (Figure 6). The highest increases in total carbohydrates were recorded after the MI and the addition of the organic amendment (26% and 29%, respectively, compared with the control). Microbial biomass reached its highest value (50% higher than the control) with the MI.

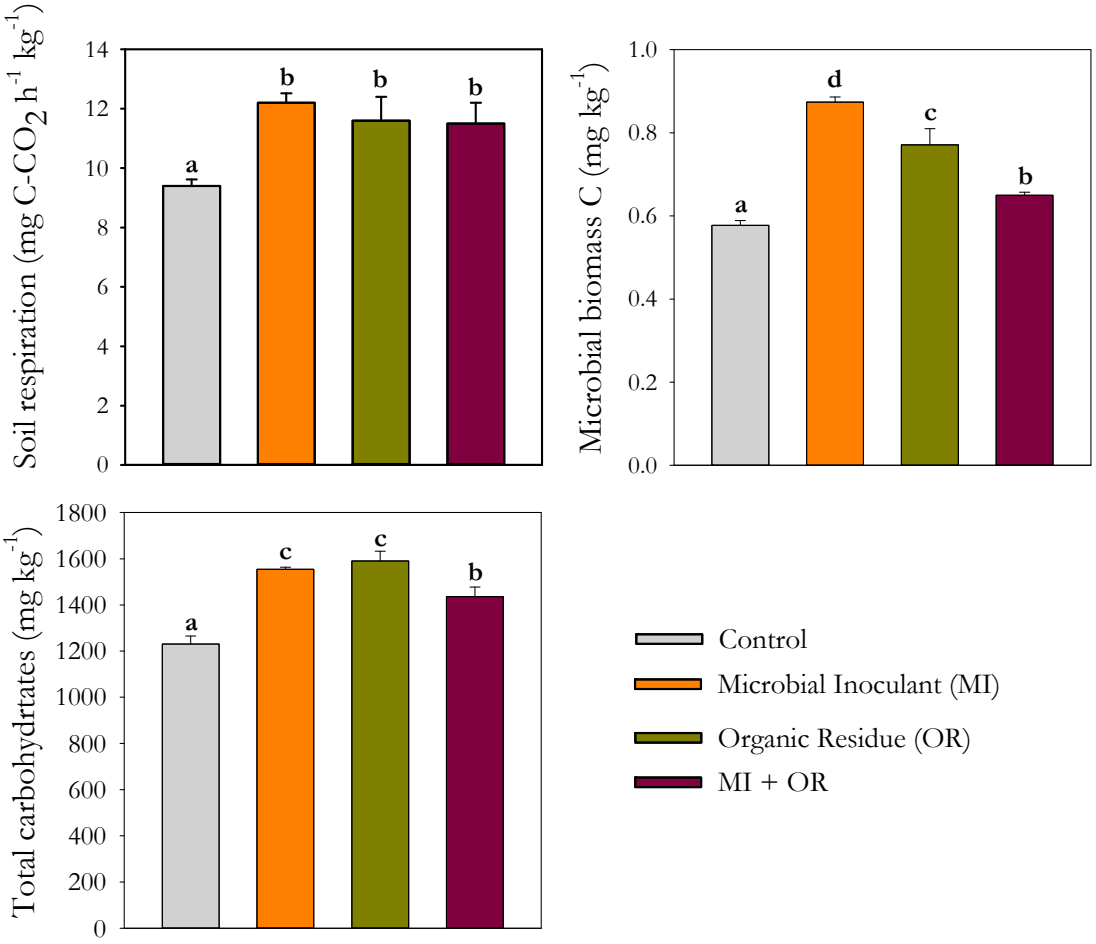


Figure 6. Carbon fractions observed on rhizosphere soil. Values are mean of five replicates. Significant differences are signed with different letters according to the Duncan's test ($P<0.05$).

4. Discussion

The results demonstrate that the inoculation with immobilised rhizobacteria was the most-effective treatment for promoting the establishment of *P. halepensis* under semiarid field conditions. Inoculation with rhizobacteria has recently been proved to be a useful strategy for the re-establishment of native shrub species in these degraded environments (Mengual et al., 2014a; Schoebitz et al., 2014). Previous afforestation practices with *P. halepensis* in semiarid environments included the use of organic amendment and ectomycorrhizal inoculation (Rincón et al., 2006; Roldán et al., 1996). In this study, we provide the first evidence of the beneficial effect of

immobilised PGPR as a microbial inoculant on the growth of this tree species in a degraded semiarid soil.

In our afforestation assay, the microbial inoculant, organic residue and combination of both treatments increased the total N, P and K concentrations in plants. This may be attributable to the nutrients incorporated with the organic residue and their solubilisation, mediated by the microbial inoculant (Tejada et al., 2009). The best results were observed after the microbial inoculation. It seems admissible that the inoculated rhizobacteria were rhizosphere-competent bacteria capable of colonising plant roots (Hozore and Alexander, 1991), which allowed to explore the available ecological niches in the rhizosphere (Kumar et al., 2011; Trivedi et al., 2012) of *P. halepensis*; consequently, they could explore a wider range for mobilisation of nutrients. The nitrate reductase values suggest that the N concentration also could have been increased by an improvement in this enzyme activity promoted by these bacterial strains.

It has been demonstrated that the use of rhizobacteria improves plant health and growth performance in degraded soils, as well as enhancing their tolerance of drought and salinity (de-Bashan et al., 2012). The accumulation of proline in plants plays a major role in the process of osmotic adjustment, helping to decrease the cell osmotic potential and thus allow higher water retention during drought periods (Medina et al., 2010). Another mechanism of protection against drought stress is the enzyme nitrate reductase, which catalyses the rate-limiting step in the nitrate assimilation pathway (Alguacil et al., 2006). A decrease in oxidative stress could also be considered a sign of improved plant health. In our study, the control plants and plants treated with organic residue, having high proline levels and low nitrate reductase activities, were the most affected by drought. The treatments that included microbial inoculation gave lower proline and higher nitrate reductase values, while the microbial inoculation produced the lowest value of oxidative stress. This suggests that the inoculated rhizobacteria were highly effective with regard to induce plant resistance to drought in these semiarid conditions. These results are in accordance with the measurements of the stem water, leaf water and leaf turgor

potentials obtained for the needles, where the greatest values were reached after microbial inoculation. Thus, the fact that the highest values of leaf turgor were found in inoculated plants suggests that the rhizobacteria may have contributed to the active osmoregulation and hence to the maintenance of leaf turgor.

The treatments which included the application of organic amendment increased the soil total organic carbon, and all the treatments tested increased the total carbohydrates. These improvements are relevant because these compounds can be used as carbon and energy sources by soil-borne microflora (Mengual et al., 2014a). In addition, the carbon compounds released by roots promote the microbial biomass in the rhizosphere soil, since the microbiota produce a cementing effect through the excretion of polysaccharide material (Roldán et al., 1994). This material has the capacity to increase the hydrophobicity of soil particles and stabilise soil aggregates (Caravaca et al., 2002c). Enzyme activities are properties sufficiently sensitive to indicate changes caused by microbial inoculations (Schoebitz et al., 2014). The β-glucosidase results recorded for the treatments involving microbial inoculation are in accordance with the microbial biomass and soil respiration values, which have frequently been used as indicators of soil microbial activity (Caravaca et al., 2002d). The soil microbial activity in semiarid areas is very low due to the low capacity of the soil organic matter for mineralisation. Due to this fact, Caravaca et al. (2002d) proposed microbial biomass, soil respiration and enzyme activities as indices of the microbiological activity in semiarid soils.

Although all the treatments increased the total N level in the rhizosphere soil, the highest value was produced by the combined treatment. It can be assumed that the addition of amendment would mediate an input of N and that *A. brasilense* and *P. dispersa* were able to fix N_2. All the treatments evaluated enhanced the protease and urease activities, which are involved in the N cycle. The application of amendments and microorganisms has been used to increase nutrient availability in the soil and improve plant growth and nutrient uptake (Caravaca et al., 2005a). In our experiment, available P in the rhizosphere soil was increased by the use of the microbial inoculant, organic residue and combined treatment; presumably, the

rhizobacteria inoculated were able to solubilise P from both the soil and the applied organic residue. Soil microflora is able to excrete organic acids and/or enzymes involved in the P cycle (i.e. phosphatases) which increase the concentration of P in rhizosphere soil (Vassilev et al., 2006). However, based on our phosphatase results, it seems that these rhizobacteria were only capable of solubilising P through their excretion of organic acids (Basak and Biswas, 2010).

The microbial inoculation, the addition of organic residue and the combined treatment increased the extractable K content in rhizosphere soil with respect to the control, which may be attributable to the input of K provided by the organic residue and also to the mobilisation of K by the soil rhizobacteria. Since the microbial inoculant treatments helped plants to compensate for deficiencies of immobile nutrients, the inoculation with immobilised rhizobacteria can be considered an effective tool for the development of biofertilisers that could substitute partially for chemical fertilisation. In this regard, the introduction of a microbial inoculant can improve nutrient availability to plants and thereby increase the efficiency of applied manures (Adesemoye and Kloepper, 2009). It is worth noting that the organic amendment had any effect on plant growth, despite improving soil and plant nutrient status and microbiological quality. In contrast, previous studies have shown the effectiveness of urban residue for improving plants performance during afforestation tasks of a degraded Mediterranean soil with *P. halepensis* (Rincón et al., 2006; Roldán et al., 1996). One possible explanation for this discrepancy is that the composted olive-mill waste used in our experiment could contain phytotoxic compounds after composting (Alburquerque et al., 2006), which would be reducing or eliminating the effectiveness of organic residue for promoting plant growth.

5. Conclusions

The microbial inoculation of *P. halepensis* seedlings with the mixture of the strains *A. brasilense* and *P. dispersa* improved the plant growth in a degraded soil under semiarid Mediterranean conditions. The lower proline accumulation and lesser oxidative damage to lipids, linked to a higher water potential in the plants

inoculated with rhizobacteria, indicate that the plants developed mechanisms to avoid oxidative damage under hydric stress. The capacity of the microbial inoculant to increase plant drought tolerance may have been related to nutrient uptake improvement and an increase in N assimilation through NR activity. Both microbial inoculation and the organic amendment were effective with regard to enhancing soil fertility and microbiological quality. However, the promotion of plant growth by the microbial inoculant was greatest when applied independently, and the lower cost of implementation of this restoration biotechnology supports its preferential use in re-afforestation tasks with *P. halepensis* in semiarid environment.

Chapter VI

Microbial inoculants and organic amendment improves plant establishment and soil rehabilitation under semiarid conditions

1. Introduction

The introduction of plants and shrubs species in degraded Mediterranean soils is a difficult task due to low soil fertility, low and irregular precipitations and a severe drought period (Caravaca et al., 2002a). Under these conditions, it is necessary to assess new methodologies to facilitate the revegetation and improve physicochemical and biological soil properties. Thus, the establishment of a plant cover with shrub species in revegetation programs has been successfully used in revegetation activities in degraded soils, principally those developed under Mediterranean ecosystems (Alguacil et al., 2003; Caravaca et al., 2003b).

Microbial inoculations and organic amendments are widely used tools to aid in the restoration of plant cover and soil quality in degraded Mediterranean areas (Alguacil et al., 2003; Azcón et al., 2009; Caravaca et al., 2003a; Medina et al., 2004). Rhizobacteria, as an important part of the soil microbiota, are known for their ability to increase the root surface area and improve nutrient uptake, biological nitrogen fixation and phosphate solubilisation (Bashan et al., 2004). Rhizobacteria may enhance plant growth by improving the supply of nutrients of low mobility from soil, such as phosphorous (Caravaca et al., 2003c) and potassium. In this regard, rhizobacteria may have a potential role in the establishment of plant species in arid environmental conditions (Benabdellah et al., 2011), although their use is quite more frequent in agricultural lands (Kohler et al., 2006, 2007).

Some recent studies showed the beneficial effects of the application of organic amendments in reclamation of semiarid soils, for example, alperujo (Kohler et al., 2008), urban refuse (Alguacil et al., 2009a) and sugar beet residue (Caravaca et al., 2005a). They have reported beneficial effects on soil quality by increasing the proliferation and development of natural populations of soil microorganisms and improving soil properties. These effects could be extended to the enhancement of the soil enzyme activities, which are considered as key factors contributing to soil activity (Caravaca et al., 2005a), fertility of soil and availability of nutrients to plants. Sugar beet containing cellulose, lignocellulose and other polysaccharides, can be

used by the inoculated microorganisms as a substrate and as carbon and energy source (Vassileva et al., 2010). Additionally, the application of the sugar beet interacts positively with some microorganisms inoculated, like arbuscular mycorrhizal fungi, and can improve both the soil quality and plant performance in degraded soils (Caravaca et al., 2004).

The main objective of this assay was to investigate the role of native rhizobacteria strains in the revegetation of a Mediterranean semiarid area and to prove whether the application of an organic residue could have a synergistic effect with the studied microorganisms. We hypothesise that the combined effects of native rhizobacteria and sugar beet residue addition can increase the plant establishment and soil properties in a degraded semiarid area.

2. Material and methods

2.1. Study site

The study area was located in Vicente Blanes Ecological Park in Molina de Segura, (southeast Spain) (Lat. 38º 12' N, Long. 1º 13' W, Elev. 392 m). The characterisation of the experimental zone is detailed in Chapter III "Matherial and Methods", section 1: Experimental areas, subsections 1.2 and 1.3.

2.2. Plants

The plant used for the revegetation experiment was *Lavandula dentata* L. that is described in Chapter III "Matherial and Methods", section 2: Biological material used to develop the assays, subsection 2.1.

2.3. Microbial inoculants and organic residue

The plant growth promoting rhizobacteria *Bacillus megaterium*, *Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp. were isolated from the experimental area and cultivated in Estación Experimental del Zaidín (EEZ). Bacteria and type of inoculum description are recorded in Chapter III "Matherial and Methods", section 2: Biological material used to develop the assays, subsection 2.2. The amendment

used was composted sugar beet residue and it characterisation is showed in Chapter III "Matherial and Methods", section 3: Organic residues.

2.4. Experimental design

A complete randomised factorial assay was established with two factors and fivefold replication in a split plot design. The first factor was the inoculation of *L. dentata* seedlings with microbial inoculant (*B. megaterium, Enterobacter* sp., *B. thuringiensis* and *Bacillus* sp.) and the second one was the addition of sugar beet residue into the soil. The experimental design was performed as follows: treatment 1, *L. dentata* without rhizobacteria treatment and soil without organic residue addition (Control); treatments 2, 3, 4 and 5, *L. dentata* inoculated with *B. megaterium*, *Enterobacter* sp., *B. thuringiensis* and *Bacillus* sp., respectively, and soil without organic residue addition; treatment 6, *L. dentata* without microbial inoculant treatment and soil with sugar beet residue (SB) addition; treatments 7, 8, 9 and 10, *L. dentata* inoculated with *B. megaterium*, *Enterobacter* sp., *B. thuringiensis* and *Bacillus* sp. soil with sugar beet residue addition (Figure 1). In later March of 2011, pots of 500 ml containing 500 g of soil from the experimental area were used for planting *L. dentata* seedlings. Sugar beet residue was added to the pots at a rate of 2% by weight (10 g of sugar beet residue) and the rhizobacteria dose per inoculation corresponded to 10^{10} CFU plant^{-1}. After fifteen days, a new inoculation was carried out for each treatment. Plants were allowed to establish in the pots for two months, and in later May, the plants were carried to the experimental field, where planting holes 15 x 15 cm wide and 15 cm deep were dug manually. The seedlings were planted at least 1 m apart between holes, with 3 m between treatment levels. At least 5 seedlings per treatment level were planted (Figure 1, Figure 2).

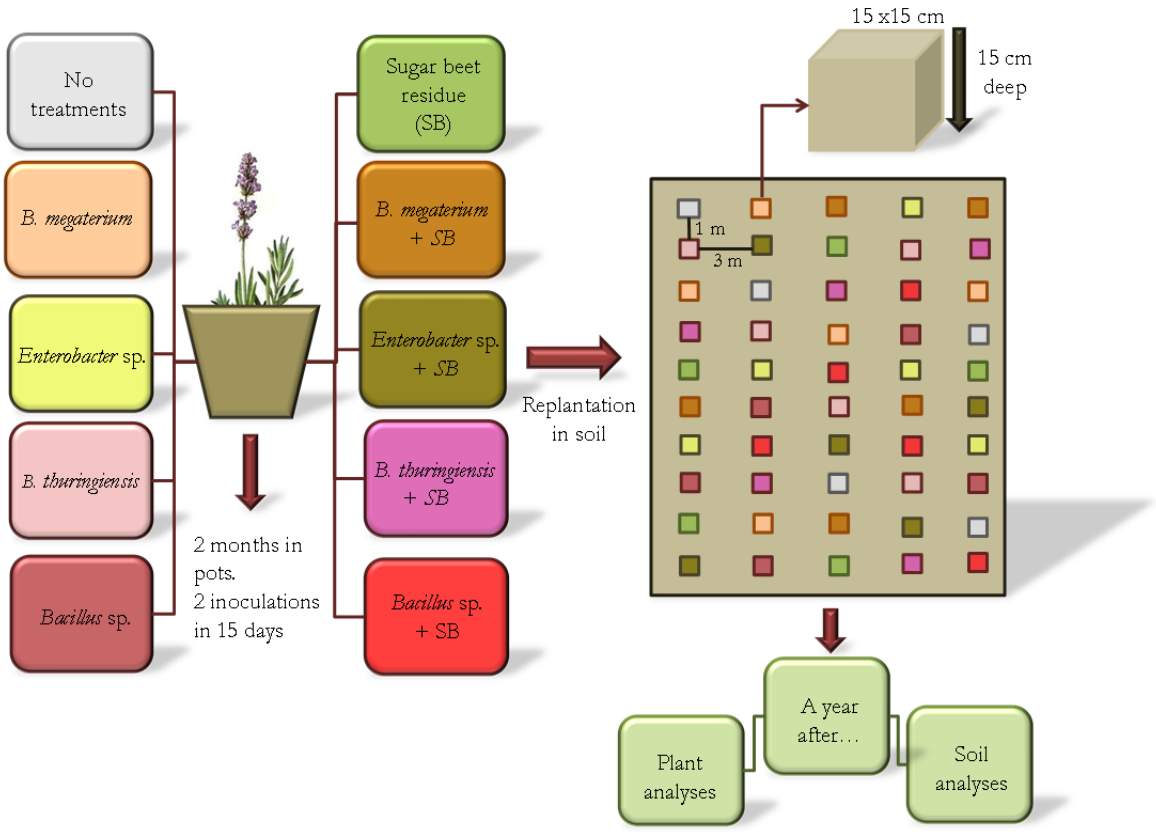


Figure 1. Diagram showing experimental design.

Figure 2. *Lavandula dentata* plantation in Vicente Blanes Ecological Park.

2.5. Sampling procedures

Samples were collected twelve months after planting, in early June 2012. Five plants per treatment, including root systems and rizhosphere soil, were collected between 0 and 15 cm depth from planting holes, and placed in polyethylene bags for transport to the laboratory. Rhizosphere soil samples were divided into two

subsamples before physico-chemical, chemical and biological analyses: one subsample sieved to < 2mm and other subsample sieved between 4 and 0.25 mm.

2.6. Plant analyses

Fresh and dry weights of shoots and roots (70ºC, 48 h) were recorded before chemical analysis. The shoot contents of nitrogen (N), phosphorus (P) and potassium (K) were determined by ICP/OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL). Plant analyses are detailed in Chapter III "Matherial and Methods", section 5: Analytical determinations on plants.

2.7. Soil physico-chemical, chemical and biological analyses

Soil pH and electrical conductivity were measured in a 1:10 (w/v) aqueous solution. Total N and total total organic carbon (TOC) were determined by dry combustion using a LECO Tru-Spec CN analyser (Leco Corp., St. Joseph, MI, USA) while available P and extractable K were determined by ICP-OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL). Water-soluble carbohydrates were determined by the method of Brink et al. (1960). In all these analyses soil sieved to < 2mm was used.

Soil respiration was calculated as the amount of CO_2 emitted during a 24 h incubation period. Soil microbial biomass C was evaluated by the Substrate Induced Respiration (SIR) method and the transformation of the amount of CO_2 emitted to microbial biomass C was done with the equation developed by Anderson and Domsch (1978). Soil respiration and soil microbial biomass C were determined with an automatic analyser (μ-TRAC 4200, SY-LAB). Glomalin related soil protein (GRSP) was determined in the easily extractable glomalin form according to Wright and Anderson (2000).

Dehydrogenase activity was determined according to García et al. (1997). Urease and N-a-benzoyl-L-arginine amide (BAA) hydrolysing protease activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al. 1980). Alkaline phosphatase activity was determined based on the production of *p*-

nitrophenol (PNP) using *p*-nitrophenyl phosphate disodium (PNPP) as substrate according to Tabatabai and Bremmer 1969. β-glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG) as substrate to obtain *p*-nitrophenol (PNP) which amount was established by spectrophotometry (Tabatabai and Bremmer 1969).

Soil analysis methodologies are detailed in Chapter III "Matherial and Methods", section 6: Analytical determination of soil physico-chemical, chemical and biological properties.

2.8. Statistical analyses

The effects of amendment addition, microbial inoculation and their interaction on measured variables were analysed by a two-way ANOVA and post hoc mean separation was performed by the Tukey honestly significant difference (HSD) test, calculated at $P < 0.05$.

3. Results

3.1. Growth parameters of *L. dentata* after microbial inoculation and amendment with organic residue

Both experimental factors, microbial inoculation (MI) and the addition of organic amendment (OA), as well as the MI x OA interaction were significant on *L. dentata* shoot and root growth (Table 1).

Table 1. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic amendment (OA) and their interaction on the measured variables.

Factors and interaction	Shoot (fw)	Shoot (dw)	Root (fw)	Root (dw)
MI	<0.001	<0.001	<0.001	<0.001
OA	0.027	<0.001	0.001	<0.001
MI x OA	<0.001	<0.001	<0.001	<0.001

NS: Not significance; fw: Fresh weight; dw: Dry weight.

One year after planting, all the treatments: microbial inoculations, addition of sugar beet and the combined treatment (MI + SB) significantly increased shoots fresh weight (except *B. thuringiensis* + SB) and shoots dry weight compared to control values (Figure 3).

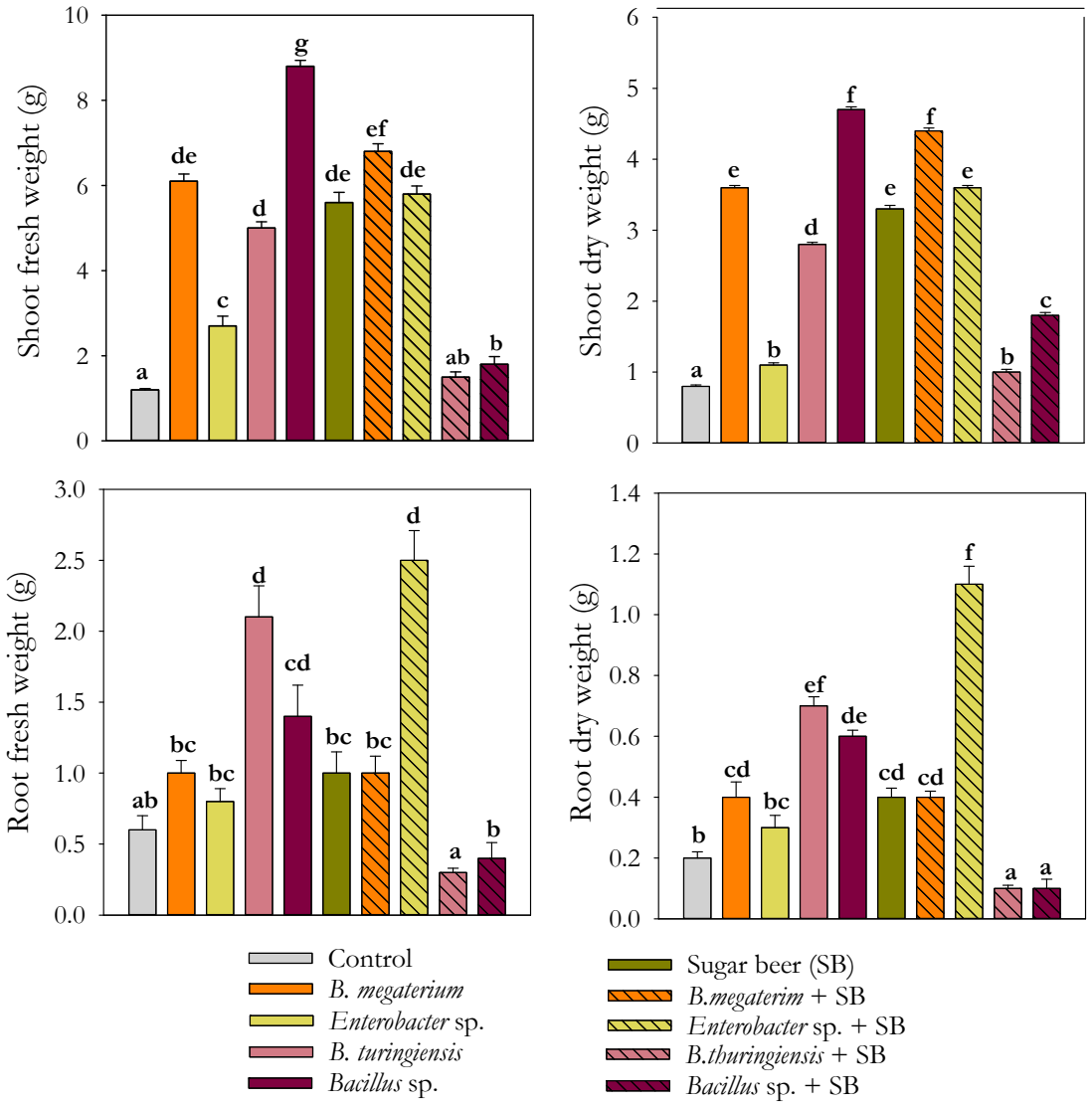


Figure 3. Shoot and roost fresh and dry weight of *L. dentata* seedlings in response to microbial inoculation and organic residue addition. Values are mean of five replicates. Significant differences according to the Tukey honestly significant difference (HSD) test at $P < 0.05$ levels were indicated by different letters.

The greatest improvement was observed on plants inoculated with *Bacillus* sp. (which increased the shoots fresh weight by 7 fold), on plants inoculated with

Bacillus sp. and on plants subjected to the combined treatment *B. megaterium* + SB (both increasing shoots dry weight by 5 fold; Figure 3). Roots dry weight showed a statistically significant increase mediated by almost all the treatments with respect to the control plants. Only the combined treatments with *B. thuringiensis* and *Bacillus* sp. produced a significant decrease while the inoculation with *Enterobacter* sp. did not show any significant change. The inoculation with *B. thuringiensis* and *Bacillus* sp. and the mixed treatment with *Enterobacter* sp. promoted an increase in roots fresh weight, while it was showed a decrease with *B. thuringiensis* + SB and *Bacillus* sp. + SB mixed treatments, compared with the control plants (Figure 3).

3.2. Nutrients uptake

The ANOVA revealed that the microbial inoculation, organic amendment and MI x OA interaction affected significantly N, P and K uptake (Table 2).

Table 2. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic amendment (OA) and their interaction on *L. dentata* shoot nutrients content.

Factors and interaction	N	P	K
MI	<0.001	<0.001	<0.001
OA	<0.001	<0.001	<0.001
MI x OA	<0.001	<0.001	<0.001

All the treatments mediated an improvement on N, P and K contents, except the combined treatment *B. thuringiensis* + SB which did not produce any change on shoot N content (Figure 4). The greatest values for these nutrients were recorded in plants subjected to the inoculation with *Bacillus* sp. and the mixed treatment *B. megaterium* + SB. N and P contents were increased by 6 fold with respect to their controls, whereas shoot K values were improved by 8 fold by both treatments (Figure 4).

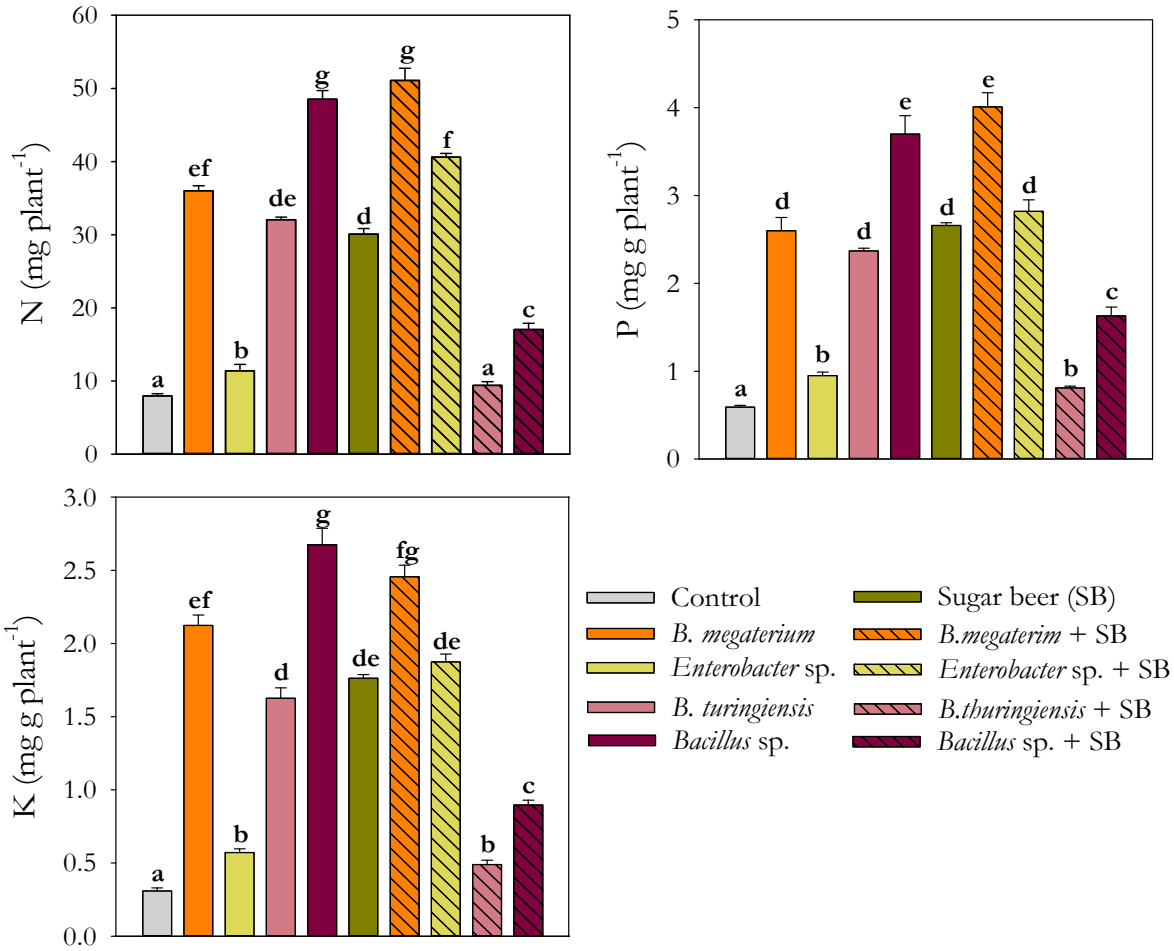


Figure 4. Nutrients content in shoot of *L. dentata* seedlings. Values are mean of five replicates. Significant differences according to the Tukey honestly significant difference (HSD) test at $P < 0.05$ levels were indicated by different letters.

3.3. Soil physico-chemical, chemical and biological analyses

The microbial inoculation, organic amendment and MI x OA interaction significantly affected the soil electrical conductivity, while only organic amendment affected pH values (Table 3). The ANOVA showed that both microbial inoculation and organic amendment factors and their interaction affected significantly N values (Table 3). Thus, the combined treatments *B. megaterium* + SB and *Enterobacter* sp. + SB showed an improvement in total N content (by 38% and 46% respectively, Table 3).

All the microbial inoculation treatments, except the inoculation with *Bacillus* sp., produced a decrease on available P content, while the addition of SB increased it by 29% (Table 3). With regard to extractable K, significant effects were recorded for microbial inoculation and MI x OA interaction (Table 3). The inoculation with *B. megaterium* and the combined treatment *B. thuringiensis* + SB mediated a decrease in K extractable content compared to the control (Table 3).

Table 3. Changes observed on physicochemical and chemical properties in rhizosphere soil of *L. dentata* in response to immobilised rhizobacteria inoculation and organic residue addition.

Treatments	pH (H_2O)	EC ($\mu S\ cm^{-1}$)	Total N ($g\ kg^{-1}$)	P available ($mg\ kg^{-1}$)	K extractable ($mg\ kg^{-1}$)
Control	8.6 ± 0.2 b	484 ± 3 a	1.3 ± 0.0 ab	10 ± 1 de	208 ± 12 cd
B. megaterium	8.4 ± 0.0 ab	490 ± 11 ab	1.6 ± 0.2 bcd	9 ± 1 abc	171 ± 5 ab
Enterobacter sp.	8.4 ± 0.0 ab	495 ± 6 ab	1.6 ± 0.2 bcd	8 ± 0 ab	202 ± 1 c
B. thuringiensis	8.4 ± 0.0 ab	480 ± 11 a	1.1 ± 0.1 a	7 ± 0 a	202 ± 5 c
Bacillus sp.	8.4 ± 0.0 ab	484 ± 4 a	1.3 ± 0.1 abc	9 ± 0 bcd	191 ± 5 bc
Sugar beet (SB)	8.3 ± 0.0 a	535 ± 14 c	1.6 ± 0.1 bcd	14 ± 0 f	234 ± 3 d
B. megaterium + SB	8.3 ± 0.0 a	503 ± 5 abc	1.8 ± 0.0 d	11 ± 0 de	195 ± 5 bc
Enterobacter sp. +SB	8.3 ± 0.0 a	525 ± 1 bc	1.9 ± 0.1 d	10 ± 0 cde	197 ± a bc
B. thuringiensis + SB	8.3 ± 0.0 a	501 ± 1 abc	1.3 ± 0.0 abc	11 ± 0. de	160 ± 4 a
Bacillus sp. + SB	8.3 ± 0.0 a	486 ± 4 a	1.7 ± 0.03 bcd	11 ± 0 d	196 ± 6 bc
Anova *P* values					
MI	NS	0.028	<0.001	<0.001	0.002
OA	0.018	<0.001	<0.001	<0.001	NS
MI x OA	NS	0.004	0.023	NS	0.001

EC: electrical conductivity. Values are mean of five replicates. Significant differences according to the Tukey honestly significant difference (HSD) test at $P < 0.05$ levels were indicated by different letters. NS: Not significance.

Concerning the factorial analysis, the ANOVA recorded that total organic carbon, soil respiration and microbial biomass were significantly affected by both

factors and their interaction and glomalin related soil protein by the organic amendment and the interaction, while only microbial inoculation significantly affected water soluble CH values (Table 4).

Table 4. ANOVA *P* values. Significance of effects of microbial inoculant (MI), organic amendment (OA) and their interaction on carbon fractions.

Factors and interaction	TOC	W-S CH	Soil respiration	Microbial biomass C	GPRS
MI	0.013	0.030	<0.001	<0.001	NS
OA	<0.001	NS	<0.001	<0.001	<0.001
MI x OA	0.001	NS	0.002	0.012	0.001

TOC: Total organic carbon; W-S CH: water soluble carbohydrates, GPRS: glomalin related soil protein.

The post hoc test showed an improvement in TOC contents mediated by all the treatments with the exception of the inoculation with *Enterobacter* sp. and *B. thuringiensis*. The inoculation with *Enterobacter* sp., *Bacillus* sp., and the combined treatments *B. megaterium* + SB and *Enterobacter* sp. + SB increased water soluble CH values (Figure 5). However, the inoculation with *B. thuringiensis* produced a significant decrease in this parameter respect to the control (Figure 5). The greatest value for soil respiration was observed with the mixed treatment *Enterobacter* sp. + SB which improved it by 48%. This parameter underwent a significant decrease when plants were inoculated with *Bacillus* sp. and no change with the combined treatment *B. thuringiensis* + SB respect to the control (Figure 5). Microbial biomass significantly decreased after the inoculation with *B. thuringiensis* and *Bacillus* sp. and with the mixed treatment *B. thuringiensis* + SB. It did not experiment any change after *Enterobacter* sp. inoculation (Figure 5). With regard to glomalin related soil protein, the concentration increased mainly after the addition of SB and the combined treatments *Enterobacter* sp + SB and *B. thuringiensis* + SB (by one fold in each case; Figure 5).

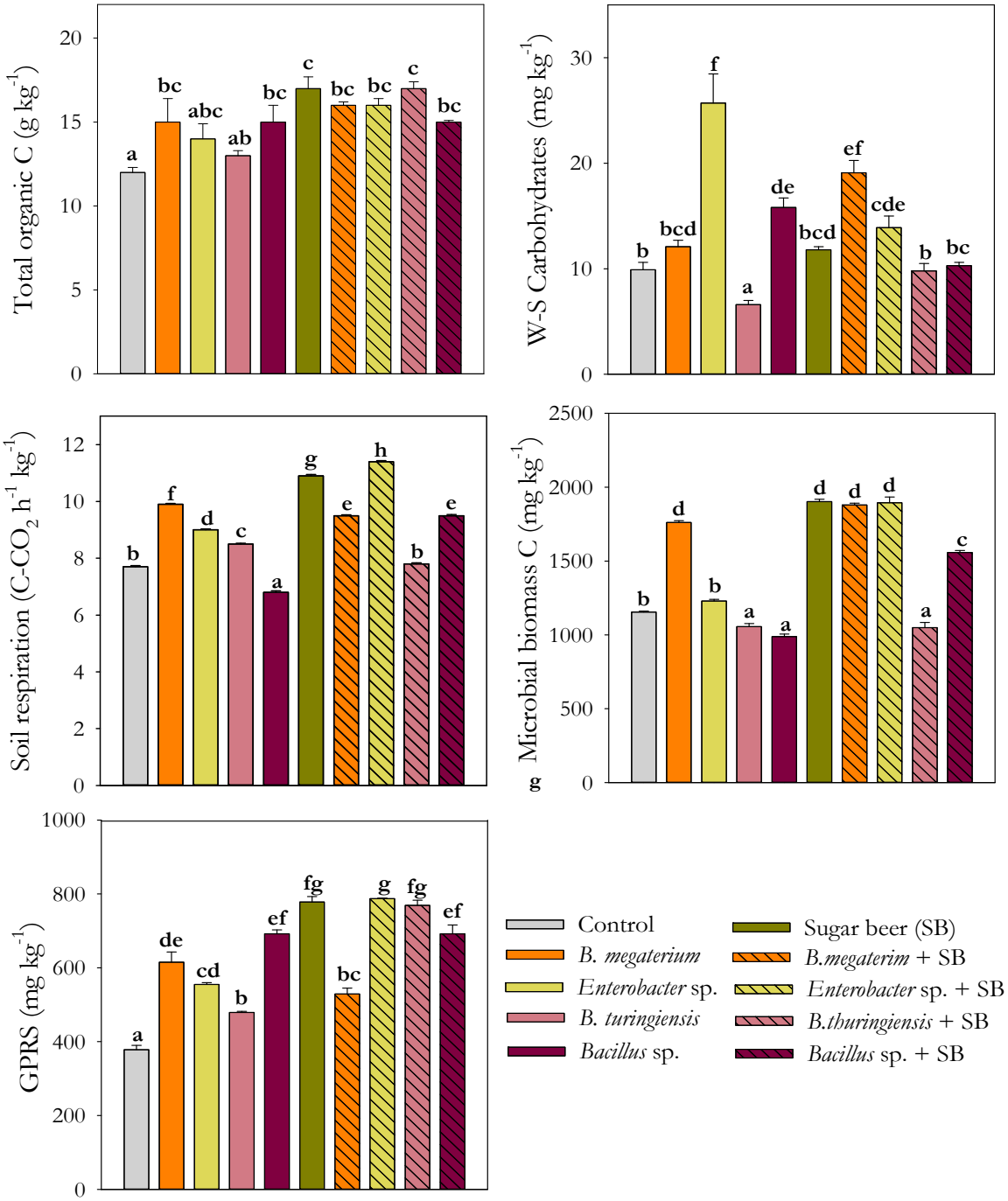


Figure 5. Carbon fractions in rhizosphere soil of *L. dentata* seedlings in response to microbial inoculation and organic residue addition. Values are mean of five replicates. Significant differences according to the Tukey honestly significant difference (HSD) test at $P < 0.05$ levels were indicated by different letters.

The MI x OA interaction significantly affected enzyme activities (urease, β-glucosidase, protease and dehydrogenase) (Table 5).

Table 5. Changes observed on enzymatic activities of rhizosphere soil of *L. dentata* in response to immobilised rhizobacteria and organic residue addition.

Treatments	Dehydrogenase (μg g^{-1} INTF)	β-glucosidase (μmol PNF g^{-1} h^{-1})	Urease (μmol NH_3 g^{1} h^{-1})	Protease (μmol NH_3 g^{-1} h^{-1})	Phosphatase (μmol PNF g^{-1} h^{-1})
Control	113 ± 3 a	0.22 ± 0.02 a	0.43 ± 0.01 ab	1.2 ± 0.0 ab	1.6 ± 0.0 abc
B. megaterium	128 ± 5 abc	0.28 ± 0.01 abc	0.52 ± 0.05 bc	1.4 ± 0.0 bc	1.7 ± 0.0 abc
Enterobacter sp.	120 ± 9 ab	0.27 ± 0.02 ab	0.50 ± 0.04 abc	1.2 ± 0.1 ab	1.2 ± 0.0 a
B. thuringiensis	115 ± 11 a	0.28 ± 0.02 abc	0.55 ±0.01 bcd	1.2 ± 0.0 ab	1.4 ± 0.1 ab
Bacillus sp.	150 ± 8 bc	0.47 ± 0.02 d	0.59 ± 0.04 cde	1.8 ± 0.1 d	1.7 ± 0.0 abc
Sugar beet (SB)	161 ± 5 c	0.49 ± 0.04 d	0.36 ± 0.02 a	1.8 ± 0.1 d	2.0 ± 0.1 bc
B. megaterium + SB	126 ± 4 ab	0.37 ± 0.02 bcd	0.74 ± 0.04 de	1.3 ± 0.1 abc	1.2 ± 0.0 a
Enterobacter sp. +SB	131 ± 5 abc	0.40 ± 0.03 bcd	0.80 ± 0.07 e	1.2 ± 0.1 ab	2.0 ± 0.0 bcd
B. thuringiensis + SB	151 ± 2 bc	0.35 ± 0.01 abcd	0.61 ± 0.04 cde	1.3 ± 0.1 abc	1.8 ± 0.0 abc
Bacillus sp. + SB	151 ± 2 bc	0.38 ± 0.04 cd	0.63 ± 0.02 cde	1.5 ± 0.1 c	1.3 ± 0.1 a
Anova *P* values					
MI	0.001	NS	<0.001	0.001	NS
OA	<0.001	<0.001	<0.001	NS	NS
MI x OA	0.003	0.003	<0.001	0.001	NS

Mean ± standard error (n=5). Significant differences according to the Tukey honestly significant difference (HSD) test at $P < 0.05$ levels were indicated by different letters. NS: Not significance.

Microbial inoculation had a significant effect on urease, protease and dehydrogenase, while the addition of organic amendment significantly affected urease, β-glucosidase, and dehydrogenase activities. None of the factors or MI x OA interaction had a significant effect on phosphatase (Table 5). Thus, phosphatase did not undergo any change with respect to the control (Table 5). β-glucosidase activity increased after the addition of SB and with almost all the combined treatments, except *B. thuringiensis* + SB, which did not differ with respect to the control.

Although the microbial inoculation factor did not affect significantly this activity, an improvement was observed after the inoculation with *Bacillus* sp. (Table 5). Urease values showed an increase mediated by the inoculation with *Bacillus* sp. and all the combined treatments (Table 5). Protease activity was improved by the inoculation with *Bacillus* sp. and the mixed treatment *Bacillus* sp. + SB. The addition of SB also increased the protease activity values (Table 5). Dehydrogenase activity was increased with the inoculation with *Bacillus* sp., the amendment with organic residue and the application of the combined treatments *B. thuringiensis* + SB and *Bacillus* sp. + SB (Table 5).

4. Discussion

The amendment of degraded soils with organic residues improves plant performance under semiarid conditions (Alguacil et al., 2009a; Caravaca et al., 2005a; Fernández et al., 2012; Kohler et al., 2008). It is well documented that plant growth promoting rhizobacteria (PGPR), under greenhouse and laboratory conditions, exert beneficial effects on plant growth and development (Adesemoye and Kloepper, 2009; Adesemoye et al., 2009; Schoebitz et al., 2009). In this field experiment, the addition of sugar beet residue and the inoculation with the native rhizobacteria, applied separately or in combined treatments, were very efficient to promote the shoot growth of *L. dentata*. Caravaca et al. (2002a) reported a synergistic effect of combining mycorrhizal fungi inoculation and organic amendments on plant performance in semiarid soils; nevertheless, in our, case the observed effects of combining rhizobacteria and sugar beet showed a function merely additive. The combination of *Enterobacter* sp. and sugar beet was the best treatment to improve root biomass. However, roots dry weight underwent a significant decrease with the combined treatments *B. thuringiensis* + SB and *Bacillus* sp. + SB. Population dynamics within the rhizosphere of the plant host and ability to colonise roots and shoots by endophytic *Enterobacter* and *Bacillus* species have been reported in crops, increasing their vegetative growth under greenhouse conditions (Rekha et al., 2007; Schoebitz et al.,

2009). Nevertheless, true root colonists are those bacteria that colonise roots in competitive conditions, i.e., natural field soils (Kennedy et al., 2004). It could be considered that *B. thuringiensis* and *Bacillus* sp. were not able to compete with autochthonous soil microbiota to colonise plant roots when the organic amendment was added. *Enterobacter* sp. seemed to be a more efficient colonist with the addition of sugar beet under field conditions.

Rhizobacteria can play a fundamental role on nutrient uptake and therefore on the biomass promotion (Adesemoye and Kloepper, 2009; Bashan et al., 2004). The increase observed in shoot K and P contents may be attributable to the mobilisation of nutrients from soil due to the secretion of organic acids mediated by the inoculants acting as plant growth promoting rhizobacteria (Basak and Biswas, 2010). The improvement observed on foliar N concentration may be explained by the fact that some *Bacillus* species, i.e. *Bacillus sphaericus*, are diazotrophic bacteria able to increase the uptake of N derived from N_2 biological fixation (Zakry et al., 2012) although, in our study, the application of the organic amendment seemed to inhibit this characteristic in *B. thuringiensis*.

With regard to changes in soil properties, the improvements observed in total organic C, water soluble CH and glomalin are quite relevant because these compounds can be used as carbon and energy sources for soilborne microflora. Glomalin is a glycoprotein, produced by arbuscular mycorrhizal fungi, capable of increasing the hydrophobicity of soil particles and to form soil aggregates (Rillig et al., 2001; Wright and Anderson, 2000). These results of total organic C, water soluble CH and glomalin are in agreement with the β-glucosidase and dehydrogenase activity values recorded. Enzyme activities are properties sufficiently sensitive to indicate changes caused by microbial inoculation (Schoebitz et al., 2014). β-glucosidase results in the treatments with residue application are in accordance with microbial biomass and soil respiration values, which have frequently been used as indicators of soil microbial activity (Caravaca et al., 2002b).

It is remarkable the low values for total N in soil recorded in this assay. When the treatments including organic residue were applied, it could be assumed that the

addition of the amendment would mediate an input on N levels. However, this only occurred after the application of sugar beet with *B. megaterium* or *Enterobacter* sp. This may be explained through the assimilation by both plant and soil microbiota of the N provided with the amendment. Besides, it could be assumed that *B. megaterium* and *Enterobacter* sp. were able to fix more N_2 than the other strains in presence of the organic residue. Actually, these two treatments yielded the highest values recorded for the urease activity, which is involved in the N cycle. In general, protease activity was low, but with the treatments including *Bacillus* sp. underwent a significant increase. It could be attributable to a greater capacity of *Bacillu*s sp. to produce this type of enzyme. In fact, the proteases obtained from certain strains of *Bacillus* have a great applicative significance on global biotechnology (Chu, 2007).

Available P content in rizhosphere soil was increased only after the amendment with sugar beet. This could suggest that the microbial strains used in this assay did not solubilise P. Furthermore, this nutrient suffered a significant decrease mediated by almost all the microbial strains when they were applied independently. However, it was observed that shoot P content increased with all the treatments, and this would indicate that, somehow, these bacteria strains were able to solubilise P in soil. To solubilise P in soil, microorganisms have to excrete organic acids or phosphatases (Rodríguez et al., 2006; Vassilev et al., 2006) and supporting in our results, the low phosphatase values recorded could point that these rhizobacteria only were capable to solubilise P by excreting organic acids (Basak and Biswas, 2010). In this sense, pH values in soil decreased in all the treatments tested; in the case of those including the organic amendment, it could be attributable to the low pH of the residue (3.0), and in the case of microbial inoculation to a limited excretion of organic acids. Similar mechanisms could be involved in the high K assimilation recorded for the established plants.

5. Conclusions

The microbial inoculation of the seedlings with native rhizobacteria strains clearly improved plant performance in the revegetation of a degraded semiarid soil.

The combined effect of the organic addition can exert an additive effect with some strains, but also can diminish the positive effect of rhizobacteria. When considering the improvement of soil quality, addition of sugar beet residue and *Enterobacter* sp. combined with sugar beet residue were the treatments that enhanced it to a greater extent. Based on these data, the application of combined treatments involving rhizobacteria inoculation and organic amendments seem to be the most appropriate method to aid in the restoration of both plant cover and soil quality in semiarid degraded areas, although an adequate selection of the rhizobacteria strains must be considered the critical point when developing this restoration technology.

Chapter VII

Isolation and characterisation of actinobacteria strains from *Rhamnus lycioides* L. rhizosphere soil

1. Introduction

Over 10-20% of arid, semi-arid and dried sub-humid areas show severe levels of degradation, which impairs their ability to provide ecosystem services (Piñeiro, 2013). The restoration of these degraded areas throughout the world is, generally, started by studies devised to reclaim the vegetation such as planting of tree/shrubs species (Ouahmane et al., 2009, Cortina et al., 2011; Mengual et al., 2014a, 2014b; Schoebitz et al., 2014) with the objective of modifying the factors limiting the natural recovering of vegetation, and to foster the natural regeneration and the resilience of ecosystems (Piñeiro et al., 2013). There is a basic need for C, N and P in plant nutrition, but soluble N and P nutrients are frequently limiting in degraded soils (Vassilev et al. 2006), so they are often added as chemical fertiliser that can contribute to contaminate rivers and ground waters because they can be washed away (Shigaki et al., 2006), or can be quickly converted in insoluble mineral complexes (Reddy et al., 2002), precipitated forms that plants cannot absorb (Rengel and Marschner, 2005). Thus, only a minimal fraction of the provided nutrients as chemical fertilisers is made available to plants (Scheffer and Schachtschabel, 1989). The inappropriate land-use practices in semiarid areas, along with limited rainfalls and long summers characterised by a dry climate and high temperatures, have a strong relationship with soil degradation and desertification (Alguacil et al., 2011a). Thus, it seems necessary to develop new ecotechnological tools to improve the ability of introduced plants to with-stand stressful environmental conditions (Oliet and Jacobs, 2012). The pre-conditioning of seedlings (including mycorrhization, microbial inoculation, fertilization and water hardening treatments (Barea et al., 2011; Trubat et al., 2011) are among the most widely employed methodologies for recovering degraded drylands. In the last 20 years, the interest on the use of these techniques has grown and there has been an increase in analyses to evaluate their effectiveness to improve seedlings establishment under field conditions (Pausas et al, 2004; Cortina et al, 2011, Vallejo et al., 2012) as well as to improve soil quality (Alguacil et al., 2003; Caravaca et al., 2003b) what has a direct effect on the

establishment and the improvement of the vegetal cover. When considering degraded semiarid Mediterranean areas, the vegetal cover is really scarce. The presence of certain plants species in sites characterised by severe hostile conditions should allow formulating a question about their survival in areas where both soil organic matter content and water availability are limited. One of the shrubs species that can be considered important due to its use to recover drier areas, and that has been encouraged by the Common Agricultural Policy of the European Union, is *Rhamnus lycioides* L., a low-growing shrub, well-adapted to water stress conditions, which belong to the natural succession in certain plant communities of semiarid Mediterranean ecosystems in the southeast of Spain (Caravaca et al., 2003a). It seems admissible to think that the growth of plants in these conditions must be favoured by a complex microbial structure capable to provide nutrients to plants and, even, improve the soil quality, what should allow promoting the establishment of indigenous vegetation. Several studies under field semiarid conditions, have been carried out with mycorrhizal arbuscular fungi (Caravaca et al., 2003a; Caravaca et al., 2005b; Alguacil et al., 2011b) or plant growth promoting rhizobacteria (PGPR) (Mengual et al., 2014a, 2014 b; Schoebitz et al. 2014) but soil microflora also include another type of bacteria, less known and with morphological interest due to their filamentous growth behaviour, having relatively large genomes compared to other bacteria (Kügler et al., 2014) what has allowed, during several years, to consider them belonging to Fungi Kingdom. Actinobacteria belong to the order Actinomycetales, a division of the Gram-positive bacteria (Doumbou et al., 2002) and are capable to mineralise nitrogen and carbon, decompose organic material, fix atmospheric nitrogen (Valdés et al., 2005), produce phytohormones-like compounds (Hamdali et al. 2008a) and behave like biocontrol agents (Tarkka et al., 2008), properties that benefit to plant growth (Hamdali et al. 2008a) and could improve soil quality, being admissible to consider them PGPR. Member of this group are known to produce a large variety of biological substances, in total by 45% of all known bioactive microbial metabolites (Kügler et al, 2014.). *Streptomyces* is in fact known as one of the major sources of bioactive natural products (Franco-Correa et

al., 2010), being historically one of the actinobacteria genus most commonly isolated from soil jointly *Micromonospora* (Basilio et al., 2003). Nevertheless, these capacities have been reported separately in various species (Hamdali et al. 2008a). It is known that actinomycetes can establish actinorhizal symbiosis with certain dicotyledons families, being among them the family *Rhamnaceae* (Mayz-Figueroa, 2004). Therefore, the aims of this study were i) to try the isolation of several strains of actinomycetes to demonstrate their presence in the rhizosphere of *Rhamnus lycioides* being part of the living soil microstructure, due to the plant relatively quick growth and its capacity to survive in extreme conditions and ii) to verify their physiological capacities in a same strain to consider them potential PGPR.

2. Material and methods

2.1. Isolation sites

Two different semiarid Mediterranean areas: Vicente Blanes Ecological Park in Rellano, Molina de Segura and Calblanque Nature Reserve in Cartagena, Province of Murcia, Spain, were selected to collect the samples consisting on rhizosphere soil to try the isolation of different strains of actinobacteria. The characterisation of the experimental zones is detailed in Chapter III "Material and Methods", section 1: Experimental areas, subsections 1.2 and 1.3.

2.2. Experimental design and sampling

The experiment was conducted using a randomised design with three replicates, separated at least 30 m each other in each area (Figure 1). A shrub species natural growing in these sites was selected: *Rhamnus lycioides* L., characterised in Chapter III "Material and Methods", section 2, Biological material used to develop the assays, subsection 2.1.

Samples were collected from the rhizosphere of *R. lycioides* in the first half of February 2012 and transported to the laboratory (Figure 1, Figure 2).

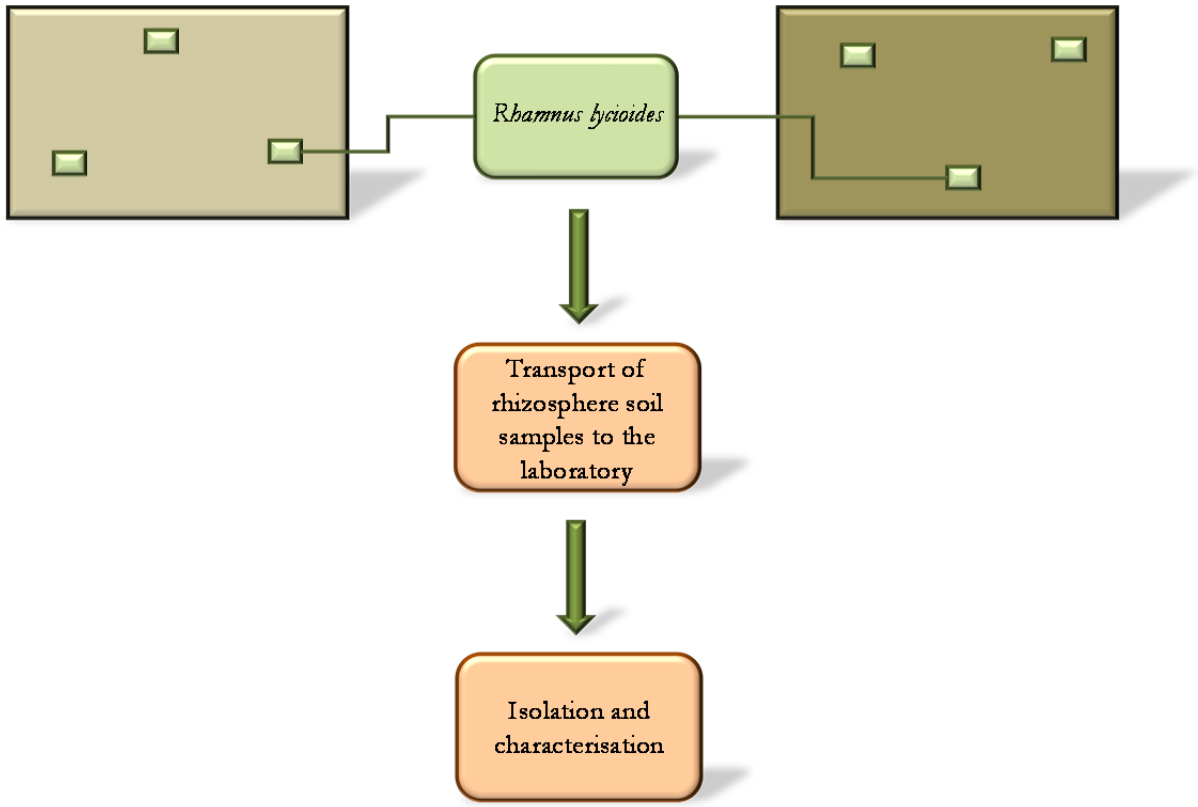


Figure 1. Diagram showing randomised sampling.

Figure 2. Roots and rhizosphere soil of *Rhamnus lycioides* in Calblanque Nature Reserve.

2.3. Isolation methodology

For actinomycete isolation, it was used the methodology described by Franco-Correa et al. (2010) and it is detailed in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.1.

2.4. Macroscopic and microscopic identification

Morphological identification is detailed in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.2.

2.5. Culturing of actinobacteria

The culturing of actinobacteria was developed according to Franco-Correa (2008), and it is described in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.1.

2.6. Growth in Nitrogen Free Media (NFB) and acetylene reduction

The fixation of nitrogen was studied by growing actinobacteria in Nitrogen Free Media (NFB) (Döbereiner and Day, 1976) and testing their capacity to reduce ethylene to acetylene according to Hardy et al. (1968).

Methodology about N_2 fixation is detailed in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.3.

2.7. Siderophore excretion

The qualitative evaluation of siderophore production was performed using a commercial kit, SideroTec AssayTM (Emergen Bio) following the manufacturer's recommendations.

2.8. Phosphate solubilising capacity

Actinomycete strains were cultivated on solid minimal medium based on the Pikovskaya (PVK) medium (Pikovskaya, 1948) supplemented with bromocresol purple as a pH indicator for determining the implication of organic acid production in this activity (Franco-Correa et al., 2010). The haloes due to the phosphate solubilisation were measured at 15th day of the plate incubation at 28ºC. Solubilisation index (SI) was calculated according to Premono et al. (1996). More details are recorded in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.5.

Once this test was assayed, the strains that showed the greatest values for phosphate solubilisation were selected to their molecular identification and to evaluate their biocontrol capacity.

2.9. Molecular identification

Molecular identification consisted in total DNA extraction and the subsequent PCR amplification of 16S ribosomal DNA using the universal primer pair fD1 and (Weisburg et al., 1991). The PCR products were purified using a Gel extraction Kit (Qiagen) (Alguacil et al., 2012) and sequenced by the section of Molecular Biology of Servicio de Apoyo a la Investigación (SAI) (Campus de Espinardo- Murcia, Spain) using the universal primers fD1 and rp2. Sequence editing was done using the program FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTn) sequence similarity search tool (Altschul et al., 1997).

Total DNA extraction, purification and amplification are explained in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.6.

2.10. Antagonism of actinomycete strains against pathogenic fungi

This assay was carried out in mixed cultures of the selected strains of actinomycetes and four pathogenic fungi *Pythium ultimum*, *Fusarium* sp., *Botrytis cinerea* and *Phytophthora parasitica*. For that, Petri dishes with nutritive agar medium were used according to method reported by (Cafaro and Currie, 2005). The level of inhibition was determined by the equation proposed by Yuan and Crawford (1995) and modified by Prapagdee et al. (2008).

This methodology is highlighted in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.7.

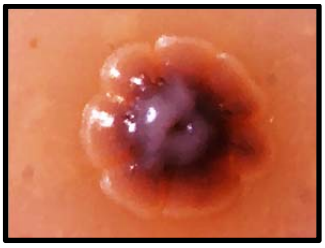
2.11. Statistical analyses

Post hoc mean separation was performed by the Tukey honestly significant difference (HSD) test, calculated at $P < 0.05$.

3. Results

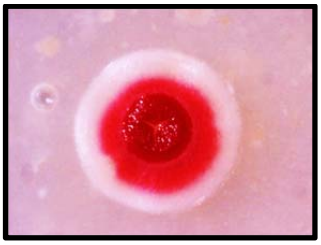
3.1. Isolation and identification of morphological characters of actinomycetes

Six wild type strains of actinomycetes were isolated from de rhizosphere of three specimens of *R. lycioides* L. located in Vicente Blanes Ecological Park, and six wild type strains were isolated from Calblanque Natural Park. After incubation time, the strains were identified according to macroscopic and microscopic characteristics (Figure 1, Table 1). Macroscopic characterisation was based mainly on the aerial and substrate mycelia development. All isolates were positive for Gram's Method and microscopy confirmed that 100% of isolates were violet dyed bacteria showing hyphal accumulations. Once purified on oat-meal agar medium and grown in Yeast Extract Peptone (YEP) broth, the obtained biomass was utilised to develop the following test (Table 1).

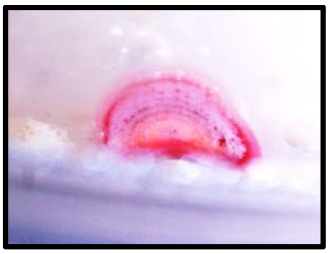


RE1 before mycelial development

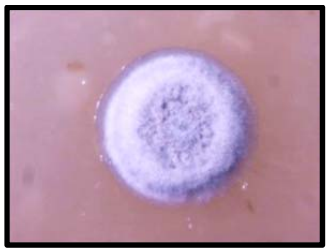
RE2 before mycelial development



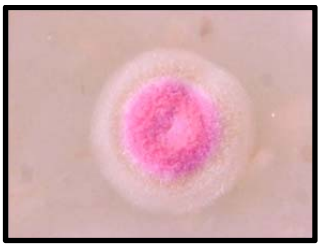
RE3 mycelial development.



RE4 mycelial development



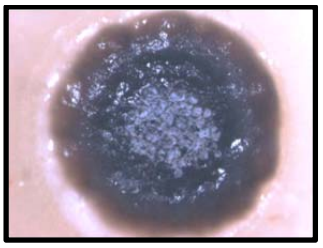
RE5 mycelial development



RE6 mycelial development

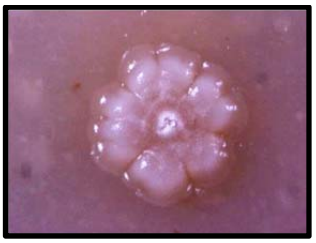
CA1 mycelial development

CA2 mycelial development



CA3 aerial mycelial development

CA4 before mycelial development



CA5 aerial mycelial development

CA6 mycelial development

Figure 3. Macroscopic characteristics of the isolated strains, being observed the colonies morphology and the aerial and substrate mycelia development on oatmeal-agar.

Table 1. Macroscopic and microscopic characterisation of the isolated strains.

Strain	Isolation site	Aerial mass colour on oat-meal agar	Substrate mycelium on oat-meal agar	Gram's Test	Presence of mycelial hyphae
RE1	Rellano	White	White	+	+
RE2	Rellano	Red	White	+	+
RE3	Rellano	Red to purple	White	+	+
RE4	Rellano	White-orange	Purple	+	+
RE5	Rellano	Beige	White	+	+
RE6	Rellano	White-pink	White	+	+
CA1	Calblanque	White	White	+	+
CA2	Calblanque	White	White	+	+
CA3	Calblanque	White	Grey	+	+
CA4	Calblanque	White	White	+	+
CA5	Calblanque	White	White	+	+
CA6	Calblanque	Grey	White	+	+

3.2. Growth in Nitrogen Free Media (NFB) and acetylene to ethylene reduction

All the strains were capable of growing in N-deficient media, what suggested that they could be nitrogen fixing bacteria (Table 2). All of them were tested by acetylene reduction activity (ARA) method and the data recorded showed that all actinobacteria strains were able to reduce acetylene to ethylene, process mediated by N_2 fixation (Table 2).

Table 2. Evaluation of the isolated strains ability to grow in N-free media and to reduce acetylene to ethylene.

Strain	Growth in solid NFB	Growth in NFB broth	Ethylene (nM ml^{-1} h)
Control	-	-	0.004 ± 0.000 a
RE1	+	+	0.016 ± 0.001 d
RE2	+	+	0.009 ± 0.001 bc
RE3	+	+	0.011 ± 0.001 cd
RE4	+	+	0.007 ± 0.001 b
RE5	+	+	0.006 ± 0.001 b
RE6	+	+	0.008 ± 0.001 bc
CA1	+	+	0.015 ± 0.003 d
CA2	+	+	0.006 ± 0.001 b
CA3	+	+	0.007 ± 0.001 bc
CA4	+	+	0.006 ± 0.000 b
CA5	+	+	0.006 ± 0.001 b
CA6	+	+	0.008 ± 0.001 bc

Significant difference according to the Tukey HSD test at $P < 0.05$ levels were indicated by different letters.

3.3. Siderophore excretion

Isolated strains, except R5 and R3, excreted siderophores proved by the change of original blue colour of the reagent to purple or pink as a consequence of the reaction of ferric cation chelation developed in presence of siderophores (Table 3).

Table 3. Qualitative evaluation (presence "+" or absence "-") of siderophores by the change of reactive blue colour to purple or pink.

RE1	RE2	RE3	RE4	RE5	RE6	CA1	CA2	CA3	CA4	CA5	CA6
+	+	-	+	-	+	+	+	+	+	+	+

3.4. Phosphate solubilising capacity

15 day after the inoculation, the Tukey HSD test disclosed that eight actinobacteria strains were capable of solubilising the three calcium, ferric and aluminum phosphates. RE6, CA5 and CA6 were not able to pull phosphorus out of ferric phosphate, although the capacity of R6 in the evaluation with calcium and aluminium phosphates was recorded. CA3 did not solubilise any phosphate source. Regarding to the efficacy to solubilise the three phosphates, the best results were recorded after the inoculation of RE1, RE2, CA1 and CA2 (Table 4).

Table 4. Phosphate Solubilisation Index (SI) of strains cultivated on PVK solid agar plates.

Strain	$Ca_3(PO_4)$ SI	$FePO_4$ SI	$AlPO_4$ SI
Control	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
RE1	3.03 ± 0.04 def	3.00 ± 0.08 f	2.40 ± 0.07 e
RE2	3.66 ± 0.09 g	1.73 ± 0.03 d	2.21 ± 0.06 de
RE3	2.54 ± 0.09 c	1.43 ± 0.06 bcd	1.47 ± 0.06 c
RE4	2.70 ± 0.04 cd	1.62 ± 0.09 cd	1.92 ± 0.10 d
RE5	3.21 ± 0.05 f	1.33 ± 0.08 bc	1.40 ± 0.03 c
RE6	0.64 ± 0.04 b	0.00 ± 0.00 a	0.39 ± 0.03 b
CA1	3.06 ± 0.05 ef	2.21 ± 0.09 e	2.40 ± 0.09 e
CA2	3.80 ± 0.07 g	1.74 ± 0.07 d	2.24 ± 0.09 de
CA3	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
CA4	2.95 ± 0.05 def	1.16 ± 0.09 b	1.88 ± 0.08 d
CA5	2.80 ± 0.09 cde	0.00 ± 0.00 a	2.18 ± 0.05 de
CA6	2.98 ± 0.06 def	0.00 ± 0.00 a	1.43 ± 0.07 c

Significant difference according to the Tukey HSD test at $P < 0.05$ levels were indicated by different letters.

According to the results obtained by the evaluation of the isolates abilities to grow in N-free media and reduce acetylene to ethylene, the qualitative analysis of siderophore production and the phosphate solubilising capacity, two strains coming from Rellano, RE1 and RE2, and two coming from Calblanque, CA1 and CA2, that showed similar behaviours, were selected to continue with the following tests.

3.5. Molecular identification

Partial SSU rDNA sequences from 4 CFU belonging to the four selected strains were amplified successfully by PCR. The sequences were grouped into the genus *Streptomyces* based on sequence similarity to those available in GenBank of ≥ 97%. The strains RE1 and RE2 displayed 99% similarity to sequences from *Streptomyces albospinus* (accession JN566023.1) and 100% similarity to sequences from *Streptomyces* sp. (accession HM210306.1), respectively. The strains CA1 and CA2 showed similarity to sequences 99% from *Streptomyces* sp. (accession JN866719.1) and 99% from *Streptomyces microsporus* (accession AB184459.2), respectively. Right after, the sequences were deposited in GenBank with their corresponding accession numbers (Table 5).

Table 5. Grouping of t selected strains into genus *Streptomyces*.

Strain	BLAST analysis	Sequence similarity	Accession number
RE1	*Streptomyces albospinus* (JN566023.1)	99%	LN610452
RE2	*Streptomyces* sp. (HM210306.1)	100%	LN610454
CA1	*Streptomyces* sp. (JN866719.1)	99%	LN610453
CA2	*Streptomyces microsporus* (AB184459.2)	99%	LN610455

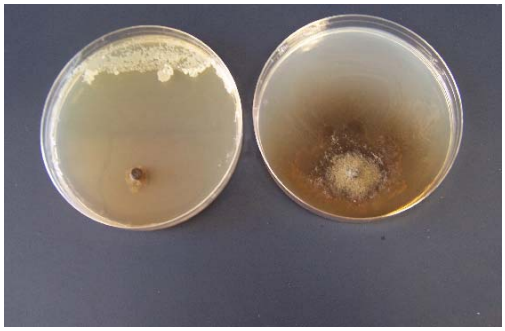
3.6. Antagonism of actinobacteria against pathogenic fungi

Actinobacteria were capable of inhibiting the growth of the tested fungi, showing a great antagonistic activity to *P. ultimum*. With respect to *P. parasitica* and *Fusarium* sp. the strains behaviour was moderate, except for RE2 against *Fusarium* that showed a high efficacy to impede the fungal development. After de inoculation with CA1 in a mixed culture with *B. cinerea*, also was observed a moderate level of inhibition, while for the remaining strains, low values were recorded (Table 6, Figure 4).

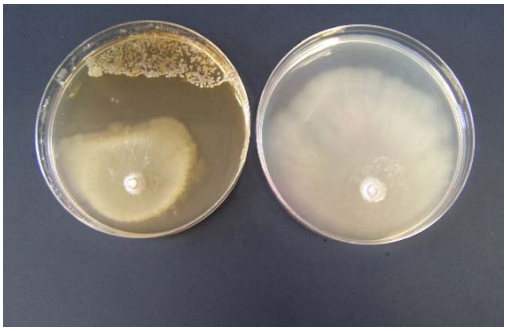
Table 6. Antifungal activity of isolated actinobacteria against *B. cinerea, Fusarium* sp., *P. parasitica* and *P. ultimum*.

Strain	*Botritis cinerea*	*Fusarium* sp.	*Phytophthora parasitica*	*Pythium ultimum*
RE1	+	++	++	+++
RE2	+	+++	++	+++
CA1	++	++	++	+++
CA2	+	++	++	+++

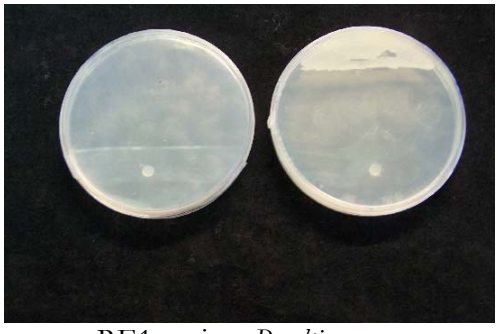
Ratings of inhibition levels: + + + = $\Delta\gamma \geq 20$ mm; + + = $\Delta\gamma \geq 10$ - 19 mm; + = $\Delta\gamma \geq 5$ - 9 mm; - = $\Delta\gamma < 5$ mm (no antifungal activity).



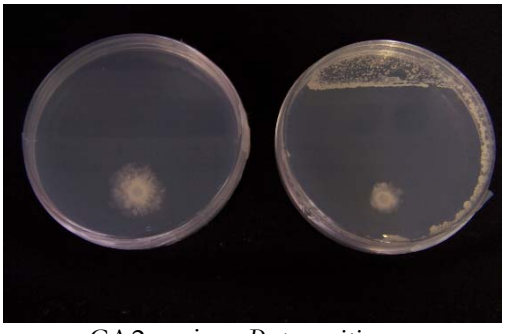
CA2 against *B. cinerea*



RE2 against *Fusarium* sp.



RE1 against *P. ultimum*



CA2 against *P. parasitica*

Figure 4. Antifungal activity of selected actinobacteria against *B. cinerea*, *Fusarium* sp., *P. parasitica* and *P. ultimum*.

4. Discussion

In the current assay, all the strain tested showed indicative characteristic to consider them actinobacteria. All the isolated developed aerial and substrate mycelia (Bergey et al., 2000) and were observed under light microscopy, being dyed in obscure blue or violet by means of Gram's Method, making clear the presence of mycelial hyphae. These results are in accordance with those recorded by Sousa et al. (2008). Once isolated strains were identified as potential actinobacteria, based on morphological characteristics, several physiological characters were assayed. With respect to siderophore excretion, almost all the strains were able to produce them, at least in a qualitative analysis. Iron is an essential element for nearly all organisms. As a transition element, iron can reversibly modify its oxidation state. Despite its relative abundance, the amount of bioavailable iron in nature is very limited because atmospheric oxygen rapidly oxidizes iron to form sparingly soluble ferric oxy hydroxides (Wang et al., 2014). Under iron starvation, most microbes excrete at least one type of siderophore to solubilise environmental iron (Haas, 2003). Siderophores are low molecular-weight chelating agents (200-2000 Da) which can facilitate the uptake of iron (Schalk et al., 2011) and it has been proved that actinobacteria and particularly the genus *Streptomyces* are able to excrete them (Wang et al., 2014).

The availability of sufficient soluble C, N and P is indispensable for plant growth but N and P nutrients are often limited in soils (Hamdali et al. 2008a). For that, it is of interest to test the ability of actinobacteria to provide macronutrients to the plant, since it should contribute to favour plant performance. Regarding to the possibility of facilitating N to the plant, it was assayed whether isolated strains were able to grow in nitrogen free media and, subsequently, if they were able to reduce acetylene to ethylene. These analyses were positive for experimental actinobacteria what confirmed they were capable to fix atmospheric nitrogen. The efficacy on this property was not greater depending of the isolation origin, being observed similar values in isolates from

both localities. Hamdali et al. (2008a) and El-Tarabily et al. (2008) described the ability of actinomycetes to solubilise phosphates what should favour plants and soils conditions. For this purpose, we verified this character by the solubilisation of three different phosphates (calcium, ferric and aluminium) on a solid medium based on Pikovskaya descriptions (1948). The importance of proving different P compounds resides in that calcium phosphate, used as universal factor to isolate and evaluate phosphate solubilising bacteria, is not a good selector concerning failure with inoculated plants. Bashan et al. (2012a) proposed a combination of two or three metal-P compounds to replace the sole calcium phosphate as an initial selection factor. The highest values were found for RE1, RE2, CA1 and CA2, capable of solubilising them with the largest efficacy. Basing in these previous premises, four strains coming from the two different isolation areas were selected to carry on the molecular identification and the evaluation as biocontrol agents. As well as in the case of other bacteria, the 16S rDNA sequencing approach is routinely used to identify actinobacteria after comparing with described sequences in an rDNA sequence database (Franco-Correa et al., 2010). The universal primers pair fD1 and rp2 used in this assay confirmed their efficacy to amplify all sequences of 16S rDNA gene of target bacteria. The four selected actinobacteria strains pertained to genus *Streptomyces* which is logical due to it is one of the more described groups (Basilio et al., 2003) in comparison to other actinobacteria, which could indicate that is one of the most abundant actinobacteria genus.

Actinobacteria are frequent coloniser of mycorrhizospheres, rhizospheres and plant roots (Kurth et al., 2013) and they are known for their antagonism against other microbial species (Huddleston et al, 1997). Particularly, disease suppression by streptomycetes owes partially to their exudation of various antimicrobials, helminticides and enzymes degrading fungal cells walls and insect exoskeleton (Tarkka et al., 2008). This antagonist interaction was verified, in our case, with the four selected strains in mixed cultures with the four selected

pathogenic fungi species, by inhibiting fungal development under laboratory conditions.

The obtained data should afford to infer that, in effect, actinobacteria are important components of soil biota, capable of appearing in the rhizosphere of plants growing in degraded semiarid areas and they have an important role as PGPR bacteria, at least, under laboratory conditions. Since the four selected strains were able to fix atmospheric nitrogen, solubilise phosphates, excrete siderophores and inhibit pathogenic fungal development under laboratory conditions, we could consider that certain species of actinobacteria could satisfy the requirements to act as PGPR under field conditions, allowing and favouring the establishment of a vegetal covering on degraded semiarid areas.

5. Conclusions

It seems admissible to consider as adequate the techniques used to carry on this assay, since we were capable to isolate, characterise and identify four actinobacteria strains that could act promoting the establishment and development of plant species in semiarid Mediterranean soils. However, this character only must be considered as a potential role because their intervention under field conditions is crucial to consider this evaluation as satisfactory.

Chapter VIII

Assessment of the potential role of *Streptomyces* strains in the revegetation of semiarid sites: the relative incidence of strain origin and plantation site on plant performance and soil quality indicators

1. Introduction

Natural revegetation tends to be slow in arid and semiarid Mediterranean ecosystems, where the scarcity of water frequently limits plant establishment and growth (Caravaca et al., 2005a; Schoebitz et al., 2014). However, other environmental factors also could provoke major differences in the plant cover regeneration, including soil type and soil nutrient availability (Caravaca et al., 2002a; Alegre et al., 2004). Several revegetation programs have been developed by using a plant cover based on autochthonous plant species, which seems the most appropriate strategy for reclaiming degraded lands (Caravaca et al., 2005a). The use of shrubs to recover dry areas has been encouraged by the Common Agricultural Policy of the European Union. The application of plant growth promoting microorganisms has been recorded as a successful tool in the reclamation of semiarid Mediterranean areas (Mengual et al., 2014a; Schoebitz et al., 2014). Revegetation practices based on microbial inoculations require the development of an inoculum whose performance is optimum under specific environmental conditions (Caravaca et al., 2003a), in order to benefit the growth, nutrient uptake, and hydric status of the host plant (Ortiz et al., 2014). Plants and microorganisms co-existing in a soil are often adapted to the same environmental conditions; so, presumably, native strains could be more effective for this purpose in semiarid Mediterranean sites (Armada el at., 2014), allowing the local biodiversity to be preserved without introducing new microbial species (Ortiz et al., 2014). Rhizobacteria are free-living bacteria which can colonise the rhizosphere and improve root system establishment (Antoun and Kloepper, 2001), improving plant health and nutrition (Puente et al., 2004; Benabdellah et al., 2011; Mengual et al., 2014a, 2014b; Schoebitz et al., 2014). Actinobacteria are one of the major components of soil microbial populations, comprising 10-50% of the soil microflora community over a broad range of soil conditions (Hamdali et al., 2008a). They are able to mineralise nitrogen and carbon, decompose organic material (Li et al., 2010), fix atmospheric nitrogen (Valdés et al., 2005), produce phytohormone-like

compounds, and behave like biocontrol agents (Tarkka el al., 2008) - properties that benefit plant growth (Hamdali et al., 2008b). Due to their multiple traits, the use of actinobacteria to favour the establishment of plant species in semiarid environments is interesting. Actinobacteria have displayed their potential as plant growth promoting rhizobacteria (PGPR) under laboratory and greenhouse conditions (Shishido and Chanway, 1998; De Vascocellos and Cardoso, 2009; Franco-Correa et al., 2010), but knowledge of their effectiveness under field conditions is scarce, being limited to assays in agricultural ecosystems (Jog et al., 2014). Meanwhile, no studies on the employment of actinobacteria to promote the establishment of plant species in revegetation programs have been conducted.

It has been proved that microorganisms that are native to a particular soil, such as arbuscular mycorrhizal fungi (Caravaca et al., 2003a; Ouahmane et al., 2006b; Ouahmane et al., 2007; Bashan et al., 2012b) or *Bacillus* strains (Armada et al., 2014; Ortiz et al., 2014), are often successful inoculants in revegetation tasks, presumably as a result of their adaptation to specific edaphic and environmental conditions (Schreiner, 2007). However, the efficacy of native strains of actinobacteria in comparison with allochthonous strains, regarding enhancement of plant growth, has not been investigated. We hypothesised that variations among actinobacterial strains from different sites could lead to distinct effects on plant growth and nutrient uptake. The aims of this study were i) to ascertain if the positive effects of actinobacteria are maintained when the strains are inoculated in soils different from their isolation source, ii) to verify the relevance of the strain origin to the ability of actinobacteria to enhance plant growth under semiarid field conditions, and iii) to unravel the mechanisms which account for the differences in the ability of native actinobacterial isolates to influence growth and nutrient uptake. In this regard, we investigated whether the actinobacterial strains stimulate plant growth directly or indirectly, by improving soil properties. To address these questions, we assessed, in a field experiment, the efficacy of actinobacterial strains isolated from two Mediterranean semiarid sites with different soil characteristics on the establishment of *Rhamnus lycioides* L. seedlings in both locations, as well as their

effect on soil chemical and microbiological properties. The information gained here will enable us to establish effective criteria for the selection of strains for use in the revegetation of semiarid environments.

2. Material and Methods

2.1. Study areas

The field assay was carried out in two different semiarid Mediterranean areas: Vicente Blanes Ecological Park in Rellano and Calblanque Nature Reserve, both in the Province of Murcia, Spain. The characterisation of the experimental zones are listed in Chapter III "Matherial and Methods", section 1: Experimental areas, subsections 1.2 and 1.3.

2.2. Plants

The plant used was *Rhamnus lycioides* L. that is described in Chapter III "Matherial and Methods", section 2: Biological material used to develop the assays, subsection 2.1. Prior to the experimental procedures, *R. lycioides* seedlings were grown for 1 year in nursery conditions with peat as substrate. At planting, *R. lycioides* seedlings reached 29 ± 1.8 cm high, with a shoot dry weight of 1.78 ± 0.2 g and root dry weight of 2.45 ± 0.5 g (n=5).

2.3. Microbial inoculants

Six strains coming from each study area were isolated from the rhizosphere of naturally established *R. lycioides* plants as it is described in Chapter VII "Isolation and characterisation of actinobacteria strains isolated from *Rhamnus lycioides* rhizosphere", section 2, subsections 2.1 and 2.2. and in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.1. They were assayed in vitro for their abilities to solubilise phosphate from calcium, aluminium and iron (III) phosphates (Premono et al., 1996; Bashan et al., 2013), to fix dinitrogen by measuring acetylene reduction activity (ARA) (Hardy et al., 1968) and to produce siderophores (SideroTec AssayTM, Emergen Bio),

techniques detailed in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsections 7.3, 7.4 and 7.5. Four of those strains with similar capacities, two coming from Rellano RE1 and RE2 and two from Calblanque CA1 and CA2, were chosen to perform our experiment (Table 1). The strains were identified using molecular methods, consisted in total DNA extraction followed by 16S rDNA amplification and sequencing. The strains RE1 and RE2 displayed 99% similarity to sequences from *Streptomyces albospinus* (accession JN566023.1) and 100% similarity to sequences from *Streptomyces* sp. (accession HM210306.1), respectively. The strains CA1 and CA2 showed a sequence similarity of 99% to *Streptomyces* sp. (accession JN866719.1) and 99% to *Streptomyces microsporus* (accession AB184459.2), respectively (Table 1). These data are recorded in Chapter VII, section 3: Results. Inocula preparation is highlighted in Chapter III "Material and Methods", section 7: Isolation, characterisation and encapsulation of actinobacteria, subsection 7.1., reaching a cells concentration of 1.2 10^8 CFU g^{-1}.

Table 1. Characterisation in vitro of the four strains of *Streptomyces* sp. selected for the field experiment.

Strain	$Ca_3(PO_4)$ SI	$FePO_4$ SI	$AlPO_4$ SI	Ethylene (nm ml^{-1} h)	Siderophore excretion	Accession number
RE1	3.03 ± 0.04 b	3.00 ± 0.08 d	2.43 ± 0.08 b	0.02 ± 0.00 b	+	LN610452
RE2	3.66 ± 0.09 c	1.73 ± 0.03 b	2.21 ± 0.06 b	0.01 ± 0.00 b	+	LN610454
RE3	3.06 ± 0.05 b	2.11 ± 0.08 c	2.39 ± 0.05 b	0.02 ± 0.00 b	+	LN610453
RE4	3.80 ± 0.07 c	1.76 ± 0.06 b	2.22 ± 0.10 b	0.01 ± 0.00 b	+	LN610455

SI: Solubilisation Index. Significant difference according to the Tukey's HSD test at $P < 0.05$ levels were indicated by different letters.

2.4. Experimental design

A full-factorial design was established with two factors and five-fold replications in a split plot design. The first factor was the origin of the strains

(strains isolated from Rellano or isolated from Calblanque plus non-inoculated controls) and the second one was the planting site (Rellano or Calblanque) (Figure 1). In late November of 2012, the seedlings were transported to the experimental sites, where planting holes 15 x 15 cm wide and 15 cm deep were dug manually. There, an amount of 7 g of microbial inoculant pellets was applied per plant. The same quantity of sterilised inoculant was applied to the non-inoculated plants. The seedlings were planted at least 1 m apart between holes, with 3 m between treatment levels (Figure 1, Figure 2).

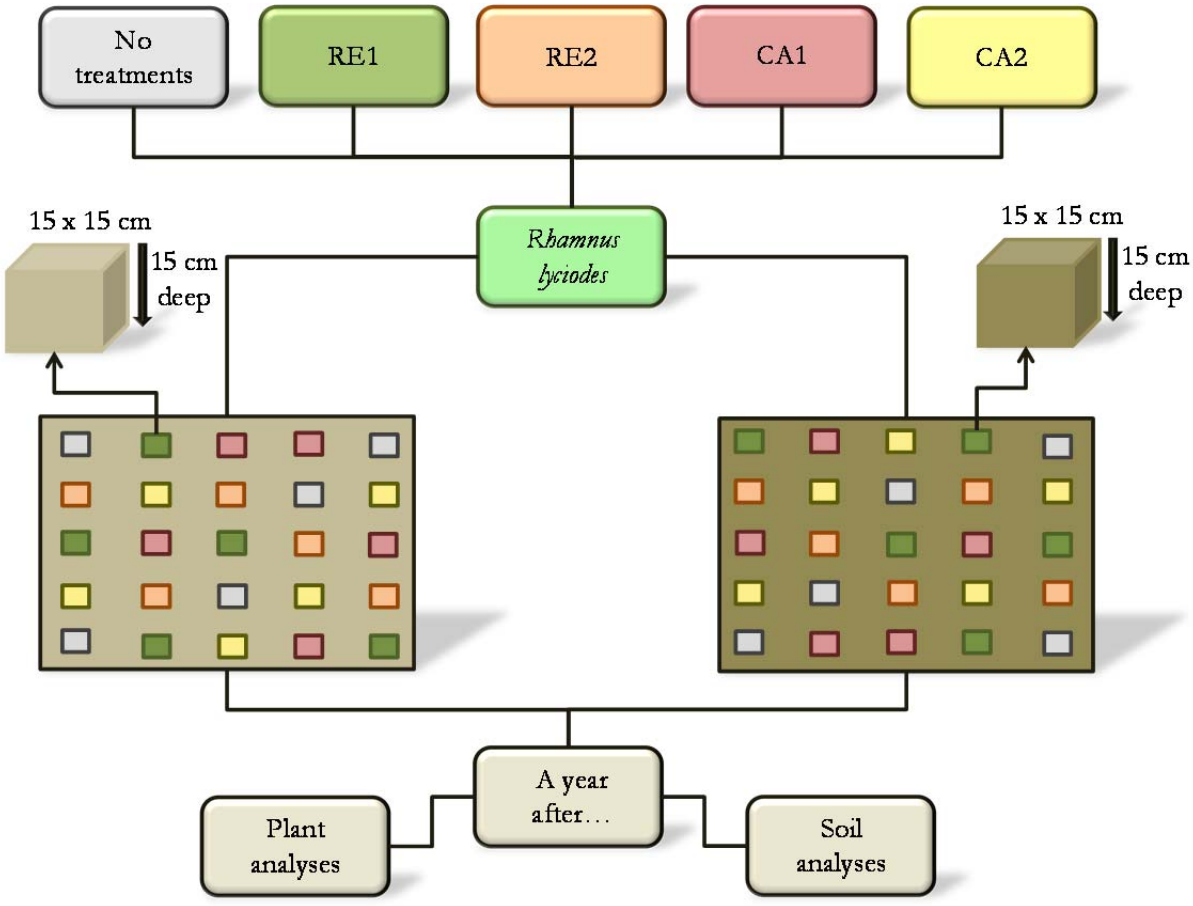


Figure 1. Diagram showing the experimental design.

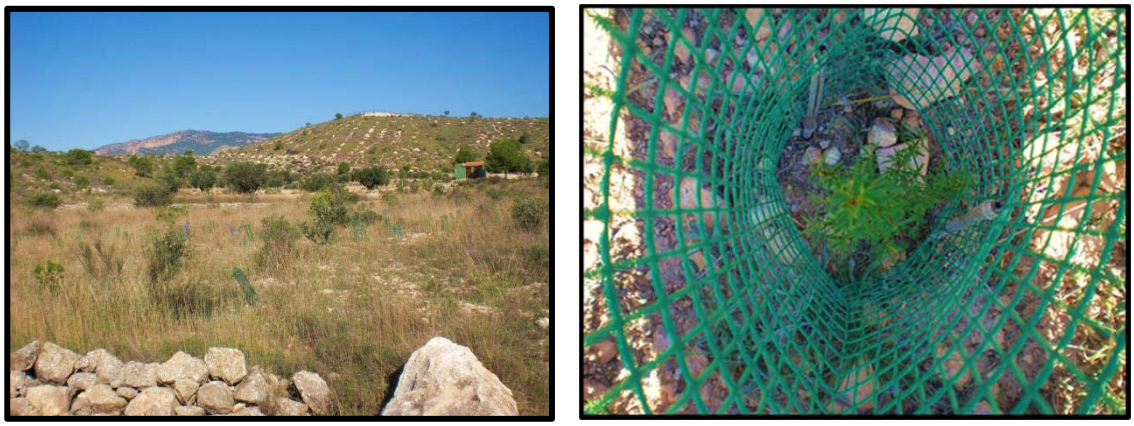


Figure 2. *Rhamnus lycioides plantation* in Vicente Blanes Ecological Park.

2.5. Sampling procedures

Twelve months after planting, in November 2013, samples were collected from each experimental area. Five plants per treatment including root systems and soil firmly adhered to the roots (rhizosphere soil) were harvested, and introduced in polyethylene bags for transport to the laboratory A total number of 60 plants and rhizosphere samples were collected. Rhizosphere soil samples were divided in two subsamples. One soil subsample was sieved at 2 mm and stored at 4ºC for microbiological and biochemical analyses and another soil subsample was allowed to dry at room temperature for chemical analyses.

2.6. Plant analyses

Fresh and dry weights of shoot and root, basal stem diameter and plant height were recorded. Shoot P and K were determined by ICP/OES spectrometry while shoot N was determined by dry combustion. Nitrate reductase activity was assayed in vivo by measuring NO_2^- production in tissue that had been vacuum-infiltrated with buffered NO_3^- solutions (Downs et al., 1993). The level of lipid peroxidation was determined by the content of malondialdehyde (MDA), a product of lipid peroxidation (Zhao et al., 1994) by the method of Minotti and Aust (1987). Plant analyses are detailed in Chapter III "Matherial and Methods", section 5: Analytical determinations on plants.

2.7. Soil physico-chemical, chemical and biological analyses

Total nitrogen (N) was determined by dry combustion. Available P and K were determined by ICP/OES spectrometry. Water soluble C (WSC) was determined in water extracts (1:10 w/v) by using an automatic carbon analyser for liquid samples. Water soluble carbohydrates (WSCH) were determined by the method of Brink et al. (1960).

Soil respiration was calculated as the amount of CO_2 emitted during a 24 h incubation period. Soil microbial biomass C was evaluated by the Substrate Induced Respiration (SIR) method and the transformation of the amount of CO_2 emitted to microbial biomass C was done with the equation developed by Anderson and Domsch (1978). Soil respiration and soil microbial biomass C were determined with an automatic analyser.

Dehydrogenase activity was determined according to García et al. (1997). Urease and N-a-benzoyl-L-arginine amide (BAA) hydrolysing protease activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al. 1980). Alkaline phosphatase activity was determined based on the production of *p*-nitrophenol (PNP) using *p*-nitrophenyl phosphate disodium (PNPP) as substrate according to Tabatabai and Bremmer 1969. β-glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG) as substrate to obtain *p*-nitrophenol (PNP) which amount was established by spectrophotometry (Tabatabai and Bremmer 1969).

Soil analysis methodologies are detailed in Chapter III "Matherial and Methods", section 6: Analytical determination of soil physico-chemical, chemical and biological properties.

2.8. Statistical analyses

The effects of the strains origin, planting site and their interaction on measured variables were analysed by a two-way ANOVA. The mean separation was performed by Duncan's multiple range test, calculated at $P < 0.05$.

3. Results

3.1. Growth parameters of *Rhamnus lycioides*

Both strain origin (SO) and planting site (PS) had a significant effect on *R. lycioides* shoot biomass and height, while root dry weight was only affected by PS (Table 2).

Table 2. Two-way ANOVA *P* values. Significance of effects of strain origin (SO), planting site (PS) and their interaction on the measured variables. NS: Not significance.

Factors and interaction	Shoot	Root	Height
SO	0.025	NS	0.006
PS	<0.001	<0.001	<0.001
SO x PS	NS	NS	NS

One year after planting, the plants inoculated with *Streptomyces* sp. strains were taller at the Calblanque site than at the Rellano site, regardless of the SO. At Calblanque, the inoculation was more effective with native strains (CA1 and CA2) than with allochthonous strains (RA1 and RA2), for increasing shoot dry weight (by about 48% and 28%, respectively, compared to control plants). However, at Rellano, the origin of the strain did not influence its efficacy regarding promotion of plant growth. Thus, in this soil the native and allochthonous strains produced similar increases in the shoot biomass of *R. lycioides* (on average, about 44% compared to control plants). Root biomass increased in response to the inoculation with both native and allochthonous strains at Calblanque (by 60% and 52%, respectively, compared to control plants). In contrast, at Rellano, only the allochthonous strains (CA1 and CA2) provoked an increase in root biomass. The microbial inoculation hardly had an effect on *R. lycioides* height. A significant increase was observed only at Calblanque, after the inoculation with the native strains (Figure 3).

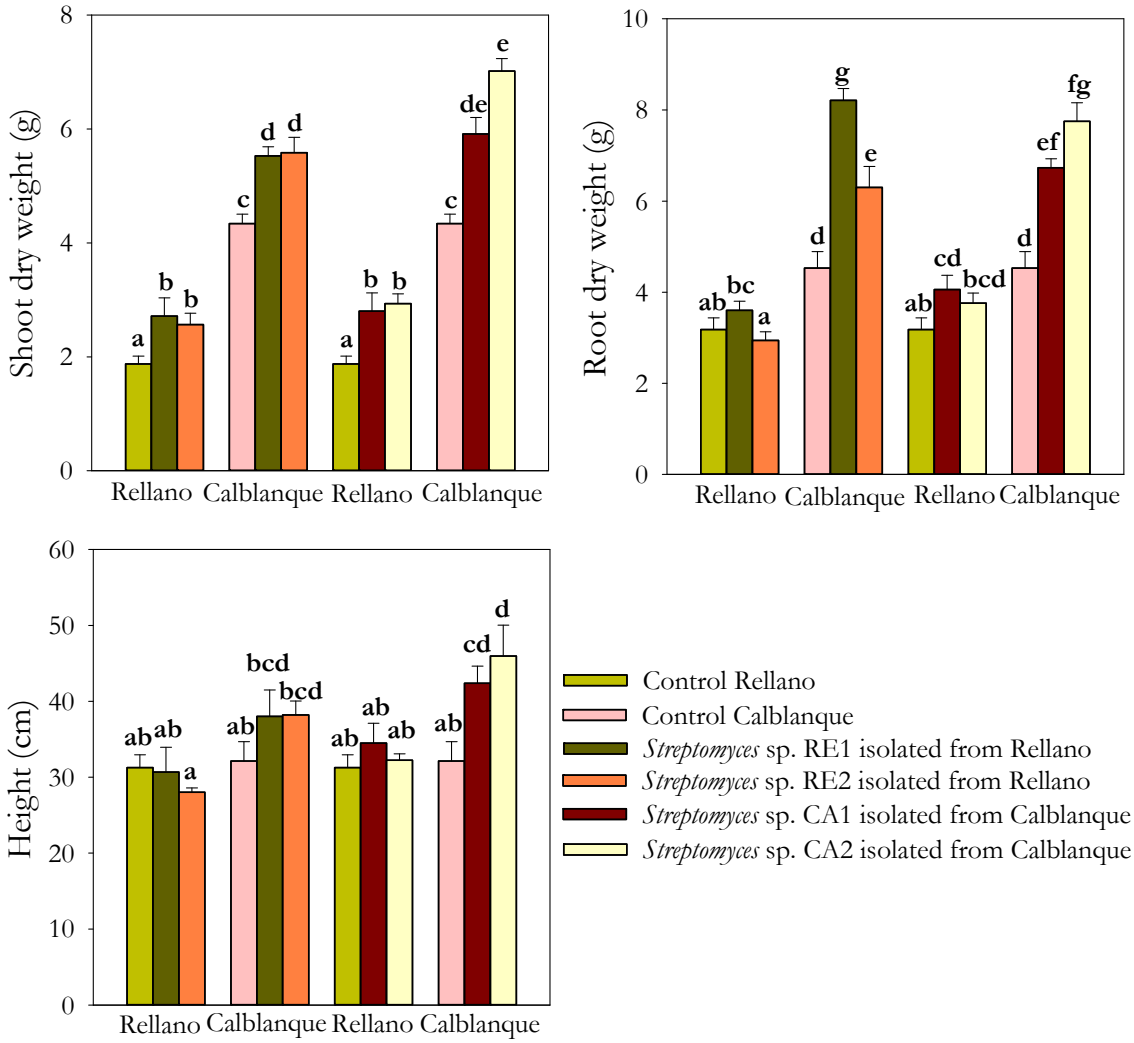


Figure 3. Shoot and root biomass and height of *R. lycioides* seedlings in response to inoculation with immobilised *Streptomyces* sp. strains. Values are means of five replicates. Significant differences according to the Duncan test at $P < 0.05$ levels were indicated by different letters.

3.2. Nutrients uptake

The ANOVA revealed a significant effect of SO and PS on NPK uptake (Table 3). The *Streptomyces*-inoculated plants grown at the Calblanque site possessed higher levels of nutrients than those grown at the Rellano site (Figure 4). As observed for the growth parameters, only the nutrient contents in shoots of *R. lycioides* seedlings grown at Calblanque were influenced by the SO (Figure 4). At

Calblanque the plants inoculated with native strains had higher NPK contents than those inoculated with allochthonous strains.

Table 3. Two-way ANOVA *P* values. Significance of effects of strain origin (SO), planting site (PS) and their interaction on nutrients uptake. NS: Not significance.

Factors and interaction	N	P	K
SO	<0.001	<0.001	<0.001
PS	<0.001	<0.001	<0.001
SO x PS	NS	NS	NS

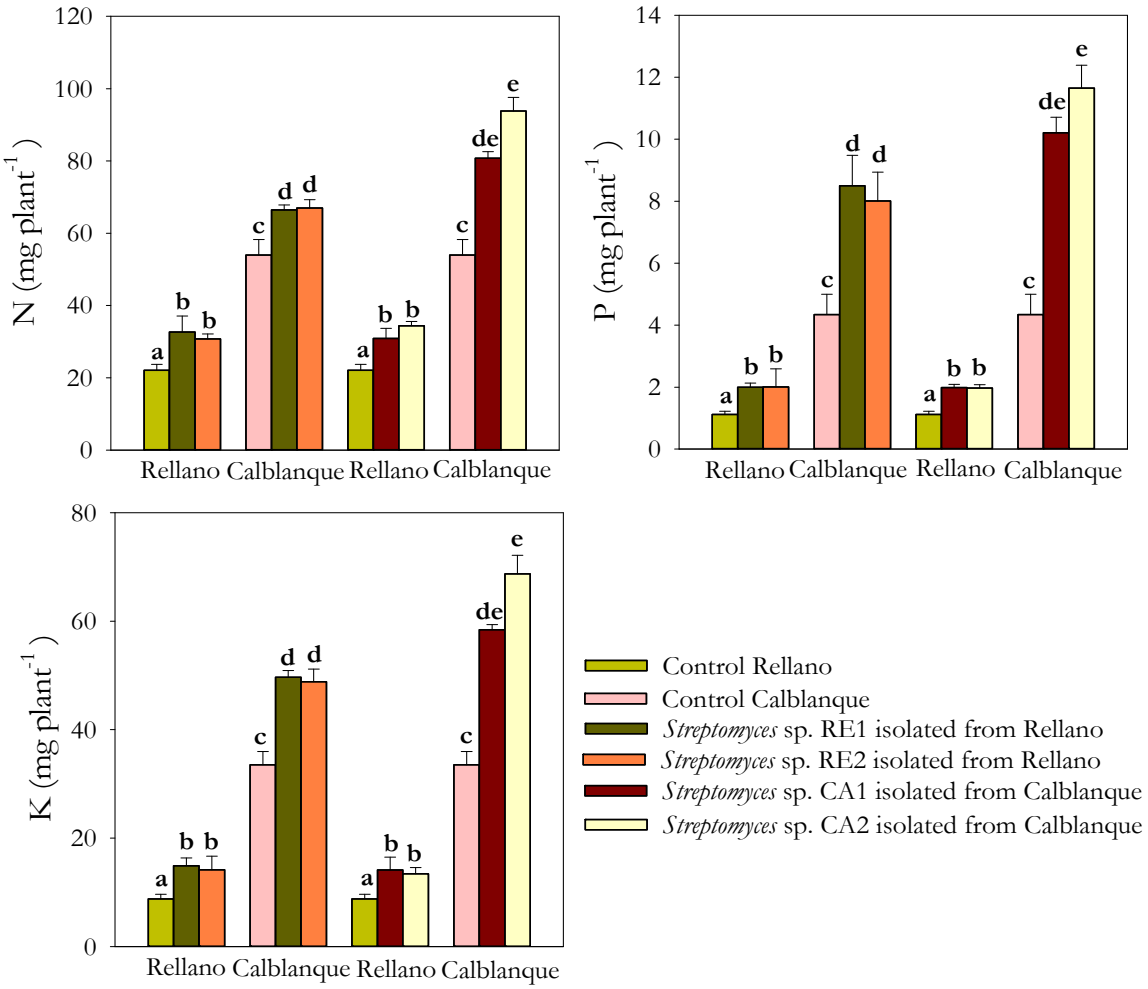


Figure 4. Nutrients content in shoot of *R. lycioides*. Values are means of five replicates. Significant differences according to the Duncan test at P < 0.05 levels were indicated by different letters.

3.3. Plant stress parameters

The PS significantly affected the nitrate reductase activity, it being higher in the shoots of *Streptomyces*-inoculated plants grown at the Rellano site than in those at the Calblanque site while a significant effect of SO and PS on the oxidative damage to lipids was observed (Table 4).

Table 4. Two-way ANOVA *P* values. Significance of effects of strain origin (SO), planting site (PS) and their interaction on plant stress parameters. NS: Not significance.

Factors and interaction	Nitrate reductase	Oxidative damage
SO	NS	0.030
PS	<0.001	<0.001
SO x PS	NS	0.006

The post-hoc test showed that the actinobacteria enhanced the nitrate reductase activity, without significant differences between native and allochthonous isolates at both planting sites (Figure 5).

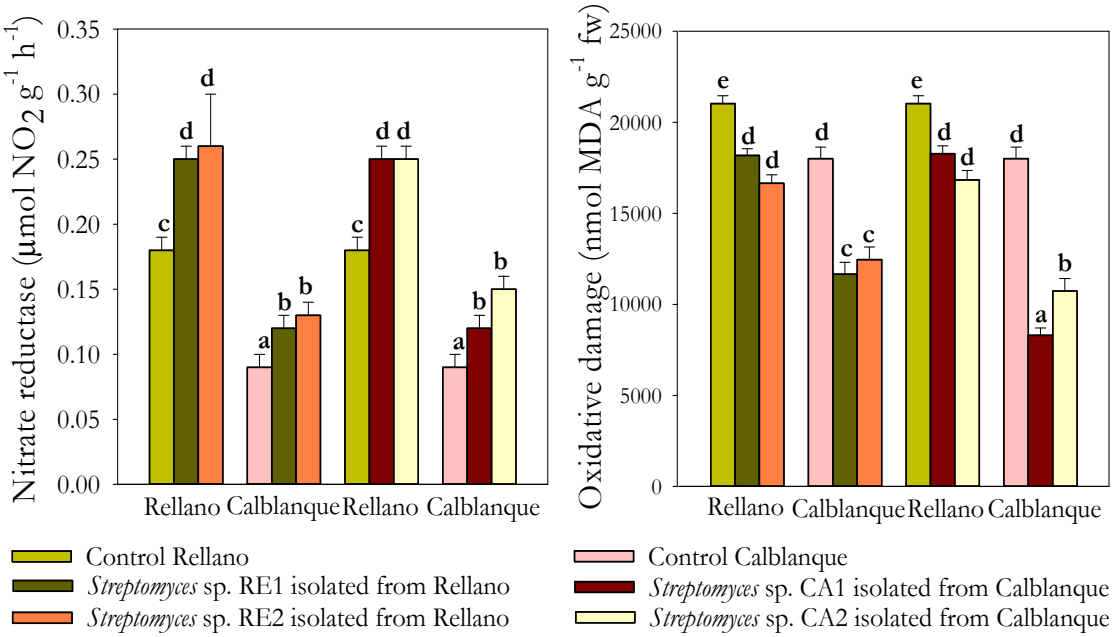


Figure 5. Nitrate reductase activity and oxidative damage to lipids after the inoculation with immobilised *Streptomyces* sp. strains. Values are means of five replicates. Significant differences according to the Duncan test at $P < 0.05$ levels were indicated by different letters.

Oxidative damage was decreased significantly by the inoculations with actinobacteria, the greatest decrease being observed in the plants inoculated with native strains (CA1 and CA2) at the Calblanque site (50%, compared to the corresponding control plants). The native and allochthonous strains provoked similar decreases in the levels of lipid peroxidation in plants grown at Rellano (Figure 5).

Soil physico-chemical, chemical and biological analyses

Whit regard to carbon fractions, the SO, PS, and SO x PS interaction had significant effects on water soluble carbon (WSC), water soluble carbohydrates (WSCH) and microbial biomass while only PS significantly affected soil respiration (Table 5).

Table 5. Two-way ANOVA *P* values. Significance of effects of strain origin (SO), planting site (PS) and their interaction on carbon fraction. NS: Not significance.

Factors and interaction	WSC	WSCH	Soil respiration	Microbial biomass
SO	<0.021	0.008	NS	0.049
PS	<0.001	<0.001	<0.001	<0.001
SO x PS	<0.001	<0.001	NS	0.012

The highest contents of WSC and WSCH were recorded at Calblanque. The levels of WSC were increased by the inoculation with native and allochthonous strains, although the highest increases were recorded at Calblanque with native *Streptomyces* sp. strains (about 48%, compared to the corresponding control) (Figure 6). However, the native and allochthonous strains produced similar increases in WSC at Rellano. In both soils, the inoculation of *Streptomyces* sp. strains native to each PS was effective regarding increases in WSCH (3-fold at Rellano and 77% at Calblanque) (Figure 6). The Duncan test indicated that the highest increase in microbial biomass C in response to the inoculations with actinobacteria was reached at the Rellano site (about 30% compared with control) (Figure 6). The native and allochthonous strains produced similar increases in microbial biomass C at

Calblanque, while the allochthonous strains provoked a higher increase in such parameters at Rellano (Figure 6).

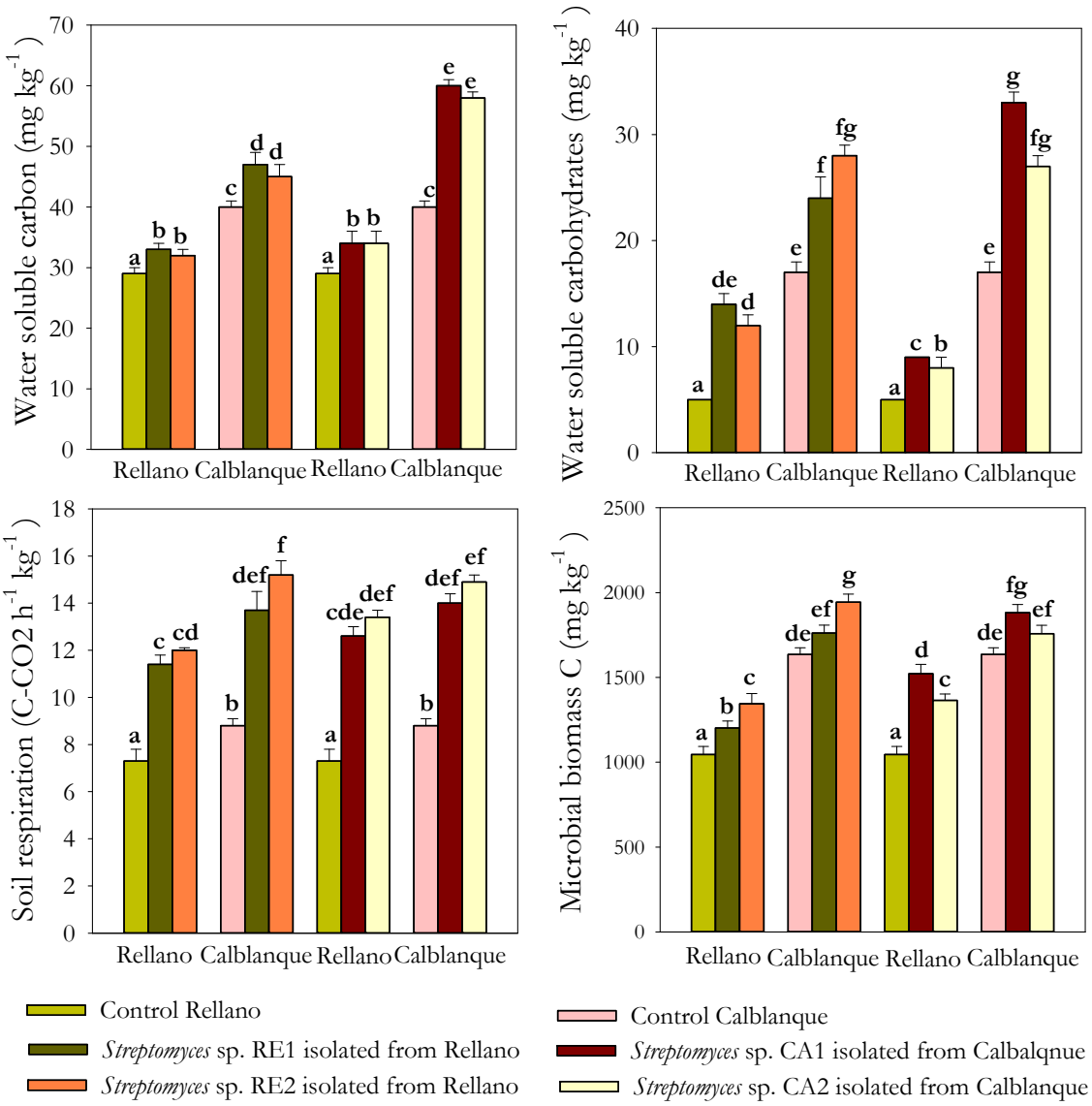


Figure 6. Carbon fraction values in response to inoculation with immobilised *Streptomyces* sp. strains. (n=5). Significant differences according to the Duncan test at $P < 0.05$ levels were indicated by different letters.

Total N and extractable K were only influenced by the PS. Neither SO nor PS affected available P. The highest contents of total N were recorded at Calblanque (Table 6).

Table 6. Soil chemical properties in response to inoculation with immobilised *Streptomyces* sp. strains.

Isolation site	Strain	Tested soil	Total N (g kg^{-1})	Extractable K (mg kg^{-1})	Available P (mg kg^{-1})
Rellano	0	Rellano	1.2 ± 0.0 a	179 ± 4 c	5 ± 1 a
	RE1		1.2 ± 0.1 a	185 ± 5 c	5 ± 0 a
	RE2		1.1 ± 0.0 a	187 ± 2 c	5 ± 0 a
	0	Calblanque	2.0 ± 0.2 b	117 ± 2 ab	6 ± 0 a
	RE1		1.9 ± 0.1 b	122 ± 5 b	6 ± 0 a
	RE2		1.8 ± 0.1 b	107 ± 3 a	5 ± 0 a
Calblanque	0	Rellano	1.2 ± 0.0 a	179 ± 4 c	5 ± 1 a
	CA1		1.2 ± 0.1 a	181 ± 3 c	5 ± 1 a
	CA2		1.3 ± 0.1 a	187 ± 4 c	5 ± 0 a
	0	Calblanque	2.0 ± 0.2 b	117 ± 2 ab	6 ± 0 a
	CA1		1.8 ± 0.1 b	117 ± 3 a	5 ± 0 a
	CA2		1.9 ± 0.1 b	111 ± 6 a	5 ± 0 a
Anova, *P* values					
Strain Origin (SO)			NS	NS	NS
Planting Soil (PS)			<0.001	<0.001	NS
SO x PS			NS	NS	NS

Mean ± standard error (n=5). Significant differences according to the Duncan's test at $P < 0.05$ levels were indicated by different letters. Significance of effects of strain origin, soil type and their interaction on the measured variables is also shown. NS: not significance.

Except for urease activity, the type of inoculated soil affected significantly all enzymatic activities, the highest levels occurring at the Calblanque site (Table 7). The SO had a significant effect on all of them except protease and dehydrogenase activities (Table 7). The Duncan's test indicated that the highest increases in the urease, protease, and dehydrogenase activities in response to the inoculations with actinobacteria were reached at the Rellano site (about 200%, 28%, 29%, respectively, compared to their respective controls), regardless of the origin of the assayed strain (Table 7). The native and allochthonous strains produced similar increases in protease activity at Calblanque, while the allochthonous strains provoked a higher increase in such parameters at Rellano. The inoculation with

native strains at Calblanque increased the urease and β-glucosidase activities to a greater extent than the inoculation with allochthonous strains. At Rellano, the actinobacteria increased the values of both of these biological parameters, compared to non-inoculated soil, although without significant differences between the native and allochthonous strains (Table 7).

Table 7. Changes in enzymatic activities in rhizosphere soil of *R. lycioides*.

Isolation site	Strain	Tested soil	Phosphatase (µmol PNF g^{-1} h^{-1})	Urease (µmol NH_4^+ g^{-1} h^{-1})	Protease (µmol NH_4^+ g^{-1} h^{-1})	β-gulcosidase (µmol PNF g^{-1} h^{-1})	Dehydrogenase (µg INTF g^{-1})
Rellano	0	Rellano	13.9 ± 0.6 a	0.17 ± 0.01 a	0.42 ± 0.01 a	4.6 ± 0.1 a	50.6 ± 1.7 a
	RE1		21.9 ± 1.0 c	0.51 ± 0.01 c	0.57 ± 0.05 b	4.8 ± 0.1 a	69.8 ± 1.2 d
	RE2		18.6 ± 0.7 b	0.50 ± 0.01 c	0.53 ± 0.01 b	4.8 ± 0.0 a	63.5 ± 1.4 bc
	0	Calblanque	26.6 ± 0.9 d	0.27 ± 0.02 b	3.09 ± 0.06 d	8.7 ± 0.3 c	154.8 ± 1.4 e
	RE1		39.5 ± 0.6 f	0.49 ± 0.04 c	3.05 ± 0.02 d	8.6± 0.2 c	182.0 ± 3.1 g
	RE2		37.8 ± 0.9 f	0.47 ± 0.02 c	3.00 ± 0.03 d	8.7 ± 0.4 c	173.3 ± 1.2 fg
Calblanque	0	Rellano	13.9 ± 0.6 a	0.17 ± 0.01a	0.42 ± 0.01 a	4.6 ± 0.1 a	50.6 ± 1.7 a
	CA1		26.1 ± 0.5 d	0.47 ± 0.03 c	0.51 ±0.03 b	4.8 ± 0.3 a	61.3 ± 2.1 b
	CA2		28.3 ± 0.6 d	0.55 ± 0.02 cd	0.54 ±0.03 b	5.5 ± 0.3 b	65.9 ± 1.3 cd
	0	Calblanque	26.6 ± 0.9 d	0.27 ± 0.02 b	3.09 ±0.06 d	8.75 ± 0.3 d	154.8 ± 1.4 e
	CA1		39.6 ± 1.2 f	0.61 ± 0.02 d	3.22 ± 0.07 d	11.2 ± 0.1 d	177.1 ± 2.4 fg
	CA2		36.6 ± 0.6 e	0.54 ± 0.02 cd	3.10 ± 0.07 d	10.0 ± 0.1 c	166.1 ± 2.8 f
Anova, *P* values							
Strain Origin (SO)			<0.001	0.004	NS	<0.001	NS
Planting Soil (PS)			<0.001	NS	<0.001	<0.001	<0.001
SO x PS			<0.001	NS	NS	0.045	NS

4. Discussion

The results of this study have revealed that inoculation with *Streptomyces* strains can be an effective tool for the revegetation of natural semiarid lands; this is a relevant result, bearing in mind that the PGPR character of actinobacteria had only been demonstrated previously in agricultural soils (Jog et al., 2014). We also found a number of different effects attributable to the experimental factors which deserve further explanation. The strains of actinobacteria differed in their ability to enhance nutrient uptake and growth of the *R. lycioides* seedlings, depending on the strain origin and planting site. The importance of selecting suitable plant growth promoting microorganisms for successful biotechnological application in the field has been highlighted (Hrynkiewicz and Baum, 2011). The use of native plant growth promoting microorganisms has been reported to be more effective (Ouahmane et al., 2007) or equally effective (Ortiz et al., 2014), in comparison to allochthonous strains. In our study, the efficacy of the actinobacterial strains native to a particular soil - as promoters of plant growth - was reliant on the fertility characteristics of the plantation site. Previous studies have shown the importance of soil characteristics such as organic matter content in shaping the re-establishment of local microorganisms (Pereira e Silva et al., 2011), even when the microorganisms originate from different soil sources (Nazir et al., 2013). In our study, the soils used for the revegetation experiment presented different levels of organic matter and microbiological activity. In the more fertile soil (Calblanque), the inoculation with native strains of *Streptomyces* sp. conferred a clear advantage, over inoculation with allochthonous strains, on *Rhamnus* growth. This result was expected as native strains of *Streptomyces* are presumably pre-adapted to the local conditions of the planting site and, probably, are more competitive colonisers of their original soil than allochthonous strains. Actinobacteria are ubiquitous inhabitants of soils, but they could show specificity with respect to the soil subjected to inoculation. Remarkably, in the less fertile soil (Rellano), *Rhamnus* shrubs inoculated with native strains had biomass yields comparable to those of shrubs inoculated with allochthonous strains.

It is worth noting that the allochthonous strains, originating from a more fertile soil, were able to establish and stimulate the growth of plants grown in a less fertile soil.

The characterisation in vitro of the plant growth promotion abilities of the four strains revealed that they had similar effects with regard to solubilising sparingly available inorganic P sources, producing siderophores, and fixing nitrogen. The ability of actinobacteria to synthesize siderophores might be especially important for the competitive abilities of rhizosphere microorganisms in soils with low nutrient concentrations (Franco-Correa et al., 2010). The increased nutrient uptake by both native- and allochthonous-inoculated plants, compared to their non-inoculated controls, indicates that the abilities of the actinobacteria manifested in vitro are preserved under field conditions. In the more fertile soil, the greater improvement of shoot biomass in *R. lycioides* plants inoculated with the native *Streptomyces* strains than in the ones inoculated with non-native *Streptomyces* strains may have been partly due to differential enhancement of nutrients uptake.

Since the revegetation experiment was carried out in semiarid conditions, where water is by far the resource most limiting to plant growth, the increased shoot biomass of inoculated seedlings could also be related to the increase in the resistance of plants to water stress induced by the actinobacterial strains. Nitrate reductase (NR) activity, which catalyses the rate-limiting step in the nitrate assimilation pathway, has been proposed as a stress index since it is highly sensitive to the metabolic and physiological status of the plant (Ruíz-Lozano and Azcón, 1996). In this study, we have found that the inoculation with actinobacteria induced an increase in NR activity, regardless of strain origin. Improvements in plant drought tolerance induced by rhizobacteria other than actinobacteria have been previously recorded (de-Bashan et al., 2012; Mengual et al., 2014a). The oxidation of membrane lipids is a reliable indication of oxidative stress (Porcel et al., 2004). In the shoot, lipid peroxidation was decreased in the *Streptomyces*-inoculated plants, compared to non-inoculated plants, which could be explained partially if the former were submitted to less oxidative stress under field conditions. In the more fertile soil, the plants inoculated with the native strains displayed oxidative stress to a lesser

extent than the plants inoculated with the allochthonous strains. This could also have contributed to the superior performance of plants inoculated with native *Streptomyces* under semiarid field conditions.

The effect of inoculants on microbial activity in the rhizosphere is decisive for maximising plant nutrient availability, since the soil microbial community in the rhizosphere plays a key role in plant nutrition and thus in plant growth. A direct measurement of the reactivation of microbial populations is the C-biomass. Also, certain C fractions, namely water soluble C and water soluble carbohydrates, are used as carbon and energy sources by soil-borne microflora (Ghani et al., 2003; Roldán et al., 2006). In this study, these fractions of C were enhanced to a greater extent by the inoculation with native *Streptomyces* strains, at both planting sites. Enzyme activities are sufficiently sensitive to indicate changes in ecosystem function resulting from microbial inoculations (Naseby and Lynch, 1997). Oxidoreductases, such as dehydrogenase, are involved in oxidative processes in soils and their activity mainly depends on the metabolic state of soil biota; thus, they are considered as good indicators of the soil microbial activity (García et al., 1997). The increase in microbial activity was also reflected by the increase in dehydrogenase activity in the rhizosphere soil of inoculated plants. The measurement of hydrolases provides an early indication of changes in soil fertility, since they are related to the mineralisation of important nutrient elements required for both plant and microbial growth (Alguacil et al., 2005). The increases observed in the hydrolases phosphatase, urease, protease-BAA, and β-glucosidase may be related to shifts in the rhizosphere microbial population, as a consequence of the inoculation treatments with actinobacteria (Conn and Franco, 2004; Trabelsi et al., 2011). It is worth noting that the greatest improvement in microbial activity in response to the inoculation with *Streptomyces* strains was recorded in the less fertile soil. The stimulation of microbial activity by the native and allochthonous isolates varied with both the planting soil and biochemical parameter. Secretion of phosphatases by phosphate solubilising bacteria is a common way of facilitating the conversion of insoluble forms of P to plant-available forms, thus enhancing plant P uptake and growth (Kim et al., 1998).

The highest increases in phosphatase activity were recorded in the rhizosphere soil of plants inoculated with allochthonous *Streptomyces* sp. strains and grown in the less fertile soil, which may indicate direct bacterial secretion of this enzyme.

5. Conclusions

The actinobacterial strains were able to promote the establishment of *R. lycioides* in soils different from that of their isolation source, indicating that their plant growth promoting abilities are preserved under different field conditions. The efficacy of native strains, with respect to allochthonous strains, was conditioned by the characteristics of the soil subjected to revegetation. For the more fertile soil, the high growth rate of shrubs inoculated with native *Streptomyces* was attributable mostly to a direct nutritional enhancement mediated by the inoculum, as well as to a concomitant improvement in plant drought tolerance. In the less fertile soil, the superior increases in soil microbial functionality suggest that the proliferation of introduced and/or native microflora could also have contributed to the improvement in plant growth, but the character (native or non-native) of the strains was not a key factor for plant establishment. The strain origin and biological fertility of the plantation site should be considered in the selection of actinobacterial strains for use in the revegetation with shrub species in semiarid environments.

Chapter IX

Conclusions

1. The inoculation of plant seedlings with rhizobacteria strains was a positive and effective tool in the field assays. They benefited the establishment and growth of the seedlings and enhanced its tolerance to both water deficit and high temperatures. However, depending of the plant type used to develop the revegetation task, the selection of the microbial inoculum was crucial since, in some cases, the reinforcement with the application of an organic residue was decisive to improve soil quality parameters.

2. The microbial inoculation of *Cistus albidus* with *Azospirillum brasilense* and *Pantoea dispersa* immobilised in clay pellets improved the performance of *C. albidus*, mainly when the microbial inoculation was combined with the addition of organic olive residue. The combination of the rhizobacteria strains with the olive residue was the most effective treatment to enhance soil properties, yielding a significant increase in C fractions, microbial biomass and enzyme activities, but the microbial inoculation and the addition of the organic amendment separately failed to do that.

3. The microbial inoculation of *P. halepensis* seedlings with the mixture of the strains *A. brasilense* and *P. dispersa* immobilised in clay pellests favoured the plant growth and improved the plant responses to hydric stress. It was indicated by the lower proline accumulation and lesser oxidative damage to lipids, linked to a higher water potential in the plants inoculated with these rhizobacteria.

4. The capacity of the microbial inoculant to increase plant drought tolerance may have been related to nutrient uptake improvement and an increase in N assimilation through nitrate reductase activity. Altough, both microbial inoculation and the organic amendment were effective respect to enhance soil quality properties, the promotion of plant growth by the microbial inoculant was greatest when applied independently, and the lower cost of implementation of this restoration biotechnology supports its preferential use in re-afforestation tasks with *P. halepensis* in semiarid environments.

5. The microbial inoculation of *Lavandula dentata* with *Bacillus megaterium*, *Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp. strains clearly improved plant performance in the revegetation of a degraded semiarid soil. The combined effect of the sugar beet residue addition did not show any synergistic effect but merely additive in some cases, being capable, even, of diminishing the positive effects of some strains. The greatest improvement of quality parameters was mediated by the addition of sugar beet residue and *Enterobacter* sp. combined with sugar beet residue.

6. Actinobacteria strains were successfully isolated from *Rhamnus lycioides* rhizosphere soil. The four *Streptomyces* strains selected showed, in vitro, the abilities that could describe them as potential PGPR.

7. In the field assay, the actinobacteria strains favoured the establishment of *Rhamnus lycioides* in the two different Mediterranean areas selected for the experiment, what demonstrated that the abilities showed under laboratory conditions were preserved under field conditions. When considering soils with different characteristcs subjected to reclamation tasks in semiarid enviromnets, it is quitre relevant to keep the strain origin and the biological fertility of plantation site in mind for an adequate selection of actinobacteria strains.

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