Caracterización de los procesos de adaptación de hongos formadores de micorrizas a ambientes afectados por la sequía y salinidad y su implicación en la mejora de la tolerancia de las plantas a tales ambientes

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Characterization of the adaptation of mycorrhizal fungi to environmnets affected by drought and salinity and their implications in the improvement of plant tolerance to such environments

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A mis padres

y hemano

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NATURALEZA, INTERÉS Y OBJETIVOS

La importancia y singularidad ecológica de los ecosistemas mediterráneos adquieren especial relevancia en el territorio español. La conjunción de influencias paleoárticas y paleotropicales han otorgado a la Cuenca Mediterránea una excepcional riqueza vegetal, entre otras, con un elevado nivel de biodiversidad y un número considerable de endemismos. Sin embargo aunque estos ecosistemas sean relativamente estables en ausencia de perturbaciones, poseen una gran fragilidad y baja resiliencia, lo que se traduce en una limitada capacidad de responder a las alteraciones del sistema.

Por sus características geomorfológicas y climáticas, así como por la intervención humana, que en zonas concretas y en periodos históricos definidos ha sido decisiva, los procesos de degradación del suelo y de la cubierta vegetal en los ecosistemas en el área mediterránea adquieren unas connotaciones especiales. Estos son los principales obstáculos para la supervivencia y reproducción de las especies vegetales que se encuentran en zonas áridas y semiáridas. España es el país europeo con más extensión de zonas con riesgo de desertificación ocupando las zonas áridas y semi-áridas casi la mitad del territorio nacional.

El estrés se debe fundamentalmente a condicionantes climáticos (marcada estacionalidad y coincidencia de estación cálida con estación seca) que produce patrones de lluvia altamente impredecibles que pueden derivar en una limitación hídrica, suelos pobres en nutrientes (nitrógeno y fósforo) con alta variación espacial y temporal y suelos con alta salinidad. El problema de la salinidad se acentúa porque las escasas lluvias anuales son insuficientes para transportar las sales solubles fuera de la zona radical de las plantas. A su vez, los altos niveles de evaporación y radiación solar, característicos de estas regiones, contribuyen a incrementar más la salinidad de los suelos.

Las plantas han tenido que desarrollar diversos mecanismos fisiológicos y moleculares para tolerar este tipo de estrés osmótico, como es la salinidad y garantizar el éxito de su adaptación en este ambiente. El conocimiento de tales mecanismos es determinante para poder explotar su potencial uso en programas de restauración y de agricultura asistida.

Una de las estrategias que han desarrollado las plantas para tolerar el estrés salino se basa en su asociación con microorganismos rizosféricos (hongos y bacterias). En particular, un componente clave de la microbiota del suelo, son los hongos formadores de micorrizas arbusculares (MA), que se postulan como uno de los factores más influyentes en el mantenimiento de la variabilidad, estabilidad, diversidad y

productividad de la cubierta vegetal. La raíces MA exploran mayores volúmenes de suelo, a mayores profundidades y distancias de lo que lo hacen las raíces de las plantas no micorrizadas, para suministrar agua y nutrientes a sus asociados vegetales. Sin duda, los ecosistemas mediterráneos y las peculiares especies de plantas que en ellos se desarrollan, ofrecen un modelo de interés prioritario para desarrollar investigaciones y estudiar el impacto de las asociaciones micorrícicas en el mantenimiento y desarrollo de dichos ecosistemas.

Como es bien conocido, las micorrizas, simbiosis hongo-raíz presente en todos los biomas y ecosistemas terrestres desde hace más de 400 millones de años, son fundamentales para que las plantas adquieran nutrientes minerales y agua del suelo, particularmente en condiciones limitantes, así como una mayor resistencia a los estreses ambientales. Se ha demostrado que la mayoría de las plantas dependen de estar micorrizadas para establecerse y prosperar. Más del 80% de las especies de plantas existentes en el Planeta forman simbiosis con micorrizas arbusculares, entre ellas las propias de ecosistemas mediterráneos. El establecimiento de la micorriza no sólo resulta ventajoso para la planta y el hongo, sino que también favorece al ecosistema en su conjunto, ya que mejora la calidad del suelo así como el desarrollo, la diversidad y la productividad de la cubierta vegetal.

La vegetación de los ecosistemas áridos y semiáridos, además de soportar condiciones adversas como largos periodos de sequía, intensas temperaturas y evaporación, a veces se asienta sobre suelos con alto contenido de sales, arenosos con alto grado de erosión, y con bajos niveles de nutrientes y de agua, entre los factores principales; lleva a pensar que las MA son un factor que permite a las plantas resistir estas condiciones adversas. Concretamente, la salinidad es uno de los principales factores abióticos que limitan la producción vegetal en muchas áreas del mundo que afecta tanto a plantas como a los microorganismos de la rizosfera, que a su vez, desarrollan mecanismos de adaptación a dicho estrés. Actualmente hay un creciente interés por conocer los mecanismos de tolerancia de las plantas frente al estrés salino y la implicación de los hongos MA a tal tolerancia.

Por todo lo anterior expuesto, el estudio de las MA de ecosistemas áridos y semi-áridos y salinizados es fundamental porque albergan importantes fuentes de inóculos de hongos MA, adaptados a esas condiciones. Los hongos MA aislados en los ecosistemas mediterráneos afectados por problemas de salinidad pueden ser utilizados como inóculo de plantas para lograr su establecimiento en condiciones naturales de estrés osmótico, siendo especialmente útiles en prácticas de restauración ambiental de ecosistemas degradados o en proceso de desertificación.

En el contexto de los aspectos conceptuales anteriormente expuestos se diseñó esta Tesis Doctoral, la cual se integra en un proyecto de investigación encuadrado en el Programa de Proyectos de Excelencia en I+D+i del PIDI (Junta de Andalucía). Aunque la estrategia de I+D+i que se propone es extrapolable a cualquier ecosistema afectado por sequía/salinidad, la propuesta se centra en un escenario de estudio definido, científicamente representativo y socioculturalmente emblemático de Andalucía, como es el Parque Natural de Cabo de Gata, Almería, donde son características sus largas cintas arenosas, salinas, dunas y aridez, con una vegetación caracterizada por su adaptación al territorio así como los hongos MA. Por todo ello, el objetivo general de la Tesis fue el *Investigar la diversidad natural de hongos MA en el ecosistema, así como los mecanismos (fisiológicos y moleculares) que rigen los procesos de adaptación de dichos hongos a la salinidad y su percusión en la tolerancia de las plantas (supervivencia, desarrollo y productividad) bajo condiciones de salinidad.*
La consecución de este objetivo general implicó el planteamiento de los siguientes objetivos específicos:

1. Analizar la diversidad de hongos MA presentes en la rizosfera de las plantas de dunas y saladares del Parque y establecer un banco de germoplasma de hongos adaptados al ecosistema.

2. Estudiar los mecanismos de adaptación/tolerancia de los hongos MA a la salinidad.

3. Determinar los mecanismos fisiológicos por lo que los hongos MA nativos del Parque incrementan la tolerancia al estrés salino de plantas glicófitas de interés agronómico y halófitas de interés en revegetación.

Aunque los objetivos propuestos pretenden avanzar en el conocimiento de la ecología y funcionamiento fisiológico en la relación hongo-planta de los hongos micorrícicos en ecosistemas semiáridos característicos de ambientes mediterráneos, la investigación propuesta presenta también un marcado carácter finalista. Este viene determinado por la posibilidad de evaluar el efecto de la micorrización dirigida sobre el desarrollo de plantas tanto para restauración de áreas degradadas como para mejora agrícola en áreas afectadas por problemas de salinidad.

INTEREST OF THE STUDY AND AIMS

The ecological importance and singularity of Mediterranean ecosystems are particularly relevant in the Spanish territory. The combination of paleotropicals and paleoartics influences has given the Mediterranean area exceptionally rich vegetation, among others, with a high level of biodiversity and a significant number of endemic species. However, although these ecosystems are relatively stable in the absence of disturbances, they have high resilience and low fragility, resulting in a limited ability to respond to disturbances in the system

Due to geomorphological and climatic characteristics, as well as human intervention in specific areas, land degradation and vegetation cover in the Mediterranean ecosystems acquires special connotations. These are the main obstacles for the survival and reproduction of plant species found in arid and semiarid areas. Spain is the European country with the greatest extent of areas at risk of desertification where the arid and semi-arid areas occupy nearly half of the national territory.

The stress is mainly due to climatic conditions (seasonality with hot and dry season together) that produces highly unpredictable rainfall patterns that can lead to a hydric limitation, soils poor in nutrients (nitrogen and phosphorus) and soils with high salinity. The salinity problem is accentuated by the limited annual rainfall which is insufficient to transport the soluble salts out of the rhizosphere of the plants. Moreover, the characteristics of these regions: high levels of evaporation and solar radiation, contribute to further increase the salinity of soils.

Plants have developed various physiological and molecular mechanisms to tolerate this type of osmotic stress, such as salinity to ensure the success of adaptation in this environment. The knowledge of such mechanisms is crucial in order to exploit their potential use in restoration programs and assisted agriculture.

One strategy that plants have developed to tolerate salt stress is based on its association with rhizospheric microorganisms (fungi and bacteria). In particular, a key component of soil microbiota, the arbuscular mycorrhizal fungi (AMF), are postulated as one of the most influential factors in the maintenance of variability, stability, diversity and productivity of the vegetation cover. The AM roots explore greater volumes of soil at greater depths and distances to supply water and nutrients to the host plant than roots of non-mycorrhizal plants. In fact, Mediterranean ecosystems together with their particular plant species, offer a model of interest to conduct research projects to study the impact of mycorrhizal associations in the maintenance and development of these ecosystems.

As is well known, the mycorrhizal fungus-root symbiosis present in all biomes and terrestrial ecosystems for over 400 million years, and they are essential for plants to acquire mineral nutrients and water from the soil (particularly under limited conditions) and greater resistance to environmental stresses. It has been demonstrated that most plants depend on mycorrhizal symbiosis to establish and develop. Over 80% of the plant species on Earth form symbiosis with arbuscular mycorrhiza, including those characteristic of Mediterranean ecosystems. The mycorrhizal establishment not only benefits the plant and the fungus, but also the whole ecosystem, improving soil quality and development and the diversity and productivity of the vegetation cover.

Vegetation of arid and semiarid ecosystems, as well as withstand adverse conditions (long periods of drought, intense temperatures and evaporation), sometimes grow on soils with high salt content, or sandy with high erosion, or low nutrient and water levels, among the main factors, suggesting that the AM symbiosis is a key factor that allows plants to cope with these adverse conditions. Specially, salinity is a major abiotic factor limiting plant production in many areas of the world, affecting both plants and microorganisms in the rhizosphere, which in turn, develop adaptation mechanisms to such stress. Nowadays there is an increasing interest in understanding the mechanisms of plant tolerance to salt stress and the involvement of AMF in such tolerance.

For all the above reasons, the study of the AMF of arid and semi-arid salinized ecosystems is critical because they are important potential sources of AMF inocula adapted to these conditions. The AMF isolated in Mediterranean ecosystems affected by salinity might be used as inoculum to achieve plant establishment under natural conditions of osmotic stress, being especially useful in environmental restoration practices of degraded ecosystems or under desertification process. The present Doctoral Thesis was outlined in the context of the conceptual topics above mentioned and it is part of a research project conducted within the Excellence Project Program in I+D+i of PIDI (Junta de Andalucía). Although the strategy of I+D+i proposed can be extrapolated to any ecosystem affected by drought/salinity, the proposal focuses on a defined scientifically representative sampling area and socioculturally emblematic part of Andalucía: the Natural Park Cabo de Gata, Almería, with characteristic long sandy ribbons, salt marshes, dunes and aridity, together with vegetation adapted to the territory and AMF. Therefore, the aim of the thesis was *To investigate the natural diversity of AMF in the ecosystem, and the physiological and molecular mechanisms that govern the processes of adaptation of these fungi to salinity and repercussion on plant tolerance (survival, development and productivity) under salinity conditions.*

To achieve this main objective, the following specific objectives were proposed:

1. To analyze the diversity of AMF present in the rhizosphere of the plants in the dunes and salt marshes of the Park and establish a germplasm bank of AMF adapted to the ecosystem.

2. To study the mechanisms of adaptation/tolerance of AMF to salinity

3. To determine the physiological mechanisms by which native AMF from the Park increase salt stress tolerance of glicophyte plants of agronomic interest and halophyte plants of revegetation interest.

Although the proposed objectives are intended to advance in the knowledge of the ecology and physiological functioning in the relationship fungus-plant of AMF in arid ecosystems typical of Mediterranean environments, the research also presents a strong proposal. The latter is determined by the ability to evaluate the effect of conducted mycorrhization in the development of both plants for restoration of degraded areas and agricultural improvement in areas affected by salinity.

INTRODUCCIÓN

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1. Ecosistemas Mediterráneos: Parque Natural Cabo de Gata

Los ecosistemas mediterráneos incluyen un importante número de comunidades bióticas que se desarrollan bajo la influencia del denominado macrobioclima mediterráneo según la clasificación bioclimática de (Rivas-Martínez, 1981). Esta unidad tipológica de rango superior se puede encontrar en cualquier altitud y valor de continentalidad en todos los territorios extratropicales de la Tierra pertenecientes a las cinturas subtropical y eutemplada, principalmente entre los 30 y 40º de latitud N y S. Característica fundamental para catalogar una zona como "de clima mediterráneo" es que en ellas existen al menos dos meses consecutivos con aridez durante el verano, es decir, en los que el valor en milímetros de la precipitación media del bimestre más cálido del trimestre estival es menor del doble de la temperatura media del bimestre más cálido del trimestre estival expresada en grados centígrados (P <2T). También es una característica del clima mediterráneo la alternancia de la estación cálida y seca con una estación fría y húmeda, con precipitaciones irregulares y esporádicas, pero frecuentemente torrenciales (López-Bermúdez and Albaladejo, 1990). Aunque se le denomine clima mediterráneo, debido a que una de las zonas más representativas con este clima son las regiones circundantes del mar Mediterráneo, hay numerosas zonas que comparten las características de este clima, con mayor representación territorial en el centro y en el occidente de todos los continentes excepto en la Antártida.

La parte árida y semi-árida de la región mediterránea, en torno a dicho Mar corresponde a la zona oriental de la Península Ibérica, las costas septentrionales de África (Argelia, Egipto, Libia, Marruecos y Túnez) y las islas de Creta, Chipre y las Baleares. En estas áreas, las precipitaciones anuales están por debajo de los 400 mm. (Wheeler and Kostbade, 1990). Además del clima, tanto el relieve, como el sustrato litológico, la cubierta vegetal y la intervención humana son factores esenciales que definen las cualidades y funcionamiento de los ecosistemas mediterráneos. Los suelos sobre los que se desarrollan los ecosistemas mediterráneos presentan características funcionales diferentes de los que se encuentran en ecosistemas templados y tropicales. Son suelos más jóvenes y menos profundos que los tropicales, (debido a tasas de meteorización de la roca sensiblemente inferiores) y han estado sujetos a una mayor tasa de erosión que los suelos de ecosistemas templados (debido a unos usos más intensivos y prolongados por parte del hombre, y quizás por la mayor recurrencia de incendios) (Gallardo *et al.*, 2009). Estas diferencias imponen las primeras restricciones a las plantas que se desarrollan sobre estos suelos. La escasa profundidad impone un límite al tamaño de los individuos, mientras que la erosión de los horizontes superficiales limita la cantidad de materia orgánica y los nutrientes que de ella se derivan (Yaalon, 1997).

Los ecosistemas de la región mediterránea son muy variados, y su vegetación climácica pueden ser bosques esclerófilos de hoja perenne, bosquetes espinosos, estepas templadas o incluso semidesiertos helados, todos ellos adaptados a soportar un periodo de aridez de hasta nueve meses. Por ello la vegetación mediterránea desarrolla principalmente dos tipos de alternativas de adaptación xerófila: la presencia de estructuras aéreas persistentes (como es el aso de los matorales) y la que expresa la vegetación de temporada, en el caso de las plantas terófitas, como por ejemplo los pastizales de especies anuales. Hay que destacar además, que la biomasa subterránea respecto de la aérea es más alta que en otros ecosistemas (Montalvo, 1992). Otra característica importante de estos ecosistemas es su excepcional riqueza florística, la cual ha sido determinada por la conjunción de factores bióticos y ambientales, como son las variaciones climáticas y los cambios de las posiciones relativas de las grandes masas continentales (Blanca and Morales, 1991; López-González, 2001). En concreto, en la cuenca mediterránea de las 25.000 plantas fanerógamas identificadas hasta el momento (alrededor del 10 % de todas las plantas conocidas en la Tierra), más de la mitad son endémicas de la región. No es de extrañar que el la cuenca mediterránea se considere uno de los lugares con mayor biodiversidad del mundo, siendo los endemismos un componente integral y fundamental (Thompson *et al.*, 2005).

En las áreas mediterráneas, las lluvias escasas e irregulares, con largos, secos y calurosos veranos, junto con la presión antropogénica y abandono de suelos agrícolas, son factores determinantes de la degradación del ecosistema (Barea *et al.*, 2006). En particular en el sureste de España, esta situación de estrés múltiple está llevando a desertificación de parte del territorio (Francis and Thornes, 1990; Albaladejo *et al.*, 1996). donde aproximadamente un 18% del territorio presenta graves procesos de erosión y desertificación, siendo la cifra en Andalucía del 36%, debido principalmente a las características accidentales del relieve, con fuertes pendientes y desniveles, que acentúan el efecto de las precipitaciones (Cornejo, 2006).

En general, es difícil pensar una revegetación natural espontánea en áreas donde las precipitaciones están por debajo de 350 mm (Barea *et al.*, 2006). La franja costera del Sureste español no es tan sólo la región de aridez más extremada de la Península ibérica, sino también de toda Europa, por lo que el estudio y conservación de zonas de especial interés ecológico, como es el Parque Natural de Cabo de Gata elegido como modelo del presente estudio, son de vital importancia debido a sus características únicas y endemismos.

1.1. Parque Natural Cabo de Gata: interés y problemática.

El Parque Natural de Cabo de Gata-Níjar, a través del Decreto 314/1.987 de 23 de Diciembre, es el primer Parque Natural marítimo-terrestre de Andalucía y el de mayor superficie y relevancia ecológica de todo el Mediterráneo occidental europeo. Su origen volcánico, sus salinas, sus dunas fósiles y la importancia ecológica de su flora y su fauna son algunas de las razones por las que se han protegido 38.000 hectáreas terrestres y 12.000 hectáreas de costa hasta una milla marítima (Figura 1).

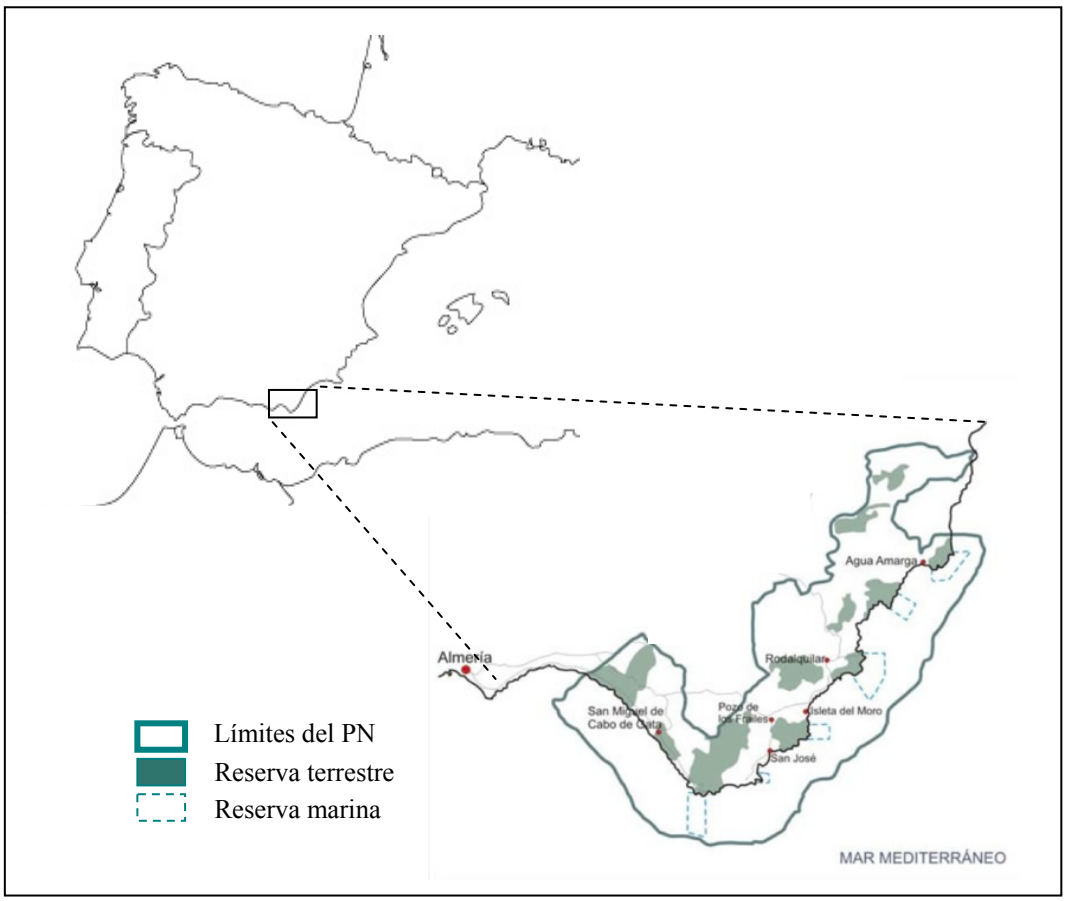

Figura 1. Localización del Parque Natural de Cabo de Gata y sus zonas protegidas

Este enclave es uno de los pocos lugares, en toda Europa, protegido por su carácter semiárido y estepario. Las condiciones climáticas de sequedad de Cabo de Gata son semejantes a las que existen en extensos territorios de África del Norte o de Oriente Medio, lo que identifica este lugar como el enclave más árido de Europa Occidental (Geiger, 1973). A pesar de ello y de su aparente aspecto desértico encierra formas de vida animal y vegetal muy peculiares, que han logrado adaptarse a extremas condiciones de aridez, caracterizada por su elevada insolación media (2960 horas), el índice de precipitaciones mas bajo de la península y la suavidad de su régimen térmico

(temperatura media entre 15 y 22ºC, no soliendo descender nunca de los 12ºC). El reparto anual de precipitaciones queda restringido a una quincena de días ligados a periodos favorables (otoño-invierno), llegando en ocasiones, a recogerse, en un solo día, más del 40% de la media anual (Asensio Grima *et al.*, 2005).

El área incluye diferentes tipos de paisajes asociados a materiales volcánicos y calizas arrecifales, distinguiéndose grandes unidades paisajísticas: Planicie, Valles, Piedemontes, Lomerios y Montañas. La vegetación es muy variada, con una alta proporción de endemismos y una alta diversidad de comunidades vegetales. De las más de 200.000 especies vegetales catalogadas en el ámbito mediterráneo, en el Parque Natural de Cabo de Gata-Nijar aparecen en torno a un millar, cantidad sorprendente para espacio tan limitado en extensión y altura con endemismos locales como el Dragoncillo del Cabo (*Antirrhinum charidemi*), Clavelinas del Cabo (*Dianthus charidemi*), la Zamarrilla del Cabo (*Teucrium charidemi*) y el Azafrán del Cabo (*Androcymbium europaeum*). La vegetación dominante en la zona de montaña está constituida por matorrales de esparto (*Stipa tenacísima*), albaida (*Anthyllis cytisoides*), tomillo (*Thymus vulgaris*) y romero (*Rosmarinus officinalis*) asociados a otras especies como palmito, (*Chamaerops humilis*) única palmera autóctona en el continente europeo y símbolo del Parque, o cornical (*Periploca angustifolia*), con una gran diversidad florística y estructural. En las zonas arenosas propias de la Planicie aparecen otras especies como *Tamarix sp*. y azufaifo (*Ziziphus loti*), siendo las formaciones de esta última las que mayor superficie ocupan en la Península. En las zonas arenosas y saladares abundan además familias de Quenopodiáceas, Leguminosas y Asteráceas, siendo una especie perteneciente a esta última familia, *Asteriscus maritimus*, de alto interés en la conservación del suelo gracias a su extenso sistema radicular (Rodríguez *et al.*, 2005). Los pastizales ocupan amplias superficies, asociadas principalmente a cultivos de secano en zonas de Piedemonte, en la Montaña, o en dunas de la Planicie (Escribano *et al.*, 2008). Respecto a las comunidades de vertebrados terrestres, cabe distinguir entre la sierra y zonas esteparias inmediatas y las salinas. En el primer dominio, según los trabajos previos a la declaración de Parque Natural, existen 54 especies. El valor de su extraordinario biodiversidad ha sido reconocido en 1997 por la U.N.E.S.C.O con la catalogación de este Parque Natural como Reserva de la Biosfera. El mundo marino protegido, tiene fondos rocosos y arenosos; la diversidad de su colonización vegetal alberga más de 1400 elementos vegetales (cabe destacar la importancia ecológica de los campos de *Posidonia oceanica*) y animales reconocidos hasta la fecha (Escribano, 2002).

Los modelos regionales de cambio climático prevén un aumento de la temperatura, una disminución de la precipitación y un mayor número de eventos torrenciales en los ecosistemas áridos (IPCC, 2007). Según esto las condiciones de aridez en el Parque se intensificarán, lo que puede incrementar la erosión y degradación

del suelo provocando una pérdida de cubierta vegetal y microbiota del suelo (Requena *et al.*, 1996). La microbiota del suelo juega un papel fundamental en la regulación de los ecosistemas terrestres, influyendo en la productividad, diversidad y estructura de las comunidades vegetales (van der Heijden *et al.*, 2008). Entre los organismos que habitan en el suelo cabe destacar por su función ecológica los hongos micorrícico arbusculares (ver punto 3). El deterioro de los sistemas suelo-planta, en cuanto que afecta a las relaciones planta-microorganismos, desencadena un círculo vicioso de efectos negativos. Si no hay plantas, se degrada la vida microbiota del suelo y si no hay propágulos, cualquier proceso natural o inducido de revegetación presenta problemas para prosperar adecuadamente (Marulanda, 2006). Por lo tanto para evitar la degradación de este tipo de ecosistemas, que por sus condiciones extremas son más sensibles, es necesario realizar más estudios que desvelen las complejas interacciones que se establecen en ellos (Martínez and Pugnaire, 2009).

2. El estrés salino

La salinización de los suelos es uno de los mayores problemas no sólo a nivel ecológico sino también agronómico. A nivel ecológico es la causante de la pérdida de comunidades naturales de plantas y acelera los procesos de degradación del suelo y desertificación (Alguacil *et al.*, 2011). La salinidad es además el estrés abiótico que afecta más negativamente al crecimiento vegetal y por tanto a la producción agrícola, además de limitar el uso de nuevas áreas potenciales de cultivo. Las plantas pueden clasificarse en base a su tolerancia a la salinidad como halófitas (tolerantes) y glicófitas (sensibles), habiendo grandes diferencias dentro de cada grupo en base al nivel de tolerancia (Greenway and Munns, 1980). Debido a que la mayor parte de los cultivos son glicófitos, el exceso de sales en el suelo ocasiona pérdidas importantísimas en las explotaciones agrícolas (Pitman and Läuchli, 2002). Los factores ambientales están estrechamente relacionados con la distribución de las zonas afectadas por salinidad. Por tanto en los climas áridos y semiáridos, caracterizados por escasez de lluvias, temperaturas extremas y alta velocidad de evaporación (Brito *et al.*, 2011), el principal factor limitante de la fertilidad de los suelos y la productividad de los cultivos lo constituye la salinidad (Evelin *et al.*, 2009). A nivel mundial hay más de 800 millones de hectáreas afectadas por la salinidad, más del 6% del área total de la superficie terrestre (Munns and Tester, 2008). En particular, en la cuenca mediterránea alrededor de 16 millones de hectáreas están afectadas por la salinidad y en la España la cifra asciende hasta 840.000 hectáreas (Serrano, 2009). Además de las causas naturales, la actividad humana, en concreto las malas prácticas de cultivo y el riego, han contribuido al incremento alarmante de sales en el suelo (Mahajan and Tuteja, 2005).

2.1. Efecto de la salinidad en las plantas

La salinidad del suelo tiene un efecto negativo en el establecimiento, crecimiento y desarrollo de las plantas (Evelin *et al.*, 2009). Las plantas que crecen en áreas afectadas por salinidad son sometidas a tres estreses fisiológicos: toxicidad iónica, estrés osmótico y desequilibrio nutricional (Munns and Tester, 2008).

- **Toxicidad iónica:** el exceso de sales conduce a una acumulación excesiva de Na^+ y Cl^- en el citosol de la célula. Aunque el Cl^- es un micronutriente esencial para las plantas implicado en la regulación de importantes funciones celulares como actividades enzimáticas, potencial de membrana, co-factor en la fotosíntesis y gradientes de pH (Marschner, 1995; White and Broadley, 2001), también puede ser tóxico en grandes concentraciones, siendo el anión más abundante en suelos salinos (Xu *et al.*, 2000). Sin embargo, la toxicidad iónica es producida principalmente por el Na^+. El Na^+ compite con el K^+ para unirse a sitios esenciales debido a la similitud en sus propiedades fisicoquímicas aunque no puede sustituir sus funciones metabólicas en el citoplasma (Tester and Davenport, 2003). El K^+ es fundamental para el mantenimiento homeostático en el citosol en plantas afectadas por estrés salino (Zhu, 2003). La alteración que provoca el Na^+ en la captación de K^+ altera la relación K^+/Na^+ de la célula, que es incluso más importante que la concentración absoluta de Na^+ (Demidchik and Maathuis, 2007). El K^+ es un ión esencial para la fotosíntesis, la síntesis de proteínas, activación de multitud de enzimas y juega un papel fundamental en el ajuste osmótico, mantenimiento de la turgencia y en los procesos estomáticos (Maathuis and Amtmann, 1999). Por tanto el exceso de Na^+ afecta a la estructura de enzimas y otras macromoléculas, daña orgánulos celulares, perturba el proceso de fotosíntesis y respiración, inhibe la síntesis de proteínas e induce deficiencias iónicas.

- **Estrés osmótico:** la salinidad del suelo dificulta la extracción de agua por parte de las raíces ya que son expuestas a un bajo potencial osmótico. Esta disminución de la capacidad de las plantas de absorber agua del suelo supone una reducción de la expansión foliar y una pérdida de turgencia, siendo más evidente la reducción del crecimiento en la parte aérea que en las raíces (Munns, 2002). Por tanto las plantas son expuestas a sequía fisiológica y tienen que mantener bajo el potencial osmótico interno ya que a altas concentraciones de Na^+ en el medio extracelular las plantas deben evitar que el agua salga de las raíces al suelo (Ruiz-Lozano *et al.*, 2012). Para mantener el potencial osmótico interno y la actividad citosólica el agua ha de pasar de la vacuola al citosol. Esto conlleva una reducción en el turgor, en la expansión celular, en la velocidad de división celular y afecta al desarrollo reproductivo (Munns and Tester, 2008).

- **Desequilibrio nutricional:** en suelos con exceso de sales el incremento de los contenidos celulares de Na^+ y Cl^- interfiere en la disponibilidad, captación, transporte y distribución no sólo de agua sino de otros nutrientes minerales necesarios para la planta como son, entre otros, K^+, Ca^{2+} y NO^{3-} (Marschner, 1995; Tuteja, 2007). El Na^+ afecta directamente en la toma de otros nutrientes debido a la interferencia que produce en la actividad de transporte iónico a nivel de la membrana celular de la raíz. Este efecto es más evidente en los transportadores y canales de K^+, afectando tanto la toma de este macronutriente como a su homeostasis. Además, cuando el Na^+ atraviesa la membrana plasmática se observa una significativa despolarización del potencial eléctrico de la misma (Shabala and Cuin, 2008). Esta despolarización dificulta la entrada de K^+ en la célula y aumenta su salida.

Además de los tres estreses fisiológicos mencionados, la salinidad también induce la producción de especies reactivas de oxígeno (abreviado ROS, por las singlas en inglés de Reactive Oxygen Species), generando **daño oxidativo** en las plantas (Ding *et al.*, 2010). El exceso de ROS puede dañar la estructura de las enzimas y otras macromoléculas de las células vegetales (Mittler, 2002). De hecho, la salinidad reduce la disponibilidad de CO_2 atmosférico debido al incremento del cierre de los estomas y el consumo de NADPH en el Ciclo de Calvin también se ve afectado. Cuando la ferredoxina se sobre-reduce durante la transferencia electrónica durante la fotosíntesis, los electrones excedentes deben ser transferidos desde el fotosistema I al oxígeno para formar el radical superóxido ($O_2^{\bullet -}$) en la llamada Reacción de Mehler, que inicia una reacción en cadena que produce más radicales de oxígeno perjudiciales (Mittler, 2002). Estos incluyen el oxígeno singlete ($^1O_2$), peróxido de hidrógeno (H_2O_2) y radicales hidroxilo ($OH^{\bullet}$). Estas ROS son citotóxicas y pueden destruir el metabolismo normal de las células produciendo daño oxidativo de lípidos, proteínas y ácidos nucleicos cuando son producidas en exceso (Miller *et al.*, 2010). La peroxidación lipídica provoca cambios en la estructura y función de las membranas, altera la compartimentación de iones y la pérdida de potencial eléctrico, inhibe el transporte de metabolitos, modifica los receptores de hormonas y la producción de mensajeros químicos (Nandwal *et al.*, 2007). A nivel de proteínas, las ROS pueden fragmentarlas o formar uniones entre ellas ya que actúan a nivel de las cadenas laterales de los residuos aminoacídicos (Therond *et al.*, 2000). El estrés oxidativo puede ocasionar también la alteración y ruptura de cadenas de ADN, originando mutaciones (Moller *et al.*, 2007). Por tanto hay una necesidad constante de mecanismos que mantengan las ROS bajo niveles no perjudiciales para las plantas ya que la acumulación de ROS depende en gran medida del balance entre su producción y retirada (Mittler *et al.*, 2004).

2.2. Sistemas implicados en la tolerancia a la salinidad en plantas

Debido al efecto negativo que produce el estrés salino en las plantas, éstas han desarrollado mecanismos bioquímicos y moleculares para paliar el efecto negativo de la salinidad (Figura 2). Para la mayoría de las plantas, el mecanismo para conferir mayor tolerancia a la salinidad es restringir la absorción de Na^+ y Cl^- y mantener la del resto de macronutrientes a niveles normales (Teakle and Tyerman, 2010).

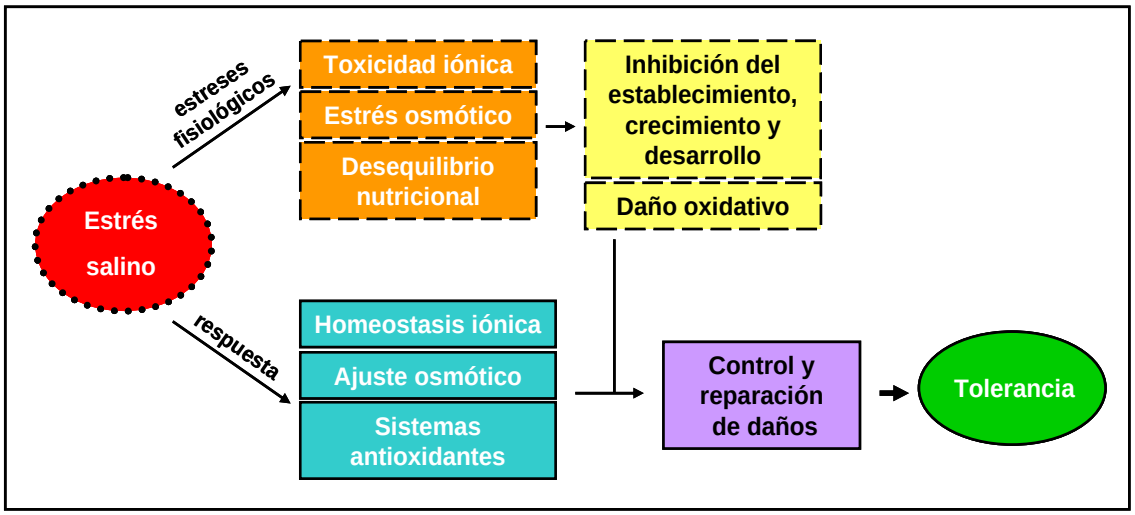

Figura 2. Efectos y respuesta del estrés salino en plantas

- **Homeostasis iónica:** además de la disminución en la toma de sales a través de la raíz, es necesario su partición a nivel celular y de tejido para que no formen concentraciones tóxicas en el citosol de las hojas durante la transpiración (Munns, 2005). También es necesaria la extrusión de iones en el apoplasto y el secuestro de iones en compartimentos intracelulares y tejidos menos sensibles (Flowers and Colmer, 2008). Para ello las plantas utilizan mecanismos que disminuyen la concentración de Na^+ en el citoplasma (especies excluidoras) y mecanismos que disminuyen la concentración en el citoplasma (especies incluidoras). Para limitar la entrada de Na^+ en la raíz, transporte y distribución hacia las hojas y su compartimentación en vacuolas, las plantas han desarrollado diversas estrategias. Esto se consigue fundamentalmente mediante sistemas de transporte de iones a través de las diferentes membranas celulares (Zhu, 2003). Los progresos en genética molecular y fisiología de las plantas apuntan como mecanismo clave en la tolerancia al estrés salino la homeostasis del Na^+ y del K^+, el cual es un proceso complejo y coordinado que conlleva el mantenimiento en el citoplasma de una alta razón K^+/Na^+ (Tester and Davenport, 2003).

- **Ajuste osmótico:** el ajuste osmótico permite evitar la pérdida de agua favoreciendo a las células vegetales mantener el turgor y los procesos que dependen de él como la expansión celular y crecimiento, la apertura de estomas y fotosíntesis, además de mantener un gradiente de potencial de agua favorable para que entre el agua en la planta. El balance osmótico en el citoplasma se consigue mediante la acumulación de osmolitos compatibles, es decir, solutos que no inhiban procesos metabólicos (Hasegawa *et al.*, 2000). Los solutos que participan en el ajuste osmótico pueden ser iones inorgánicos, principalmente K^+, o compuestos orgánicos como azúcares (principalmente glucosa y fructosa, pero también trehalosa, rafinosa, fructanos), polialcoholes (glicerol, inositoles metilados), metabolitos cargados (glicina betaína) y aminoácidos como la prolina (Flowers and Colmer, 2008). El coste energético que requiere la síntesis de osmolitos orgánicos es en parte responsable de la reducción del crecimiento.

- **Sistemas antioxidantes:** actualmente se está avanzando en el conocimiento de las ROS producidas en plantas como moléculas asociadas a la transducción de señales que se desencadenan ante situaciones de estrés (Foyer and Noctor, 2009). Bajo condiciones normales, las ROS son producidas en bajos niveles en orgánulos como cloroplastos, la mitocondria o los peroxisomas. Sin embargo durante el estrés salino, la tasa de producción de ROS se ve dramáticamente incrementada debido a la acumulación de NADPH y ATP que no son consumidos. Por tanto son necesarios mecanismos de protección y reparación de los daños producidos por el exceso de ROS en condiciones de estrés. Para ello las plantas han desarrollado sistemas eficientes de secuestración de ROS, que incluyen tanto enzimas antioxidantes (localizadas en diferentes compartimentos celulares) como pequeñas moléculas no enzimáticas que funcionan directamente como sistema de defensa antioxidante o indirectamente como cofactores de distintas actividades enzymáticas (Scheibe and Beck, 2011).

3. Las Micorrizas

La mayoría de las plantas que crecen sobre la corteza terrestre viven asociadas, en forma de simbiosis mutualista, con ciertos hongos del suelo, constituyendo las llamadas micorrizas, término que deriva del griego *mykos* (hongo) y *riza* (raíz). Este término fue utilizado por primera vez por el botánico alemán A.B. Frank a finales del siglo XIX (Frank, 1885), haciendo referencia a la simbiosis observada entre las raíces de ciertos árboles con un micelio fúngico a la que ya, en aquel momento, supuso la función de

favorecer las condiciones hídricas de la zona adyacente al sistema radical, además de un importante papel nutricional.

Sin embargo, no fue hasta los años 50 cuando se comenzó a poner de manifiesto la importancia real y el significado de estas asociaciones, así como su presencia en la práctica totalidad de los sistemas suelo-planta (Barea and Jeffries, 1995). El hongo, habitante común de los suelos, contacta las raíces y coloniza biotróficamente la corteza de las mismas, sin causar daño a la planta y sin llegar a activar por completo su reacción de defensa (Smith and Read, 2008). Una vez que el hongo ha colonizado la raíz, desarrolla un micelio externo que coloniza el suelo que rodea la raíz y ayuda a la planta a adquirir nutrientes minerales y agua, además de conferirle una mayor resistencia a los estreses ambientales (Barea *et al.*, 2012). Según esto, en la mayoría de los casos, el órgano de captación de nutrientes de la planta sería la micorriza y no la raíz propiamente dicha (Harley and Smith, 1983). A su vez la planta hospedadora proporciona al hongo simbionte (heterótrofo) compuestos carbonados procedentes de la fotosíntesis, así como un nicho ecológico protegido donde poder completar su ciclo de vida (Brundrett, 2004).

Un aspecto importante de las micorrizas es su universalidad ya que se encuentran prácticamente en todos los ecosistemas terrestres (Allen, 1991). Actualmente se estima que el 90% de las plantas que crecen sobre la Tierra están micorrizadas (Smith and Read, 2008). Sólo en unas pocas familias botánicas hay especies que no forman micorrizas, tal es el caso de las crucíferas, quenopodiáceas y ciperáceas (Barea and Honrubia, 1993). Las plantas y sus micorrizas han co-evolucionado desde hace 400-460 millones de años según registros fósiles (Fósil Rhynie, datado en 370 millones de años) y estudios sobre filogenia y evolución (Redecker *et al.*, 2000).

A pesar de muchas similitudes en cuanto a función y, en algunos casos morfología, se pueden reconocer cinco tipos de micorrizas en base a las estructuras formadas y a la naturaleza de los simbiontes implicados (Barea, 1998). Estos cinco tipos de micorrizas se encuadran en 3 grupos tróficos: ectotróficas, ectendotróficas y endotróficas. Este último grupo trófico está formado por las endomicorrizas ericoides, orquidoides y arbusculares. El objeto de esta investigación se centra en este último grupo: los “hongos formadores de micorrizas arbusculares”, comúnmente llamadas por el acrónimo MA (Smith and Read, 1997).

3.1. Las Micorrizas Arbusculares

Las micorrizas arbusculares (MA) constituyen el tipo de simbiosis más extendido en la naturaleza. Se estima que está presente en el 80-85% de las plantas, encontrándose en la práctica totalidad de las especies de interés agronómico e industrial (Jeffries and Barea, 2012). La principal característica morfológica de estos hongos son los arbúsculos, estructuras típicas de la colonización que el hongo desarrolla en el interior de las células de la corteza de la raíz por ramificación dicotómica repetida de sus hifas.

El largo periodo de vida en común de estos hongos y plantas simbiontes ha condicionado el elevado grado de mutualismo y dependencia que los simbiontes muestran entre sí. De hecho, la mayoría de las plantas son "micotróficas", necesitan estar micorrizadas para prosperar, mientras que el hongo MA es un "simbionte obligado"(Barea and Azcón-Aguilar, 2012) Esta es una de las principales limitantes del estudio de este grupo de hongos, ya que no es posible su multiplicación en condiciones axénicas (Bago and Cano, 2005).

Las bases fundamentales sobre las que se establece la simbiosis son nutritivas: los hongos MA reciben fotosintatos de la planta a cambio de una mejora en la toma de nutrientes, fundamentalmente fósforo, que es el elemento limitante en el ecosistema terrestre (Bucher, 2007), pero también potasio, calcio, magnesio, azufre, hierro, zinc, cobre, manganeso (Boomsma and Vyn, 2008), así como agua del suelo, dada la mayor accesibilidad del micelio externo del hongo a recursos del suelo más distantes del sistema radical (Ferrol and Pérez-Tienda, 2009). No obstante, la asociación genera otros beneficios, entre los que destacan una mayor resistencia de la planta micorrizada al ataque de patógenos del sistema radical (Hooker *et al.*, 1994; Pozo *et al.*, 2009), a la presencia de metales pesados en el suelo (del Val *et al.*, 1999; González-Guerrero *et al.*, 2009), al estrés hídrico (Augé, 2001; Ruíz-Lozano and Aroca, 2010) o a condiciones extremas de pH del suelo (Clark *et al.*, 1999; Oliveira *et al.*, 2005; Cornejo *et al.*, 2008). Además, la red de hifas extrarradicales y ciertas sustancias secretadas por el hongo durante la asociación favorecen la formación de agregados estables en el suelo y, por tanto, la conservación de la estructura física del mismo (Wright and Upadhyaya, 1998; Jeffries and Barea, 2012). Es por esto que las MA desempeñan un papel fundamental en la supervivencia y desarrollo de las plantas, sobre todo, en suelos sometidos a condiciones de estrés (sequía, salinidad, cambios bruscos de temperatura, deficiencia de nutrientes), como los que caracterizan a los ecosistemas mediterráneos (Requena *et al.*, 2001; Sánchez-Castro *et al.*, 2012).

3.2. Clasificación y filogenia de los hongos MA

Los primeros intentos de clasificar los hongos MA datan de finales del siglo XIX y comienzos del XX y se basaron exclusivamente en criterios morfológicos referenciados en las esporas. En estos estudios estos hongos se incluyeron en la familia Endogonaceae dentro del phylum Zygomycota (Gerdemann and Trappe, 1974). En los últimos años la sistemática del grupo de hongos formadores de MA ha sufrido varias modificaciones, sobre todo por la incorporación de técnicas moleculares en el estudio de la filogenia de estos hongos. Actualmente se ha demostrado la naturaleza monofilética de este grupo de hongos mediante el análisis de 18S ADNr. Esto ha permitido incluirlos en un nuevo phylum, denominado Glomeromycota (Schüβler *et al.*, 2001). En la actualidad este phylum es motivo de debate y posee dos posibles clasificaciones que se muestran a continuación (Tablas 1 y 2).

Tabla 1. Clasificación de los hongos MA según **Krüger *et al.* 2012.**

CLASE	ORDEN	FAMILIA	GÉNERO
Glomeromycetes	*Glomerales*	*Glomeraceae*	*Glomus*
			Funneliformis
			Septoglomus
			Rhizophagus
			Sclerocystis
		Claroideoglomeraceae	*Claroideoglomus*
	Diversisporales	*Diversisporaceae*	*Diversispora*
			Redeckera
		Pacisporaceae	*Pacispora*
		Acaulosporaceae	*Acaulospora*
		Gigasporaceae	*Scutellospora*
			Gigaspora
			Racocetra
	Archaeosporales	*Ambisporaceae*	*Ambispora*
		Archaeosporaceae	*Archaeospora*
		Geosiphonaceae	*Geosiphon*
	Paraglomerales	*Paraglomeraceae*	*Paraglomus*

Tabla 2. Clasificación de los hongos MA según **Oehl *et al.* 2011.**

CLASE	ORDEN	FAMILIA	GÉNERO
Glomeromycetes	*Glomerales*	*Glomeraceae*	*Glomus*
			Funneliformis
			Septoglomus
			Simiglomus
		Entrophosporaceae	*Claroideoglomus*
			Albahypha
			Viscospora
			Entrophospora
	Diversisporales	*Diversisporaceae*	*Diversispora*
			Redeckera
			Otospora
			Tricispora
		Sacculosporaceae	*Sacculospora*
		Pacisporaceae	*Pacispora*
		Acaulosporaceae	*Acaulospora*
			Kuklospora
	Gigasporales	*Scutellosporaceae*	*Scutellospora*
			Orbispora
		Dentiscutataceae	*Fuscutata*
			Dentiscutata
			Quatunica
		Racocetraceae	*Cetraspora*
			Racocetra
		Gigasporaceae	*Gigaspora*
Archaeosporomycetes	*Archaeosporales*	*Ambisporaceae*	*Ambispora*
		Archaeosporaceae	*Archaeospora*
			Intraspora
		Geosiphonaceae	*Geosiphon*
Paraglomeromycetes	*Paraglomerales*	*Paraglomeraceae*	*Paraglomus*

3.3. Ciclo de vida

En los hongos MA no se ha demostrado una reproducción sexual reconocida sino que producen esporas de resistencia multinucleadas, pudiendo llegar hasta los 20.000 núcleos por espora (Ferrol *et al.*, 2004). El ciclo de vida se inicia partiendo de los propágulos de estos hongos, que se mantienen en el suelo en forma de esporas, redes de micelio, o colonizando raíces activas o fragmentos de éstas que permanecen en el suelo (Sánchez-Castro, 2009) La figura 3 resume el ciclo de vida de las micorrizas arbusculares.

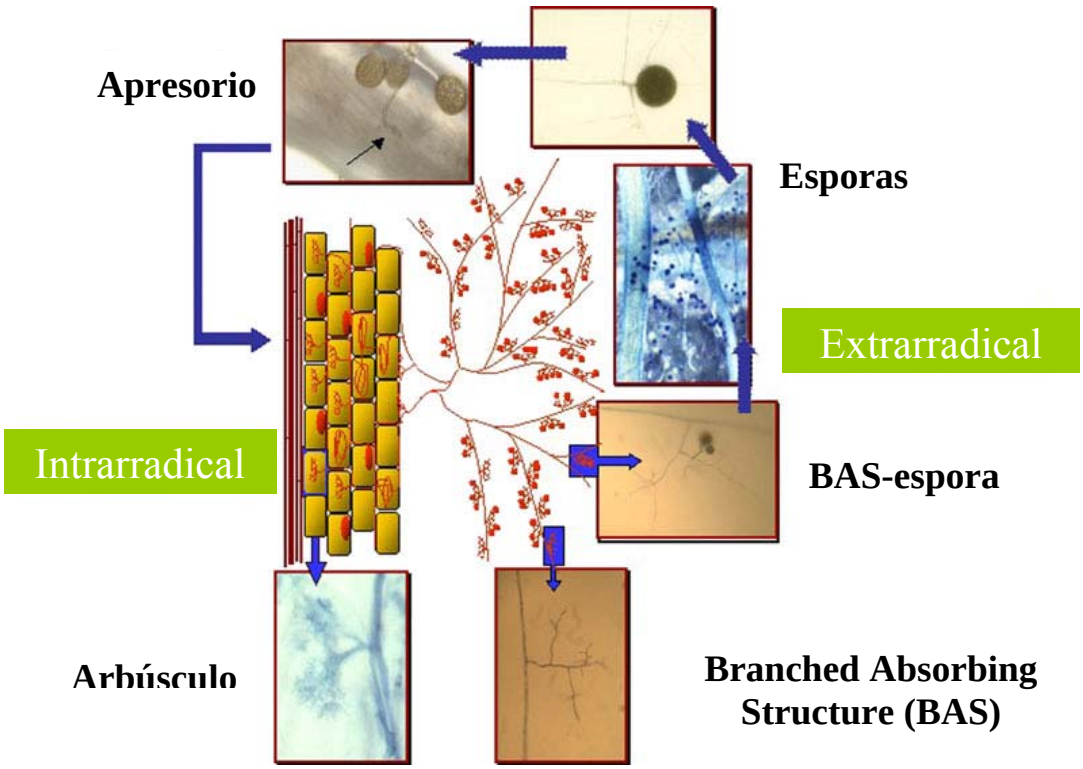

Figura 3. Ciclo de vida de los hongos formadores de MA (Tomado de Porcel *et al.* 2012).

Las MA tienen dos componentes bien diferenciados: la "fase extrarradical" del hongo en la que se incluyen el micelio externo, esporas y ocasionalmente células auxiliares, y la "fase intrarradical" que incluye hifas intra e intercelulares, arbúsculos y, en algunas especies, vesículas (Figura 4). La colonización de la raíz sólo ocurre en la epidermis y el parénquima cortical, ya que el hongo nunca llega a penetrar en el cilindro vascular ni las zonas meristemáticas (Barea *et al.*, 2008).

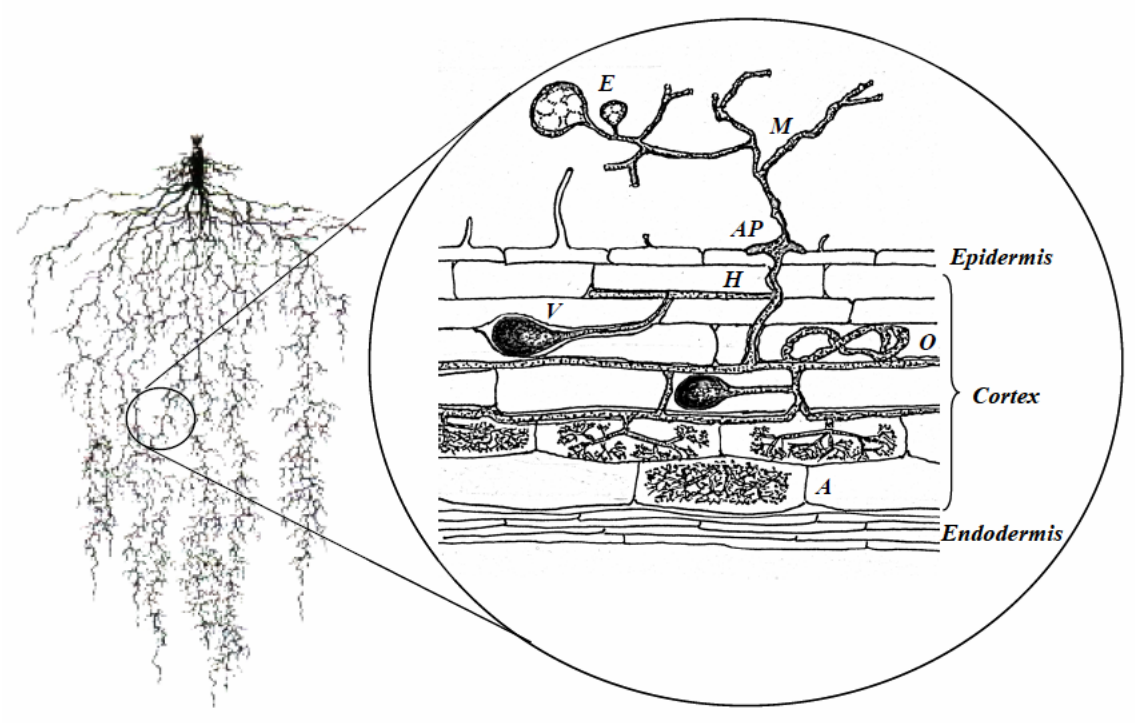

Figura 4. Esquema de las estructuras anatómicas de las MA. Abreviaturas: **A**, arbúsculo; **AP**,apresorio; **E**, espora; **H**, hifa intercelular; **M**, micelio extraradical; **O**, ovillo; **V**, vesícula. (Tomado de Palenzuela and Barea, 2002).

El proceso de formación de las MA tiene lugar tras una sucesión de interacciones entre el hongo y la planta, que van a dar lugar a una integración morfológica y funcional de ambos simbiontes (Gianinazzi-Pearson *et al.*, 2004). Como resultado, la planta acepta la colonización por parte del hongo sin mostrar una reacción de defensa persistente (Dumas-Gaudot *et al.*, 2000; Pozo *et al.*, 2002). El establecimiento de la simbiosis es el resultado de un continuo diálogo molecular y un esfuerzo mutuo para aceptar el intercambio de señales de reconocimiento tanto de la planta como del hongo (Gollotte *et al.*, 2002; Vierheilig and Piché, 2002). La identificación y clonación de los genes de la planta implicados en el programa simbiótico en MA (Gianinazzi-Pearson and Brechenmacher, 2004) y el conocimiento de los programas de señales involucrados en la formación y funcionamiento de la simbiosis (Harrison, 2005) son objeto de gran interés en la actualidad.

3.4. Significado de los hongos MA en el sistema suelo-planta

La mayoría de las plantas dependen de estar micorrizadas para establecerse y prosperar adecuadamente. Ello es debido a que los hongos MA realizan importantes acciones en los sistemas suelo-planta, como son las siguientes:

- **Mejora del enraizamiento de las plantas**

Mediante la producción de fitohormonas, vitaminas y otras sustancias fitoactivas por parte de los hongos (Parniske, 2008; Faure *et al.*, 2009).

- **Incremento del suministro de nutrientes a las plantas**

El micelio externo del hongo abarca una superficie mayor que las raíces de las plantas. Es por ello que pueden captar nutrientes de zonas a las que la raíz no tiene acceso. Además, nutrientes con poca movilidad y presentes en bajas concentraciones en el suelo se absorben más eficientemente en plantas micorrizadas, acelerando los ciclos biogeoquímicos de los nutrientes minerales, particularmente N y P (Ferrol and Pérez-Tienda, 2009).

- **Mejora de la estructura del suelo**

Las hifas de los hongos MA están implicadas en la formación de agregados hidroestables en el suelo, lo cual contribuye directamente en la mejora de la calidad del suelo (Rillig and Mummey, 2006; Jeffries and Barea, 2012). La glomalina, una glicoproteína producida por el micelio externo del hongo desempeña un importante papel en el proceso de agregación (Rillig, 2004; Bedini *et al.*, 2009).

- **Protección a la planta frente a estreses abióticos**

La simbiosis MA contribuye a incrementar la resistencia y tolerancia de las plantas a la salinidad, sequía, estado de deficiencia o exceso de nutrientes, exceso de metales pesados, degradación del suelo, etc (Barea *et al.*, 2012).

- **Protección a la planta frente a patógenos**

Se ha descrito que las micorrizas reducen los síntomas de patógenos cuando se trata de enfermedades que afectan al sistema radical (Cordier *et al.*, 1996; Slezack *et al.*, 2000). Para que se manifieste esta protección, es imprescindible que la simbiosis esté establecida antes de que se produzca el ataque del patógeno (Pozo *et al.*, 2010)

- **Favorecen la diversidad de las comunidades de plantas y la sucesión vegetal**

Cada planta muestra un nivel de compatibilidad mayor con determinados ecotipos de hongos micorrícicos, por lo que la conservación de la diversidad de estos hongos

beneficia la diversidad y sucesión de las plantas (van der Heijden *et al.*, 1998; Hart and Klironomos, 2002).

4. Efecto de la salinidad en las micorrizas arbusculares

La salinidad puede afectar negativamente a los hongos MA disminuyendo su capacidad de colonización, la germinación de las esporas y el crecimiento de las hifas.
Varios estudios han demostrado que la salinidad puede disminuir la colonización de las raíces por parte de los hongos MA (Duke *et al.*, 1986; Giri *et al.*, 2007; Sheng *et al.*, 2008), probablemente debido al efecto directo de la sal sobre el hongo (Juniper and Abbott, 2006), lo que indica que la salinidad podría llegar a suprimir la formación de las MA (Tian *et al.*, 2004; Sheng *et al.*, 2008). Los diferentes niveles de colonización MA de las plantas bajo condiciones de salinidad se puede deber al diferente comportamiento de cada especie de hongo MA, incluso en ecosistemas similares (Klironomos *et al.*, 1993) o a la influencia de diferentes condiciones ambientales (Carvalho *et al.*, 2001). El grado en el que la colonización MA se ve afectada por la salinidad es mayor al inicio del proceso de colonización, cuando las esporas necesitan de hidratación para ser activadas y producir el tubo de germinación. El exceso de sal en el suelo dificulta la toma de agua y por tanto la hidratación de las esporas, siendo en este momento cuando el exceso de sal retrasa la germinación (McMillen *et al.*, 1998). El crecimiento de las hifas también se ve reducido con el incremento de sal aunque se ha sugerido que el crecimiento de las hifas puede considerarse más sensible al exceso de sal que la germinación de las esporas, ya que la germinación se retrasa pero no necesariamente se reduce mientras que el crecimiento de las hifas sí se ve reducido (Cantrell and Linderman, 2001; Juniper and Abbott, 2006; Jahromi *et al.*, 2008). La reducción en el crecimiento de las hifas y retraso de la germinación de las esporas sugiere que si el estrés salino persiste habría una reducción en el grado de colonización debido a que la habilidad de infección de los propágulos se vería reducida.

Sin embargo, y en desacuerdo con lo anteriormente mencionado, hay estudios que demuestran que la colonización MA no se reduce en presencia de sal (Levy *et al.*, 1983; Hartmond *et al.*, 1987). Más recientemente, Yamato *et al.* (2008) y Wu *et al.* (2010) demostraron que el grado de colonización de hongos MA no se vio reducido incluso a altos niveles de salinidad. De hecho, las MA están ampliamente distribuidas en ambientes salinos (Aliasgharzadeh *et al.*, 2001; Carvalho *et al.*, 2004; Yamato *et al.*, 2008; Wilde *et al.*, 2009), lo que sugiere que las especies de hongos MA aisladas en ambientes salinos deberían estar adaptadas a tales condiciones y esto explicaría el que no disminuyera su capacidad colonizadora en presencia de sal en el medio.

5. Simbiosis MA y tolerancia al estrés salino en plantas

Además de los mecanismos intrínsecos de adaptación a la salinidad desarrollados por las plantas, en condiciones naturales crecen asociadas a las MA (Smith and Read, 2008). En la mayoría de los casos estudiados, la asociación MA-planta confiere a la planta hospedadora un incremento en la tolerancia ante determinados estreses abióticos (Barea *et al.*, 2012). Aunque algunos autores hayan señalado que el exceso de sales en el suelo tiene un efecto negativo en las MA (Smith and Read, 1997; Juniper and Abbott, 2006), muchos otros han demostrado que las MA incrementan la tolerancia de las plantas al estrés salino (Al-Karaki *et al.*, 2001; Garg and Manchanda, 2009; Hajiboland *et al.*, 2010).

La mejora en la tolerancia de las plantas colonizadas por MA al estrés salino se puede deber en parte al resultado de una mayor eficiencia tanto en la toma de nutrientes (Cantrell and Linderman, 2001; Asghari *et al.*, 2005) como de agua (Augé, 2001; Aroca *et al.*, 2007); en la mejora del balance iónico (Zandavalli *et al.*, 2004; Giri *et al.*, 2007); en una mayor producción de osmolitos (Sheng *et al.*, 2011); en un aumento de la fotosíntesis (Sheng *et al.*, 2008) y en la protección de actividades enzimáticas (Giri and Mukerji, 2004; Rabie and Almadini, 2005) entre otros mecanismos.

Todos estos estudios han sugerido varios mecanismos para explicar la mejora en la tolerancia de las plantas al estrés salino:

- mejor eficiencia para la toma de agua y nutrientes, gracias a la red de micelio que se extiende más allá de la zona de absorción directa de la planta
- barrera selectiva durante la toma de nutrientes o transferencia a la planta
- mayor conductancia hidráulica de la raíz y ajuste osmótico
- incremento de la capacidad antioxidante
- mantenimiento de la relación K^+/Na^+ y menor acumulación de Na^+ en las hojas

Sin embargo los mecanismos que permiten a las plantas colonizadas por MA tener mayor tolerancia a la salinidad están todavía lejos de ser comprendidos en su totalidad. El mayor problema para resolver los mecanismos implicados en la mejora en la tolerancia al la salinidad, es que la tolerancia salina es un mecanismo complejo y codificado por familias de genes, lo que implica a varios mecanismos tanto fisiológicos como bioquímicos y que además varían entre especies (Mian *et al.*, 2011). Por lo tanto los estudios del impacto de la colonización MA en la tolerancia de las plantas al estrés salino se encuentran con este problema, entre otros. Además se debe prestar especial atención a los aislados de MA en ambientes salinos, ya que deberían de estar fisiológica

y genéticamente adaptados a dichas condiciones y por tanto mejorar la eficiencia de las plantas sometidas a estrés salino (Ferrol *et al.*, 2004). De hecho cada vez más estudios demuestran la importancia de usar inóculo MA nativo de ambientes salinos tanto en la mejora de la producción agrícola como en programas de revegetación en áreas afectadas de salinidad (Marulanda *et al.*, 2003; Moora *et al.*, 2004; Cho *et al.*, 2006; Alguacil *et al.*, 2011).

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CAPÍTULO 1

CHAPTER 1

Diversity of arbuscular mycorrhizal fungi in the rhizosphere of *Asteriscus maritimus* (L), a representative plant species in arid and saline Mediterranean ecosystems

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Diversidad de hongos micorrícico arbusculares en la rizosfera de *Asteriscus maritimus* (L), una planta representativa de ecosistemas Mediterráneos áridos y salinos

Resumen

El uso de los hongos MA adaptados a la salinidad puede ser un elemento crucial en la recuperación de áreas salinas, ya sea en entornos naturales o en terrenos agrícolas que se han salinizado debido a un uso no adecuado del suelo o bajo un escenario de cambio climático. Por lo tanto, la búsqueda de hongos MA adaptados a la salinidad es fundamental para explorar esas posibilidades. A pesar de su importancia, existe poca información sobre la distribución y abundancia de las diferentes asociaciones de micorrizas en ambientes salinos en zonas europeas del Mediterráneo. En el presente estudio se investiga la comunidad de hongos MA en la rizosfera de una especie de planta representativa adaptada a zonas salinas mediterráneas, *Asteriscus maritimus* (L.). Se tomaron muestras de la rizosfera de veinte plantas de *A. maritimus* en dos zonas diferentes del Parque Natural de Cabo de Gata: una duna salina y una marisma. Un total de 30 morfotipos de esporas pertenecientes a tres clases, cinco órdenes, nueve familias y 13 géneros fueron encontrados en la duna y en la marisma. En la rizosfera de la marisma la densidad de esporas fue seis veces mayor que en la duna, a pesar de que la diversidad fue bastante similar en ambos sitios. Una nueva especie de hongo MA se ha descrito en la duna y publicado recientemente, y cuatro especies no pudieron ser identificadas de forma inequívoca, lo que sugiere posibles nuevas especies que aún necesitan un análisis más detallado antes de su publicación. Estos resultados deben ser tomados en cuenta en el diseño de inóculos eficaces en programas de revegetación.

Palabras clave: biodiversidad, micorriza arbuscular, Mediterráneo, salinidad, nativo, inóculo

Diversity of arbuscular mycorrhizal fungi in the rhizosphere of *Asteriscus maritimus* (L), a representative plant species in arid and saline Mediterranean ecosystems

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Abstract

The use of AM fungi adapted to salinity could be a critical issue for success in recovering saline areas either in natural environments or in agricultural lands that became salinized due to a non appropriate land use or under a climate change scenario. Therefore looking for salinity-adapted AM fungi is fundamental to explore these possibilities. Despite its important role, there is little information on the distribution and abundance of the different mycorrhizal associations in saline environments in European Mediterranean areas. In the present study the community of AM fungi is investigated in the rhizosphere of a representative plant species adapted to saline Mediterranean areas, *Asteriscus maritimus* (L.). Samples of the rhizosphere of twenty *A. maritimus* plants were taken in two different areas of the Cabo de Gata Natural Park: a saline dune and a salt marsh. A total of 30 spore morphotypes belonging to three classes, five orders, nine families and 13 genera were found in the dune and salt marsh. In the rhizosphere of the salt marsh spore densities were six times higher than in the dunes, although the diversity was quite similar in both systems. One new AM fungal species has been described in the dune and recently published, and four species could not been unequivocally identified, suggesting possible new species that still need some further analysis before publication. These results should be taken into account when designing effective inocula for revegetation programmes.

Key words: biodiversity, arbuscular mycorrhiza, Mediterranean, salinity, native, inoculum.

1. Introduction

Seasonal aridity characterises Mediterranean environments. Impoverished soils, extreme temperatures, irregular rainfall, soil misuse and overgrazing for cattle-raising are several of the factors which contribute to the eroded landscapes (Vallejo et al. 1999). This multiple stress situation is a constraint for plant development because damage to soil and plants in arid and semiarid areas is not easily repaired due to the fragility of these ecosystems (Pascual et al. 2000). The latter is promoting desertification of great areas in Southeast Spain which leads to increases in salinity (Francis and Thornes 1990; López-Sánchez and Honrubia 1992; Albaladejo et al. 1996). Disturbance of the vegetation cover is the first visible indication of a desertification process and it is known to occur concomitantly with generalised damages of physical-chemical and biological soil properties (Requena et al. 2001). Moreover, salinity is recognized as a severe global ecological problem being one of the most influential abiotic factors limiting plant growth and yield (Porcel et al., 2012).

In these areas, where conditions are unfavourable for plant growth, arbuscular mycorrhizal (AM) fungi play an essential role (Trappe 1981). AM fungi are ubiquitous soil inhabitants belonging to the phylum Glomeromycota which establish mutualistic symbiotic associations with most land plants (Smith and Read 2008). The symbiosis between plant and AM fungi is one of the plant strategies for growing under a variety of stress conditions (Entry et al. 2002). The ecological impact of AM fungi is particularly relevant for arid and semi-arid ecosystems where they would enable greater plant tolerance of environmental stresses characteristic of these ecosystems (Requena et al. 1996; Allen 2007).

It is well documented that AM fungi improve plant growth and health: they facilitate nutrient uptake (van der Heijden et al. 2006), confer drought and salt tolerance (Ruíz-Lozano and Azcón 2000; Cantrell and Linderman 2001; Marulanda et al. 2009), protect plants against pathogens (Slezack et al. 2000; Pozo et al. 2009), have positive impacts on soil aggregate stability and water infiltration (Rillig and Mummey 2006), and prevent soil erosion (O'Dea 2007). Because of the key ecological functions played in soil and plants by AM symbiosis, a diverse community of AM fungi is necessary for the development and maintenance of plant diversity (Jeffries and Barea 2001), contributing to plant community productivity in different ecosystems (van der Heijden et al. 1998). Therefore, as AM fungi play important roles in the vigour of plant communities and the restoration of disturbed ecosystems (Renker et al. 2004), assessment of the native species composition is an important issue in several contexts and the basis to produce AM inoculum for selected plant species to be used in the revegetation processes (Ferrol et al. 2004). Moreover, the use of AM fungi adapted to salinity could be a critical issue for success in recovering saline areas either in natural environments or in agricultural lands that became salinized due to inappropriate land use. Therefore looking for salinity-adapted AM fungi is fundamental to explore these possibilities (Porcel et al., 2012; Estrada et al., 2012). Despite its important role, there is little information on the distribution and abundance of the different mycorrhizal associations in saline environments in European Mediterranean areas.

The aim of the present work was to analyse the diversity of AM fungal species in sensitive ecosystems affected by desertification and salinity in Southeast Spain, selecting two ecosystems: salt marshes and dunes. Cabo de Gata Natural Park is the most arid ecosystem in Europe (Geiger 1973) and was selected as the target area. AM fungi were screened in the rhizosphere of *Asteriscus maritimus* (L.), an halophyte member of the Asteraceae family, highly mycotrophic (Schaede 1962), and native of lands surrounding the Mediterranean Sea, especially Spain (Lendínez et al. 2011). Moreover, as the plant spreads its stems over the soil they protect the soil from erosion (Alcaraz et al. 1997). It is found both in salt marshes and dunes. However, differences in the AM fungal communities associated with this plant can be assumed dependent on the ecosystems. For this purpose AM fungi were studied either directly in samples from

the target soil or from one year bait plant cultures established to increase AM fungal spore population, to reveal their maximum complement possible.
To identify the different AM fungal species in natural saline sites, morphological and developmental criteria were applied. The morphological identification of AMF can accurately be done with spores (Oehl et al. 2011), while the fungal structures within the roots (intraradical hyphae, arbuscules, vesicles,) may vary only within the family or order but not at the species level (Dodd et al. 2000). The molecular characterization of AMF in roots is faced with the problem that only about 5% of the total DNA of a root belongs to the fungi (Toth et al. 1991). Our primary purposes were to understand the species composition of AM fungi in the rhizosphere of *A. maritimus*, and to elucidate the distribution patterns of AM fungi communities related to habitats.

2. Material and Methods

2.1. Site description

The study was carried out in a representative saline Mediterranean ecosystem in the Natural Park Cabo de Gata in Almería (Andalucía, Spain). The climate is dry and hot, with an average annual temperature of 18.5ºC, irregular rainfall occurring mostly in autumn with a mean annual rainfall less than 200mm and an annual solar hours of 2.960. Two habitats were selected: a natural sand dune (36º44´41´´N 02º07´26´´W) and a salt marsh (36º45´24´´N 02º13´17´´W).

2.2. Plant and soil sampling

Most of the halophytes in saline sites belong to the *Chenopodiaceae*, *Juncaceae*, *Cyperaceae* or *Brassicaceae* families which are non-or weakly mycorrhizal. For the present study the halophyte *Asteriscus maritimus*, belonging to the Asteraceae family was selected as target plant which is known to be colonized by AMF since long time (Mason 1928).
Soil samples were taken from the rhizosphere of twenty *A. maritimus* (L.) plants at both sites in February 2010. Plants were randomly collected with their intact root systems up to 40 cm soil depth, ten of them growing in the natural sand dune system and the other ten plants in the salt marsh. Root fragments in these samples were rinsed cleansed with tap water and then cleared in 10% KOH and stained with 0.05% trypan blue to confirm AM colonization (Phillips and Hayman 1970). The samples were further used to analyse spore populations and selected chemical soil parameters (pH, soil organic carbon, total nitrogen, soil electrical conductivity).

2.3. AM fungal bait and pure cultures

AM fungal bait cultures were also established and maintained as described in Palenzuela et al. (2008) and Palenzuela et al. (2011) by transplanting each *A. maritimus* plants with their surrounding rhizosphere soils into 1 L pots and transferring them to the greenhouse at EEZ in Granada immediately after sampling. The pots were irrigated three times per week and fertilized every four weeks with Long-Aston nutrient solution (Hewitt 1952). The trap cultures were used for propagation of indigenous AM fungal communities to be checked after one year. With the main species present in these habitats pure cultures were established at the EEZ collection for future experiments and rehabilitation programs of saline areas using the benefits of native AM fungi.

2.4. AMF spore isolation and identification

For the analyses of AM fungal spores, 50 g of each rhizospheric soil were taken in March 2010, and 25 g in March 2011 from each trap cultures. AM fungal spores, both from the field collected soil and from the trap cultures, were isolated, identified and counted separately for each location. They were separated from the soil samples by a wet sieving and decanting method, followed by sucrose centrifugation process (Sieverding 1991). After centrifugation, the supernatant was poured through a 50 µm mesh and quickly rinsed with tap water. Turgid spores (suggesting viability) were counted under a dissecting microscope using up to 90-fold magnification. For identification of AMF species, spores (about 50-70% of the total numbers) were picked under the dissecting microscope and mounted in polyvinyl alcohol-lactic acid-glycerine (PVLG) (Koske and Tessier 1983) or PVLG mixed 1:1 (v/v) with Melzer's reagent (Brundrett et al. 1994) for permanent slides. The spores were then examined using a compound microscope at up to 400-fold magnification. The AMF species identification was based on current species descriptions and identification manuals (e.g. Schenck and Pérez 1990); International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu/fungi/taxonomy/speciesID.htm); Department of Plant Pathology University of Agriculture in Szczecin, Poland (http://www.agro.ar.szczecin.pl/~jblaszkowski/)), and on own type specimen analyses of almost all AMF species described. In this study, an AMF "species" is either a clearly identified species based on its spore morphology, or a species as yet unknown to us. We follow the glomeromycotean classification of Oehl et al. (2011) recently published in the new journal of the International Mycological Association on request. Photographs were taken with a Leica DFC 290 digital camera on a Leitz Laborlux S compound microscope using Leica Application Suite Version V 2.5.0 R1 software.

2.5. Species richness and specific density

The species richness is defined as the total number of species found in a community. The specific density (Di) is the proportion of individuals of one particular species in the sample (i) relative to the total number of individuals (N).

$$Di = ni / N$$

In the present work species richness and specific density have been evaluated at the dune and salt marsh.

3. Results

3.1. Root colonization by AMF

All surveyed samples were colonized by AMF and formed typical arbuscular mycorrhizal structures. Intra- and intercellular hyphae, vesicles and arbuscules were abundant in the root tissues.

3.2. Soil analysis

At the dune site, the soil was alkaline with pH 8.2, organic carbon 15.3 g kg^{-1}, total nitrogen 1.9 g kg^{-1}, available P 27.0 mg kg^{-1}, and soil electrical conductivity 0.5 dS m^{-1}. At the salt marsh, the soil was alkaline with pH 8.7, organic carbon 2.6 g kg^{-1}, total nitrogen 0.3 g kg^{-1}, available P 47.0 mg kg^{-1} and soil electrical conductivity 3.95 dS m^{-1}.

3.3. AMF spore densities at different ecosystems

The AMF spore densities differed among the sites. Salt marsh had regularly higher spore densities than the dune, both at the rhizosphere soil and in the bait cultures. In March 2010, the spore densities were 1.2 and 7.2 g^{-1} soil at the dune and the salt marsh, respectively. After one year in the trap cultures, the spores densities were more than doubled, with 2.8 and 17.8 spores at the dune and the salt marsh. The relation between dune and salt marsh remained the same, being the spore density around six times higher at the salt marsh than at the dune.

3.4. AMF species richness

In total 30 AMF species could be distinguished from the field samples across all sites and both years of survey. They belong to all three current classes, to all five current orders, to nine families and thirteen genera currently known in the Glomeromycota (Table 1). The majority of the isolated species (16) were of the order Glomerales (3

Funneliformis species, 9 *Glomus* species and one *Septoglomus* species of the Glomeraceae and two species of *Claroideoglomus* and one *Entrophospora* species of the Entrophosporaceae). One of them, *Glomus rubiforme*, had formerly been assigned to *Sclerocystis*. Of the Diversisporales, four *Diversispora*, two *Pacispora* and one *Acaulospora* were detected. Of the Gigasporales, two *Racocetra* and one Scutellospora were found. Finally, two *Archaeospora* and one *Ambispora* species of the Ambisporales and one *Paraglomus* species of the Paraglomerales were detected. Four species (*Glomus* sp. BEV1, *Gl.* sp. BEV2, *Entrophospora* sp. BEV3 and *Diversispora* sp. BEV4) could not be unequivocally identified as a known species and might represent new species.

3.5. AMF species richness in sand dune

In the field samples of March 2010, 16 AMF species belonging to 9 genera were found in the sand dune. After one year of bait culturing, 15 AMF species reproduced spores in the cultures belonging to 10 genera. From field and bait cultures, a total of 23 AMF species were detected belonging to 11 AMF genera were identified. Of these species, 7 were found recovered only from the field samples and 8 only from the bait cultures, while 9 species were detected both in the field samples and the bait cultures (Table 1).
In 2010, the species detected (in order of decreasing specific density), were: *Se. constrictum*, *Diversispora* sp. BEV4, *Cl. claroideum*, *Fu. coronatus*, *Gl. badium*, *Gl. intraradices*, *Gl. macrocarpum*, *Gl.* sp. BEV2, *Sc. calospora*, *Gl. rubiforme*, *Ra. persica*, *Ac. scrobiculata*. After one year of bait culturing, the species recovered from the sand dune (in order of decreasing specific density, see Figure 1) were: *Se. constrictum*, *Fu. coronatus*, *Cl. claroideum*, *Pa. dominikii*, *Gl. intraradices*, *Cl. etunicatum*, *Di. clara*, *Di. versiformis*, *Fu. mosseae*, *Gl. microaggregatum*, *Sc. calospora*, *Di. aurantia*, *Par. occultum*, *Ar. trappei*, *Pa. franciscana*, *Ac. scrobiculata*. In the field and in the bait cultures, *Se. constrictum* was the most abundant species.

3.6. AMF species richness in salt marsh

In the salt marsh system, a total of 24 AMF species, also belonging to 11 AMF genera, were detected (Table 1). In the field samples from the salt marsh, 20 morphospecies were identified in 2010 belonging to 10 AMF genera, and after one year of bait culturing, 12 AMF species had reproduced spores belonging to 7 genera. Of these species, 11 were found identified only from the field samples and 4 only from the bait cultures, while 9 species were recovered from both the field samples and the bait cultures.
In 2010, the species detected (in order of decreasing specific density, see Figure 1), were: *Se. constrictum*, *Diversispora* sp. BEV4, *Gl.* sp. BEV2, *Entrophospora* sp. BEV3, *Gl. intraradices*, *Cl. claroideum*, *Fu. coronatus*, *Sc. calospora*, *Gl. macrocarpum*, *Fu. mosseae*, *Fu. geosporus*, *Gl. badium*, *Cl. etunicatum*, *Ra. persica*. After one year of bait

culturing, the species recovered from the sand dune (in order of decreasing specific density, see Figure 1) were: *Par. occultum*, *Gl. intraradices*, *Fu. coronatus*, *Cl. claroideum*, *Cl. etunicatum*, *Se. constrictum*, *Fu. mosseae*, *Di. versiformis*, *Gl. microaggregatum*, *Di. aurantia*, *Fu. geosporus*, *Ar. myriocarpa*, *Ar. trappei.* In the field samples, *Se. constrictum* was the most abundant species, but not in the trap cultures *Par. occultum* had the highest specific density.

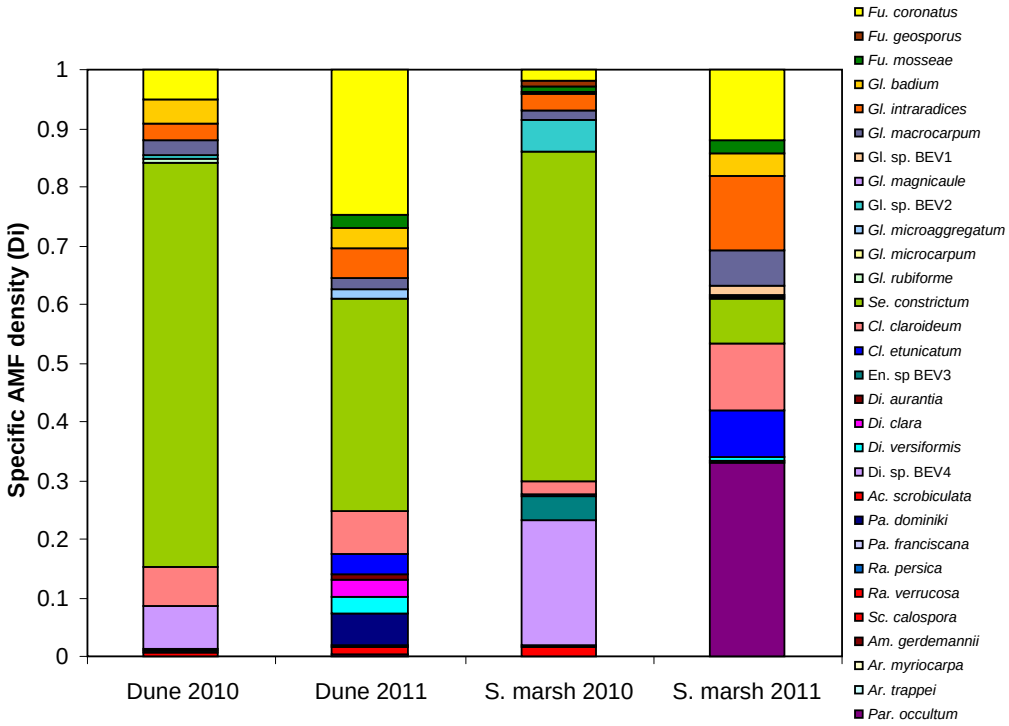

Figure 1. Specific density (Di) of AMF found in dune and salt marsh at two consecutive years 2010 and 2011 (field-collected soil and bait cultures respectively).

3.7. AM fungal differences in both ecosystems

AMF species and genus richness was similar in both ecosystems (Table 1) with 23 respective 24 out of 30 AMF species and each 11 out of 13 AMF genera detected, respectively. *Ambispora* and *Entrophospora* were the two genera found in the study area that were not detected in the dune systems but only in the salt marsh system. On the other hand, *Acaulospora* and *Pacispora* species were not found in the salt marsh while they had been revealed from the dune (Table 1). Other remarkable difference between both scenarios was the presence of *Fu. geosporus* and *Entrophospora* sp. BEV3 only in the salt marshes. Also *Gl. microcarpum*, *Gl.* sp. BEV1, *Ra. verrucosa*, *Am. gerdemanni* and *Ar. myriocarpa* were only revealed from the salt marshes, while, beside *Ac. scrobiculata*, *Pa. dominikii* and *Pa. franciscana*, also *Gl. magnicaule* and *Gl. rubiforme* were only detected in the dune but not in the salt marsh.

Table 1. Presence and species richness of AMF at dune and salt marsh directly from field-collected soil samples in 2010 and from trap cultures in 2011.

	Sand Dune		Salt Marsh	
AMF species	**In field 2010**	**Trap culture 2011**	**In field 2010**	**Trap culture 2011**
GLOMERALES				
Glomeraceae				
Funneliformis coronatus	x	x	x	x
Fu. geosporus			x	x
Fu. mosseae		x	x	x
Glomus badium	x		x	
Gl. intraradices	x	x	x	x
Gl. macrocarpum	x		x	
Gl. sp. BEV1			x	
Gl. magnicaule	x			
Gl. sp. BEV2	x		x	
Gl. microaggregatum	x	x	x	x
Gl. microcarpum			x	
Gl. rubiforme	x			
Septoglomus constrictum	x	x	x	x
Entrophosporaceae				
Claroideoglomus claroideum	x	x	x	x
Cl. etunicatum		x	x	x
Entrophospora sp. BEV3			x	
DIVERSISPORALES				
Diversisporaceae				
Diversispora aurantia		x		x
Di. clara		x		
Di. versiformis		x		x
Di. sp. BEV4	x		x	
Acaulosporaceae				
Acaulospora scrobiculata	x	x		
Pacisporaceae				
Pacispora dominiki	x	x		
Pa. franciscana	x	x		
GIGASPORALES				
Racocetraceae				
Racocetra persica	x		x	
Ra. verrucosa			x	
Scutellosporaceae				
Scutellospora calospora	x	x	x	
ARCHAEOSPORALES				
Archaeosporaceae				
Ambispora gerdemannii			x	
Archaeospora myriocarpa			x	x
Ar. trappei		x		x
PARAGLOMERALES				
Paraglomeraceae				
Paraglomus occultum		x		x
AMF species richness site^{-1} year^{-1}	**16**	**16**	**20**	**23**
Total AMF species richness site^{-1}	**23**		**24**	
AMF species richness of study	**30**			

4. Discussion

To restore Mediterranean ecosystems, a number of ecophysiological features enhancing plant performance under drought, salinity and heat, conditions to be exacerbated by climate change, are frequently looked for (Vallejo et al. 2005). AM fungi are among some other soil microorganisms that enhance plant performance under stressed conditions. Knowledge of the plant diversity and their association with AM fungi on saline areas is of crucial importance for their efficient use in the conservation and management of endangered ecosystems and agricultural lands affected by salinization.

The data presented in this paper show the AM fungal species found in the rhizosphere of *A. maritimus* from the Cabo de Gata Natural Park, Southeast Spain, which it is a typical Mediterranean ecosystem affected by salinization and desertification. The vegetal species selected have a high ecological value in Mediterranean ecosystems. Results obtained by Rodríguez et al. (2005) suggested that *A. maritimus* could be considered a useful species in re-vegetation programmes, landscaping and xerogardening, due to its tolerance to severe water stress and high salinity levels in the irrigation water. In addition to that, Caravaca et al. (2005) pointed out its capability to protect the soil from erosion because of the high percentage of stables aggregates found in the rhizosphere of *A. maritimus*.

The two sampling areas under study, dune and salt marsh, have in common high levels of salinity and the ability to accommodate common species of plants, as the target species of this study *A. maritimus*, although they differ in many physical, chemical and biological characteristics. Thus it is not rare to detect common morphotypes of AM fungi in both zones although also exclusive morphotypes can be found.

Spores were isolated directly from the field samples and from one year old bait cultures, since it is well known that the use of successive and extended trap cultures detects higher number of species than extraction of spores directly from field soil (e.g. Ferrol et al. 2004). Actually, low AMF species richness was often reported for arid and semiarid ecosystems by extractions of spores from field soil, reflects limitations in the sporulation patterns under field conditions (e.g. Stutz and Morton 1996; Stutz et al. 2000), but this might depend also on the sampling and isolation effort (Bashan et al. 2007). Nevertheless, we found high AMF species richness at both sites, which was certainly reasoned in the concomitant extensive analyses of field and bait culture samples

In both, salt marsh and dune, similar spore morphotypes corresponding to the following species were recognized: *Funneliformis coronatus, Fu. mosseae, Glomus badium, Gl. intraradices, Gl. macrocarpum, Gl.* sp. BEV2, *Gl. microaggregatum, Septoglomus constrictum, Claroideoglomus claroideum, Cl. etunicatum, Diversispora aurantia, Di. versiformis, Diversispora* sp. BEV4, *Racocetra persica, Scutellospora calospora, Archaeospora trappei, Paraglomus occultum.*

However, morphotypes assigned to species such as *Fu. geosporus, Gl.* sp. BEV1, *Gl. microcarpum, Entrophospora* sp. BEV3, *Ra. verrucosa, Ambispora gerdemannii* and *Archaeospora myriocarpa* were only found in plants from the salt marsh, while *Gl. magnicaule, Gl. rubiforme, Di. clara, Acaulospora scrobiculata, Pacispora dominiki* and*, Pa. franciscana* were only detected in the rhizosphere of *A. maritimus* grown in the dune.

Since the plant species is not an experimental variable, the reasons for similarities and differences of the presence of morphotypes should be based on the differential characteristics between both areas. Specifically, the levels of pH, available phosphorus, and salinity, have been described as factors that influence the distribution of AM fungi (Johnson-Green et al. 2001; García and Mendoza 2008).

Similar reasons could also explain the higher density of AMF spores found in the soil sampled in the *A. maritimus* rhizosphere of the salt marsh compared to the rhizospheric samples of the dune at both years of sampling. Another criterion of variability between the dune and the salt marsh is the spore density of specific species that occurs in some cases.

Regarding the similarities and discrepancies at the AMF species found in this study, relevant facts deserve discussion. For instance, it is noteworthy that fungi belonging to the family Glomeraceae were the predominant sporulators in field and bait conditions and also presented the highest number of diversity of morphotypes. These species may be more adapted in adjusting patterns of sporulation to environmental stress conditions (Jacobson 1997). A similar restriction in species composition to mostly fungi belonging to *Glomus* spp. was previously reported in arid and semiarid ecosystems (Jacobson 1997; Stutz et al. 2000).

Previous work indicated that saline soils contain up to 80% of all spores belonging to one single AMF species, *Glomus geosporum* (= *Fu. geosporus*; Carvalho et al. 2001; Hildebrandt et al. 2001; Landwehr et al. 2002; Grzybowska 2004; Sonjak et al. 2009). In agreement with the bibliography, *Fu. geosporus* has been found in the salt marsh, both at field site and trap culture. However, we found a higher AMF species diversity than those published and *Fu. geosporus* was not the most abundant species. In the field samples, *Se. constrictum* was the most abundant species in both habitats while in the bait cultures it only remained predominant in the dune but in the salt marsh *Par. occultum* took its place. Both fungi have been widely described in arid and saline environments: *Se. constrictum* (Requena et al. 1996; Beena et al. 2001; Kowalchuk et al. 2002; Ferrol et al. 2004; Rodríguez-Echeverría and Freitas 2006; Wilde et al. 2009; Hammer et al. 2011), and the genus *Paraglomus* (Ferrol et al. 2004; Tapia-Goné et al. 2008; Alguacil et al. 2011).

Some Glomeraceae species, *Gl. intraradices*, *Fu. mosseae* and *Cl. etunicatum* for instance, have been found in saline environments (Aliasgharzadeh et al. 2001; Landwehr et al. 2002; Tapia-Goné et al. 2008; Sonjak et al. 2009; Wilde et al. 2009; Camprubí et al. 2010; Yang et al. 2010; Hammer et al. 2011). In the Glomerales order,

three species could not been unequivocally identified, suggesting possible new species; two of them belonging to the Glomeraceae family, *Gl.* sp. BEV1 and *Gl.* sp. BEV2, and the other to the Entrophosporaceae family, *Entrophospora* sp. BEV3. The order Diversisporales gathers the other unidentified species, *Diversispora* sp. BEV4, and a new species that have been recently published, *Di. clara* (Estrada et al. 2011). For further identification studies, bait cultures have been established to propagate those unknown fungi.

In this study, only one Acaulosporaceae, *Ac. scrobiculata*, could be found in the dune, where the genus *Pacispora* was very common and widely represented but not in the salt marsh. Controversially Wilde et al. (2009) found a species of the genus *Pacispora*, *Pa. scintillans*, in the rhizosphere of *Puccinelia maritima* in Terschelling salt marsh.

Our results also report the occurrence of *Racocetra persica* among other Gigasporales morphotypes. This species has been recovered in the Northwestern coast of Italy (Turrini et al. 2008) and had previously been described from other sand dunes (Koske and Walker 1985; Blaszkowski and Tadych 1997; Rodríguez-Echeverría and Freitas 2006; Camprubí et al. 2010). Contrary to some results, we did not find any *Gigaspora* species in our samples while other authors found several morphotypes (Selvaraj and Kim 2004; Rodríguez-Echeverría and Freitas 2006; Tapia-Goné et al. 2008). The latter may be explained according to Turrini et al. (2008) who pointed out that *Gigaspora* species are rare in ecosystems with anthropogenic disturbance.

All the information from this study should be considered when designing the AM fungal inoculum composition for revegetation programs both in natural and agricultural lands that have become salinized. Schwartz et al. (2006) discussed the use of appropriate inoculum in restoration programmes and showed that the application of inappropriate inoculum could result in the upcoming or survival of invasive plant species and the out-competition of native fungal strains. Thus, the use of autochthonous fungi should be favoured. Several studies showed the better benefit of native than non native AMF isolates, which appear to be physiologically and genetically adapted to the stress conditions of the target environment, in the inoculation strategies used in revegetation programs of degraded ecosystems (Herrera et al. 1993; Ferrol et al. 2004; Moora et al. 2004; Renker et al. 2004; Oliveira et al. 2005; Querejeta et al. 2006; Alguacil et al. 2011). In addition to that, it may be advisable to use not only one single AMF species for restoration practices but a mix of a locally adapted AMF community in order to be able to support a diverse plant cover (Klironomos 2003; Vogelsang et al. 2006).

This study confirms the existence of a rich diversity of AM fungi in the rhizosphere soils of Spanish saline Mediterranean ecosystems. As inadequate inocula should be avoided due to possible negative effects on plant species and the plant community, there is a need to analyze the fungal community associated with target plant species in its natural environment. In restoration programmes, indigenous inocula should be favoured over exotic fungi, as the consequences of introduction of non-native fungi on plant species and plant community structure cannot be precisely predicted. Therefore, this

information should be considered when designing the AM fungal inocula composition for revegetation programs in order to maximize the potential benefits that these microorganisms can provide for the establishment of the selected plant species to the threatened ecosystem.

5. Conclusions

The study reveals a high taxonomic diversity of AM fungi in the rhizosphere of *A. maritimus*. The Glomerales order were the predominant sporulators under field and bait conditions and also presented the highest number of diversity of morphotypes. Common AM fungi morphotypes both in dunes and salt marshes were found, suggesting the importance, not only of soil characteristics but the host plant. The genus *Pacispora* was very common and widely represented in dunes but not in salt marshes and we could not identify any spore belonging to *Gigaspora* genus. One new species, *Diversispora clara*, has been recently described and published and four other species are still under study. The diversity work performed supports the potential for exploiting the natural AM fungal diversity as inoculum sources to be used in revegetation programmes in arid and saline ecosystems.

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CAPÍTULO 2

CHAPTER 2

Diversispora clara (*Glomeromycetes*) – a new species from saline dunes in the Natural Park Cabo de Gata (Spain)

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Diversispora clara (*Glomeromycetes*) – una nueva especie de dunas salinas del Parque Natural Cabo de Gata (España)

Resumen

En las dunas del Parque Natural Cabo de Gata (Almería, Andalucía, sur de España) se encontró una nueva especie de *Diversispora* (*Glomeromycetes*) en la rizosfera de *Asteriscus maritimus*, una especie vegetal especialmente adaptada a ambientes salinos. La nueva especie de hongo formador de micorrizas arbusculares forma esporas blancas brillantes que tienen un tamaño de 79-130 × 75-125 micras y tienen una pared que consta de tres capas. La hifa sustentoria es como la típica de muchas *Diversispora* spp., de pared delgada, hialina y cilíndrica (o rara vez constreñida) y flexible y frágil por debajo de los septos que separan el contenido de esporas y de hifas. Los septos se forman regularmente en la base de las esporas o, con menor frecuencia, en la hifa sustentoria a corta distancia de la base de las esporas. El análisis filogenético del ITS y parte del gen ribosómico 28S confirman que *D. clara* forma un clado independiente y monofilético dentro de *Diversispora*.

Palabras clave: *Glomus*, *Glomeromycota*, Europa, ambientes extremos, ADNr

Diversispora clara (*Glomeromycetes*) – a new species from saline dunes in the Natural Park Cabo de Gata (Spain)

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Abstract

A new species of *Diversispora* (*Glomeromycetes*) was found in saline sand dunes of the Natural Park Cabo de Gata (Almería, Andalucía, Southern Spain) in the rhizosphere of *Asteriscus maritimus*, a plant species especially adapted to saline environments. The new fungal species forms brilliant white spores that are 79-130 × 75-125 µm and have one wall consisting of three layers. The subtending hyphae are, as typical for many *Diversispora* spp., thin-walled, hyaline, and cylindrical (or rarely constricted) and flexible and fragile below the septa separating the spore and hyphal contents. The septa form regularly at the spore bases or, less frequently, in subtending hyphae at short distances from the spore base. Phylogenetic analyses of the ITS and partial 28S ribosomal gene confirm that *D. clara* forms a monophyletic, independent clade within *Diversispora*.

Key words: *Glomus*, *Glomeromycota*, Europe, extreme environments, rDNA.

Introduction

During recent studies on arbuscular mycorrhiza (AM) fungal diversity in sand dune systems of the Cabo de Gata Natural Park in Almería (Spain), a brilliant-white new glomeromycotean fungus was recovered from the rhizosphere of *Asteriscus maritimus*, a plant species characteristic of saline Mediterranean environments with elevated soil electrical conductivity. The new fungus formed spores in AM fungal bait cultures predominantly in the *Asteriscus maritimus* rhizosphere. The aims of the present study were to analyze this particular fungus applying combined morphological and molecular tools and to describe its characteristics.

Material and methods

Soil and plant sampling

Soil samples were taken in February 2010 from the rhizosphere of ten *Asteriscus maritimus* plants growing in a natural sand dune system of the Natural Park Cabo de Gata in Almería (Andalucía, Spain). The site is located at 36°44´41´´N 02°07´26´´W. The samples, air-dried in the laboratory, were used to analyze selected chemical soil parameters (pH, soil organic carbon, total nitrogen, soil electrical conductivity) and spore populations The ten plants and the surrounding rhizosphere soil were also extracted and used as bait cultures for propagation of AM fungal communities indigenous to the natural sand dune system. At the site, the soil was sandy and with pH

(H_2O) 8.2, organic carbon 15.3 g kg^{-1}, total nitrogen 1.9 g kg^{-1}, available P 27.0 mg kg^{-1}, and soil electrical conductivity 0.5 dS m^{-1}.

AM fungal bait cultures

Bait cultures were established and maintained as described in Palenzuela et al. (2008, 2011) by transplanting 10 *Asteriscus maritimus* plants with their rhizosphere soils into 1 L pots and transferring them to the greenhouse at EEZ in Granada immediately after sampling. The pots were irrigated three times per week and fertilized every four weeks with Long-Aston nutrient solution (Hewitt 1966). Pure cultures of the new fungus were initiated in a mixed-culture of three *Allium porrum* L. and three *Hieracium pilosella* L. plantlets in 750 mL pots grown together at three locations in the pots. The plant rhizosphere was inoculated with 20 spores per pot and pot location. A sterile mixture of Terragreen (American aluminum oxide, Oil Dry US special, type III R; Lobbe Umwelttechnik Iserlohn, Germany) and Loess (mixture 3:1; with pH-KCl 6.2; organic carbon 0.3%; available P (Na-acetate) 2.6 mg kg^{-1}; available K (Na-acetate) 350 mg kg^{-1} was chosen as culture substrate (Oehl et al. 2002). So far, pure cultures of the new fungus have not been obtained.

Morphological analyses

AM fungal spores were separated from the soil samples by a wet sieving process (Sieverding 1991). The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acidglycerol (PVLG; Koske and Tessier 1983); a mixture of PVLG and Melzer's reagent (Brundrett et al. 1994); a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain 1990). The spore structure terminology follows Oehl et al. (2003, 2005, 2011a) for species with glomoid or diversisporoid spore formation. Photographs (Figs. 1-10) were taken with a Leica DFC 290 digital camera on a Leitz Laborlux S compound microscope using Leica Application Suite Version V 2.5.0 R1 software. Specimens mounted in PVLG and the PVLG+Melzer's mixtures were deposited at the herbaria Z+ZT (ETH Zurich, Switzerland), GDA-GDAC (University of Granada, Spain), and URM (Federal University of Pernambuco, Recife).

Molecular analyses

Five spores isolated from the trap cultures were surface-sterilized with chloramine T (2%) and streptomycin (0.02%) (Mosse 1962) and crushed with a sterile disposable micropestle in 40 µL milli-Q water (Ferrol et al. 2004). PCRs of the crude extracts were obtained in an automated thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Foster City, California) with a pureTaq Ready-To-Go PCR Bead (Amersham

Biosciences Europe GmbH, Germany) following manufacturer's instructions with 0.4 µM concentration of each primer. A two-step PCR amplified the partial SSU, ITS1, 5.8S, ITS2 and partial LSU rDNA ribosomal fragment using the SSUmAf/LSUmAr and SSUmCf/LSUmBr primers consecutively (Krüger et al. 2009). The second PCR products were separated electrophoretically on 1.2% agarose gels stained with Gel Red™ (Biotium Inc., Hayward, CA, U.S.A.) and viewed by UV illumination. The band of the expected size was excised with a scalpel and the amplified DNA was isolated from the gel with the QIAEX II Gel Extraction kit (QIAGEN, Valencia, CA, USA), cloned into the PCR2.1 vector (Invitrogen, Carlsbard, CA, USA), and transformed into one shot TOP10 chemically competent *Escherichia coli* cells. After plasmid isolation from transformed cells, cloned DNA fragments were sequenced with vector primers (White et al. 1990) in both directions by Taq polymerase cycle sequencing on an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST (Altschul et al. 1990). The new sequences were deposited in the EMBL database under the accession numbers FR873629-FR873633.

PHYLOGENETIC ANALYSES: The AM rDNA ITS1+5.8S +ITS2 fungal sequences were aligned in ClustalX (Larkin et al. 2007) and edited with BioEdit (Hall 1999) to obtain a final alignment with *Acaulospora laevis* Gerd. & Trappe and *A. lacunosa* J.B. Morton as outgroups. Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (Milne et al. 2004). Bayesian (two runs over 1 × 106 generations with a burn in value of 2500) and maximum likelihood (1000 bootstrap) analyses were performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guindon & Gascuel 2003) respectively, launched from Topali 2.5, using the GTR + G model. Neighbor-joining (established with the model cited above) and maximum parsimony analyses were performed using PAUP*4b10 (Swofford 2003) with 1000 bootstrap replications.

Results

***Diversispora clara* Oehl, B. Estrada, G.A. Silva & Palenz., sp. nov.** **Figs 1-10**

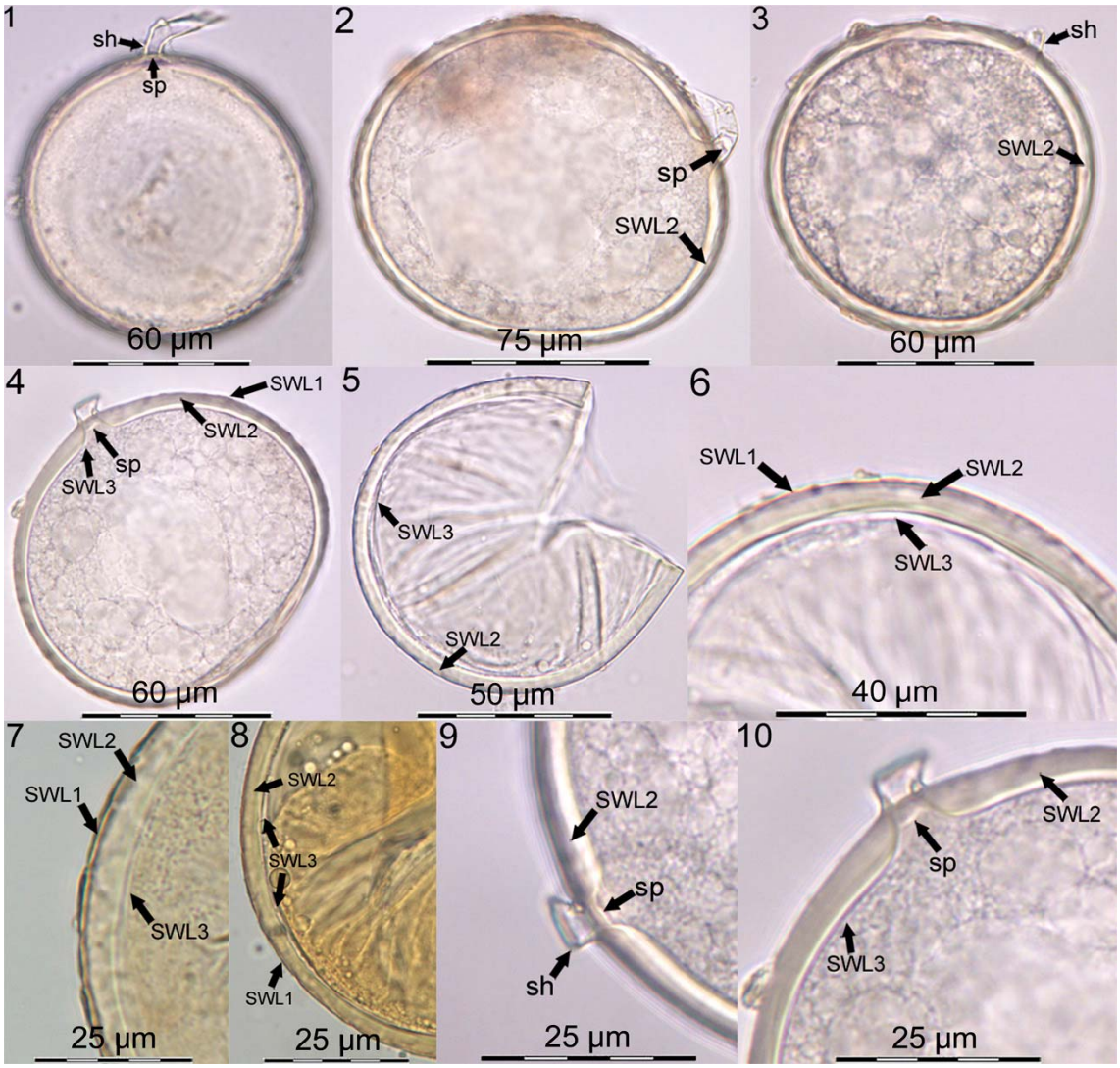
MycoBank MB 561583

Sporae albae, 79–130 × 75–125 µm, tunica tribus stratis, 4.3–8.4 µm. Stratum exterior hyalinum; stratum medium, album ad rarum ochreo-album, 2.8–5.4 µm crassum; stratum interius album. Hypha adhaerenta, 8–13 µm in diametrum. Tunica hyphae 1.0–1.8 µm crassa. Strata medium interiusque septo porum occludentes. Strata exterior mediumque flava colorantes Melzeri.

TYPE: Spain. Andalucía, Almería, Cabo de Gata Natural Park, sand dune, from the rhizosphere of *Asteriscus maritimus* (L.) Less. (*Asteraceae*), isolation date

February 2011, by B. Estrada (**holotype**, ZT Myc 3796 [permanent slide 20-2001]; **isotypes**, ZT Myc 3797 [permanent slides 20-2003 to 20-2012], GDA-GDAC [permanent slides 20-2013 to 20-2018], URM [permanent slides 20-2019, 20-2020]).

ETIMOLOGY: *clara* (Latin = clear, bright, brilliant, light), referring to the brilliant white spores.



FIGS 1-10. *Diversispora clara*: 1-4. Uncrushed spores with a triple-layered spore wall (swl1-3), a single, cylindrical subtending hypha (sh), and a septum (sp) arising directly or at some distance from the base. Figs 5–6. Spores crushed to show triple layered spore wall (swl1-3). FIGS 7-8. Spore wall structure in PVLG + Melzer's reagent. The swl1 or swl2 (or both) stain light to dark yellow in Melzer's, while no such staining reaction is seen on swl3. However, the yellow stain is usually most noticeable in the spore cell contents. FIG. 9. Septum at spore base formed by swl2 (swl3 is not visible in uncrushed spore). FIG. 10. Septum at spore base formed by swl3.

SPORES (Figs 1-4) singly formed in rhizosphere soils, terminally on subtending hyphae, globose (80-110 µm diam.) to subglobose (79-130 × 75-125 µm), one-walled, brilliant to creamy white.

SPORE WALL 4.3-8.4 µm thick, three-layered (swl1, swl2, swl3; Figs 5-7); outer layer (swl1) hyaline, 0.8-1.5 µm thick, evanescent and thus often absent in mature spores; second layer (swl2) bright to (rarely) creamy white, laminated, 2.8-5.4 µm thick (Fig. 7) in uncrushed spores (sometimes ≤ 6.6 µm in crushed spores when pressure is applied on the cover slip); inner layer (swl3) brilliant white, 0.7-1.5 µm thick (usually tightly adherent to swl2 and difficult to observe when < 1.0 µm; see Figs 5-6); both swl1 and swl2 generally light to dark yellow in Melzer's reagent (Figs 7-8, with spore cell contents often a more intense yellow than the surrounding cell wall layers (Fig. 8).

SUBTENDING HYPHAE (sh) generally singly on spores; brilliant white (Figs 1-3), cylindrical or (rarely) slightly constricted at the spore base, 4.0-8.0 µm broad tapering to 3.2-6.1 µm within 100 µm of spore base, although the distance may appear shorter (4.0-10(-25) µm) because the sh walls taper from 1.0-1.8 µm thick to 0.6-1.2 µm within the first 10 µm from the base causing the flexible fragile portion to break from the mature spore (Figs 3, 4, 9) at a point where the septum separates the spore contents from the hyphal contents. Spore pores at the spore bases or in the sh normally closed by a septum (Figs 1-2, 4) arising from swl2 (Fig. 9), swl3 (Fig. 10), or both layers (not shown); pores rarely open (Fig. 3).

DISTRIBUTION: Known only in the natural sand dune system of the Cabo de Gata Natural Park in Andalucía in the rhizosphere of *Asteriscus maritimus*.

MOLECULAR ANALYSES: Phylogenies derived from ITS (Fig. 11) and 28S (data not shown) rDNA analyses cluster the new fungus within the *Diversisporaceae* in a well-separated clade adjacent to several other *Diversispora* species, *Otospora bareae,* and *Tricispora nevadensis* (Oehl et al. 2011b).

Discussion

Our morphological analyses, in particular those of the subtending hyphae and spore bases, clearly support the new fungus in *Diversispora*, with many characters identical to those of other *Diversispora* species (e.g., *D. spurca, D. eburnea;* see Oehl et al. 2011a,b) such as the thin-walled, hyaline, cylindrical (or rarely constricted) subtending hyphae that appear flexible and fragile behind the septum that closes the pore at the spore base or in short distance from the base. In *Diversispora* species with pigmented spores, the subtending hyphae regularly change color conspicuously, becoming hyaline to white behind the septum (Oehl et al. 2011a). This color change,

however, was not confirmed for *D. clara*, since the new fungus generally does not form pigmented spores.

Molecular analyses confirm the species as new: in the phylogenetic tree, *D. clara* clusters in a independent, monophyletic clade within a polyphyletic *Diversispora*. These sequence analyses also confirm unequivocally using morphology to identify diversisporoid species within the *Glomeromycota* (Oehl et al. 2011a,c).

Including *D. clara*, there are now 14 *Diversispora* spp. known in the *Glomeromycetes* (Oehl et al. 2011a,b). The 9 species that form significantly pigmented, yellow brown to brown or orange to orange brown spores are *D. arenaria* (Błaszk. et al.) Oehl et al., *D. aurantia* (Błaszk. et al.) C. Walker & A. Schüssler, *D. epigaea* (B.A. Daniels & Trappe) C. Walker & A. Schüssler, *D. insculpta* (Błaszk.) Oehl et al., *D. przelewicensis* (Błaszk.) Oehl et al., *D. pustulata* (Koske et al.) Oehl et al., *D. tenera* (P.A. Tandy) Oehl et al., *D. trimurales* (Koske & Halvorson) C. Walker & A. Schüssler, and *D. versiformis* (P. Karst.) Oehl et al. (Oehl et al. 2011a,b). *Diversispora celata* C. Walker et al. forms triple-layered, ochre to ivory to pinkish cream spores (Gamper et al. 2009). Only *D. spurca* (C.M. Pfeiff. et al.) C. Walker & A. Schüssler, *D. Eburnea* (L.J. Kenn. et al.) C. Walker & A. Schüssler, and *D. gibbosa* (Błaszk.) Błaszk. & Kovács form hyaline to subhyaline spores that are, however, never brilliantwhite as observed for *D. clara*. Moreover, *D. spurca* and *D. eburnea* have bilayered spore walls (Kennedy et al. 1999), *D. gibbosa* has a five-layered wall (Błaszkowski 1997), and *D. clara* has a three-layered wall.

In the past, large-spored or sporocarpic AM fungi were described from sand dune systems, such as *Gigaspora*, *Scutellospora*, *Pacispora* or *Glomus* species that were generally easy to isolate and recognize from field samples (Koske & Gemma 1995, Gemma et al. 1989, Błaszkowski 1994). Likewise in the current Cabo de Gata Natural Park sand dune system, where *Funneliformis coronatus* (Giovann.) C. Walker & A. Schüssler, *F. mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüssler, *Scutellospora calospora* (T.H. Nicolson & Gerd.) C. Walker & F.E. Sanders, *Racocetra persica* (Koske & C. Walker) Oehl et al., and *Glomus macrocarpum* Tul & C. Tul. were identified (Estrada, unpublished). When sand dune AM fungal communities were maintained and reproduced in bait cultures, small-spored *Glomus* spp. were also sometimes detected (e.g. Błaszkowski et al. 2009a,b, 2010). It was supposed that species with small, quickly degrading spores were difficult to recover or identify only from field samples. This might also be true for *D. clara*, even though its laminated wall structure is clearly persistent. It will be interesting to see whether future taxonomists will be able to identify the new species directly from field samples, now that the existence and morphology of this unique, brilliant-white, conspicuous but small-spored species is known. Morphological spore and molecular root and spore analyses will hopefully tell us more about the ecology and biogeography of this fungus that is thus far known only from a single *Asteriscus maritimus* rhizosphere in the Natural Park Cabo de Gata of Almería in southern Spain.

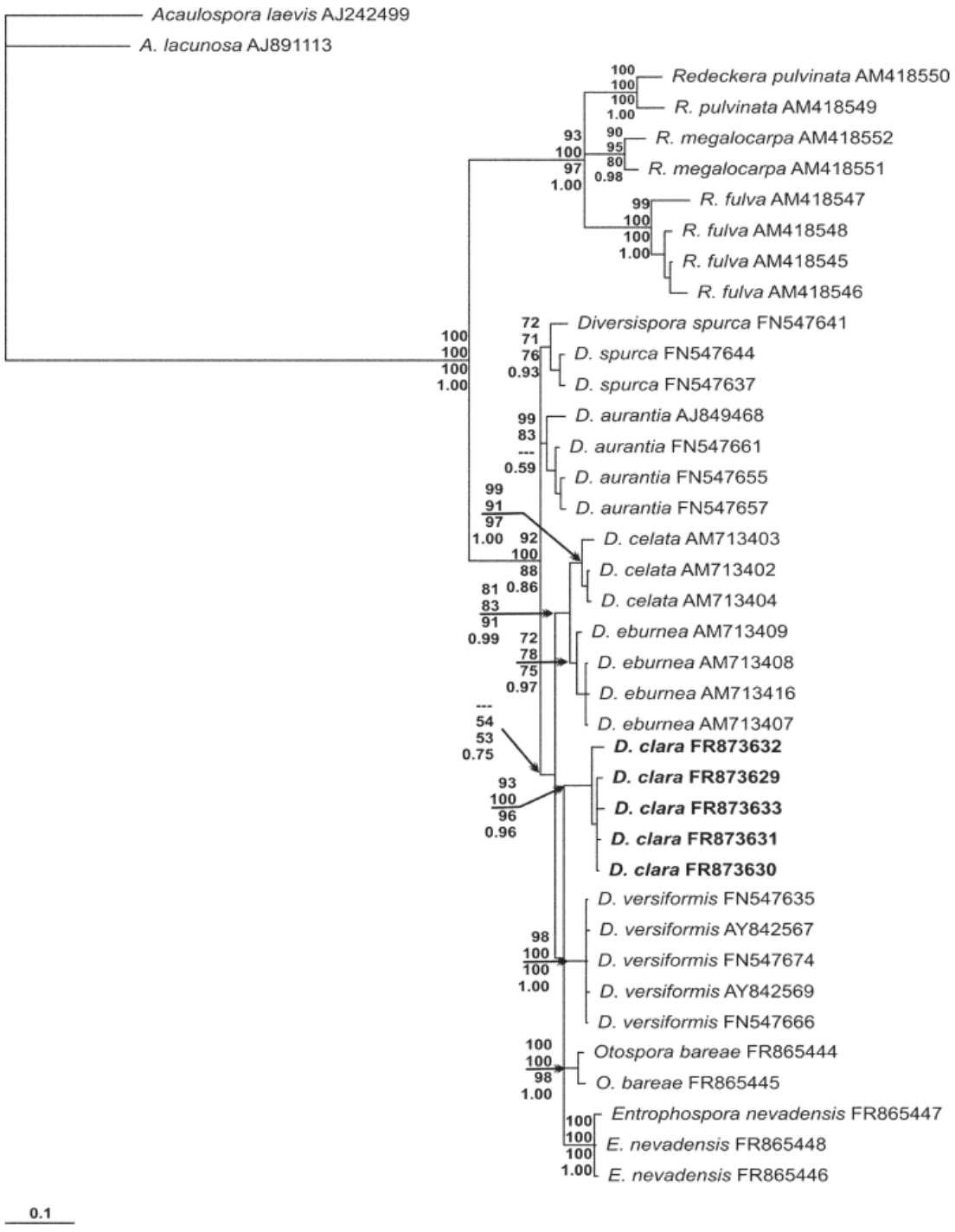


FIG. 11. *Diversisporaceae.* ITS rDNA-based phylogenetic tree rooted by *Acaulospora laevis* and *A. lacunosa*. Sequences are labeled with database accession numbers. Support values are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. *Diversispora clara* sequences are in bold. Only topologies with ≥ 50% bootstrap values are shown. (Consistency Index = 0.72; Retention Index = 0.84).

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CAPÍTULO 3

CHAPTER 3

A native *Glomus intraradices* strain from a Mediterranean saline area exhibits salt tolerance and enhanced symbiotic efficiency with maize plants under salt stress conditions

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Un aislado de *Glomus intraradices* nativo de un área Mediterránea salina muestra tolerancia a la salinidad y elevada eficiencia simbiótica en plantas de maíz bajo condiciones de estrés salino

Resumen

Los hongos micorrícicos arbusculares (MA) se ha visto que existen de forma natural en ambientes salinos y se ha sugerido que las diferencias en el comportamiento de estos hongos y su eficiencia puede ser debida al origen y la adaptación del hongo MA. Estos resultados invitan a buscar especies de MA aisladas en ambientes salinos y comparar sus mecanismos de tolerancia a la salinidad con aquellas especies de áreas no-salinas. Para ello un aislado de *G. intraradices* (Gi CdG) perteneciente a una región con graves problemas de salinidad y afectada por la desertificación se ha comparado con un aislado de colección de la misma especie, que se usa habitualmente como hongo modelo. Un experimento *in vitro* comprobó la capacidad de ambos hongos MA de crecer bajo niveles crecientes de salinidad en el medio y un experimento *in vivo* comparó su eficiencia simbiótica con las plantas de maíz cultivadas bajo condiciones de estrés salino. El aislado Gi CdG se desarrolló mejor bajo condiciones de salinidad e indujo considerablemente la expresión de los genes *GintBIP*, *Gint14-3-3* y *GintAQP1*, si bien mostró una inducción más baja del gen *GintSOD1* que la cepa de colección *G. intraradices*. El aislado Gi CdG también estimuló el crecimiento de las plantas de maíz más que la cepa de colección bajo dos niveles de salinidad. La mayor eficiencia simbiótica de Gi CdG fue corroborada por el aumento de la eficiencia del fotosistema II y la conductancia estomática y la menor pérdida de electrolitos mostrada por plantas de maíz bajo las diferentes condiciones probadas. La mayor tolerancia a la salinidad y la eficiencia simbiótica exhibida por el aislado Gi CdG en comparación con el *G. intraradices* de colección puede ser debido a una adaptación del hongo a los ambientes salinos. Tal adaptación puede estar relacionada con la importante inducción de la expresión de los genes que codifican para chaperonas o para acuaporinas del hongo. El presente estudio muestra que los hongos MA aislados en zonas afectadas por salinidad pueden ser una herramienta poderosa para mejorar la tolerancia de los cultivos a condiciones de estrés salino.

Palabras clave: Adaptación, micorriza arbuscular, monoxénico, salinidad, eficiencia simbiótica

A native *Glomus intraradices* strain from a Mediterranean saline area exhibits salt tolerance and enhanced symbiotic efficiency with maize plants under salt stress conditions

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Abstract

Aims:
Arbuscular mycorrhizal (AM) fungi have been shown to occur naturally in saline environments and it has been suggested that differences in fungal behaviour and efficiency can be due to the origin and adaptation of the AM fungus. These findings invite to look out for AM fungal species isolated in saline environments and compare their salt-tolerance mechanisms with those of species living in non-saline areas.
Methods:
A fungal strain of *G. intraradices* (Gi CdG) isolated from a region with serious problems of salinity and affected by desertification, has been compared with a collection strain of the same species, used as a model fungus. An *in vitro* experiment tested the ability of both AM fungi to grow under increasing salinity and an *in vivo* experiment compared their symbiotic efficiency with maize plants grown under salt stress conditions.
Results:
The isolate Gi CdG developed better under saline conditions and induced considerably the expression of *GintBIP*, *Gint14-3-3* and *GintAQP1* genes, while it showed a lower induction of *GintSOD1* gene than the collection *G. intraradices* strain. The isolate Gi CdG also stimulated the growth of maize plants under two levels of salinity more than the collection strain. The higher symbiotic efficiency of Gi CdG was corroborated by the enhanced efficiency of photosystem II and stomatal conductance and the lower electrolyte leakage exhibited by maize plants under the different conditions assayed.
Conclusions:
The higher tolerance to salinity and symbiotic efficiency exhibited by strain Gi CdG as compared to the collection *G. intraradices* strain may be due to a fungal adaptation to saline environments. Such adaptation may be related to the significant up-regulation of genes encoding chaperones or genes encoding aquaporins. The present study remarks that AM fungi isolated from areas affected by salinity can be a powerful tool to enhance the tolerance of crops to saline stress conditions.

Key words: adaptation, arbuscular mycorrhiza, monoxenic culture, salinity, symbiotic efficiency

Introduction

Soil salinity exists naturally on Earth, but inadequate cultivation practices, mainly due to excess irrigation (Zhu 2001; Tester and Davenport 2003; Flowers 2004) have also exacerbated growing concentration of salts in the rhizosphere (Mahajan and Tuteja 2005). This is a major concern in some parts of the world, particularly in arid and semiarid areas, where evaporation greatly exceeds precipitation and salts dissolved in the ground water reach and accumulate at the soil surface through capillary movement (Kohler et al. 2010). Excessive soil salinization affects negatively the establishment, growth and development of most plants and also of rhizosphere microbiota (Rietz and Haynes 2003), leading to huge losses in plant productivity and diversity (Evelin et al. 2009). Some estimations suggest that salinization of arable land will result in 30% land loss within next 25 years and up to 50% within next 40 years (Wang et al. 2003).

Salinity stress in plants depends on three main components: an initial osmotic stress due to the reduction in the osmotic potential of the soil solution reduces the amount of water available to the plant, causing physiological drought. This obliges the plant to maintain lower internal osmotic potentials in order to prevent water movement from roots into the soil (Feng et al. 2002; Jahromi et al. 2008). Secondly, the accumulation of toxic ions, such as sodium and chloride, negatively affects cellular metabolism (Munns et al. 2006). The toxic effects include disruption to the structure of enzymes and other macromolecules, damage to cell organelles and plasma membrane, disruption of photosynthesis, respiration and protein synthesis (Porcel et al. 2012). Finally, salinity produces nutrient imbalance in the plant caused by decreased nutrient uptake and/or transport to the shoot leading to ion deficiencies (Marschner 1995; Adiku et al. 2001).

Salinity also induces an increase in the production of reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$). These cytotoxic ROS can destroy normal metabolism through oxidative damage of lipids, proteins, and nucleic acids when they are produced in excess (Miller et al. 2010). Thus, efficient mechanisms are needed to prevent the possible oxidative damage to cellular components.

As a consequence of all the above mentioned processes, salt stress affects plant metabolism, including growth, photosynthesis, protein synthesis, and energy and lipid metabolisms (Ramoliya et al. 2004). However, plants have evolved several biochemical and molecular mechanisms to cope with the negative effects of salinity (Türkan and Demiral 2009). In addition, besides the intrinsic adaptation mechanisms developed by plants, in their natural environment they are associated to both saprophytic and endophytic microorganisms, which can improve plant performance under stressful conditions (Barea et al. 2005; Aroca and Ruiz-Lozano 2009).

Arbuscular mycorrhizal (AM) fungi are widespread microorganisms associated symbiotically with the roots of 80% of terrestrial plants (van der Heijden et al. 1998;

Smith and Read 2008). In the AM symbiosis, plants get nutrients and water resources that are less available to the plant roots from the fungi, while the fungi receive carbon compounds from the plant and find a niche to complete their life cycle (Koide and Mosse 2004). At the same time, AM symbiosis enhances plant tolerance to different abiotic stresses with osmotic components such as drought and salinity (Augé 2001; Ruíz-Lozano 2003; Ruíz-Lozano et al. 2006; Jahromi et al. 2008). Although it is clear that AM fungi mitigate growth reduction caused by salinity, the mechanism involved remains unresolved (Ruiz-Lozano et al. 2012). Moreover, some studies reveal that salinity affects directly the fungal development, reducing fungal mycelia formation and host root colonization (Poss et al. 1985; Juniper and Abbott 2006; Giri et al. 2007; Sheng et al. 2008). Contrary to those reports, a few other studies reported no reduction or even increasing fungal development (Hartmond et al. 1987; Aliasgharzadeh et al. 2001; Yamato et al. 2008). This may be related to evolved mechanisms that allow specific AM fungi to have a higher tolerance to salinity. In fact, mycorrhizal fungi have been shown by several workers to occur naturally in saline environments (Juniper and Abbott 1993) and Copeman et al. (1996) suggested that differences in fungal behaviour and efficiency can be due to the origin of the AM fungus (Ruíz-Lozano and Azcón 2000). These results invite to look out for AM fungal species isolated in saline environments and compare their salt-tolerance mechanisms with those of species living in non-saline areas (Porcel et al. 2012).

The main problem to elucidate the mechanisms that allow specific AM fungi to tolerate salinity is that salt tolerance is a multigenic and complex trait which involves many physiological and biochemical mechanisms that vary between species (Mian et al. 2011). Thus, when examining putative salt tolerant AM fungi, several aspects involved in the protection against damage caused by ROS, altered water content or protein inactivation should be evaluated. In this regard, it is known that to overcome the oxidative damage generated by stresses such as salinity, all living organisms have developed antioxidant systems to efficiently scavenge ROS excess. Little is known about the antioxidant responses in the AM fungi, but studies have demonstrated that AM fungi possess ROS scavenging systems. These include genes encoding for superoxide dismutases (SOD) (Lanfranco et al. 2005; González-Guerrero et al. 2010) or glutaredoxins (GRXs) (Benabdellah et al. 2009), although their involvement in the fungal responses to salinity have never been studied. On the other hand, aquaporins are proteinaceous pores present in the membranes of all living organisms that facilitate the transport of water and other small and neutral solutes (Forrest and Bhave 2007; Maurel et al. 2008). An aquaporin gene has been described in the AM fungus *G. intraradices* which may have a role in the transport of water from mycelium growing under osmotically favourable conditions to salt-stressed mycelium (Aroca et al. 2009). Finally, protection mechanisms to prevent protein inactivation by salinity can include the activity of 14-3-3 proteins or that of chaperone-like proteins such as luminal binding proteins (BiP). 14-3-3 proteins are binding proteins that regulate the activities of a wide

array of targets via direct protein–protein interactions (Bridges and Moorhead 2004). In plants, these proteins have a role in regulation of development and stress response (Chung et al. 1999; Roberts 2003), including salinity (Wang et al. 2002; Xu and Shi 2006). A gene encoding for a 14-3-3 protein has been described in *G. intraradices* (Porcel et al. 2006), but its involvement in the fungal response to salinity has not been investigated so far. Luminal binding proteins (BiPs) are molecular chaperons present in all kingdoms and their role in the endoplasmic reticulum is to transiently bind to unfolded proteins to prevent intramolecular and intermolecular interactions that can result in permanent misfolding or aggregation, with the subsequent loss of their function (Gething and Sambrook 1992; Hendershot et al. 1996). A gene encoding for a BiP protein has been described in *G. intraradices* but no information is available on its possible biological function, although it was postulated that it could be similar to that of animals or plants (Porcel et al. 2007).

In this study, a strain of *G. intraradices* isolated from Cabo de Gata Natural Park (Almería, Spain), a region with serious problems of salinity and affected by desertification, has been compared with a collection strain, used as a model fungus. Bago et al. (1998b) highlighted monoxenic cultures as an appropriate tool for studying the extraradical phase of AM symbiosis. In the present work we took advantage of the *in vitro* monoxenic culture of AM fungi in order to study under increasing salinity levels the differences in development and in expression of putative stress-responsive genes of two strains of the AM fungus *G. intraradices*. In a parallel study, we conducted an *in vivo* experiment with the same AM fungi and a host plant of agronomic importance such as maize, in order to test their symbiotic efficiencies under increasing salinity levels.

Materials and methods

Identification of the mycorrhizal strain isolated from Cabo de Gata Natural Park

AM fungal spores were separated from the soil samples by a wet sieving process (Sieverding 1991). The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG) (Koske and Tessier 1983); a mixture of PVLG and Melzer's reagent (Brundrett et al. 1994); a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain 1990). For identification of the AMF species, spores were then examined using a compound microscope at up to 400-fold magnification as described by the glomeromycotean classification of Oehl et al. (2011) recently published in the new journal IMA Fungus. The species was clearly identified based on its spore morphology as *Glomus intraradices* (Schenk and Smith 1982), which has been recently reassigned to *Rhizophagus intraradices* (N.C. Schenck and G.S. Sm.) C. Walker & A. Schuessler

2010. Spores presented a globose form, from 65 to 145 µm diameter, and light colour between white and yellow (some old spores had a brownish colour). Up to four layers were observed in some of the samples and some of them presented a hyphal constriction at the base of the spore.

In addition to the morphological identification, a molecular identification was also carried out. For that, spores isolated from the bait cultures were surface-sterilized with chloramine T (2%) and streptomycin (0.02%) and crushed with a sterile disposable micropestle in 40 µL milli-Q water (Ferrol et al. 2004). A two-step PCR was conducted to amplify the AM fungal DNA from the spores. The first PCR step was performed with the universal eukaryote primers NS1 and NS4 region of the small subunit ribosomal gene and the second with the specific AM fungal primers AML1 and AML2 (Lee et al. 2008). The amplified DNA was purified using the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Helthcare, UK). DNA fragments were sequenced on an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST program (Altschul et al. 1990).

The BLAST analysis unambiguously placed *Rhizophagus intraradices* as the closest relative of our *G. intraradices* CdG strain, with sequence accession number FR750209 (Kruger et al. 2012) having a 99% identity.

The AM fungal strain has been incorporated to the collection of Zaidin Experimental Station, Granada, Spain, under accession EEZ 195.

In vitro mycorrhizal cultures

The two *Glomus intraradices* strains used in this study were established in monoxenic culture as described by St-Arnaud et al. (1996). For that, the clone DC2 of carrot (*Daucus carota* L.) Ri-T DNA transformed roots were cultured in two-compartment Petri dishes with the AM fungal strain DAOM 197198 of *G. intraradices* Smith and Schenck [recently reassigned to *G. irregulare* by Stockinger et al. (2009) and then as *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler comb. nov.] or with the strain *G. intraradices* isolated from Cabo de Gata (CdG) Natural Park in Almería (Southeast Spain; located at 36°44´41´´N, 02°07´26´´W).

The root compartment of each plate was filled with sterile minimal medium, as described by Chabot et al. (1992) referred to as “M” throughout this report, to the top of the division wall. The medium had been autoclaved for 20 minutes at 120ºC. The hyfal compartment of each plate was filled in the same manner except that M medium used did not contain any sucrose (referred to as “M-C” in this work). In addition, prior to the sterilization of the M-C medium NaCl had been added to the medium to obtain 0, 75 or 150 mM concentrations of salt. The media were allowed to solidify at room

temperature. There were six different treatments, with 50 replicate plates per treatment, totalling 300 plates.

A piece of M agar (approximately 0.5 cm^2) was cut from the root compartment of each Petri dish and replaced with fungal inoculum, which consisted of a piece of agar of the same size obtained from stock monoxenic cultures containing spores and hyphae of the collection *G. intraradices* strain or the strain *G. intraradices* CdG. The specimens, kept in continuous monoxenic cultures, were provided by the culture collections of Zaidin Experimental Station, Granada, Spain**.** Three to four pieces of transformed carrot roots (2.5 cm length each), grown in M medium, were placed on top of the fungal inoculum in the root compartments. Two weeks after inoculation plates were checked and if roots were crossing onto the distal compartments, they were aseptically moved back to their proximal compartments. This check was subsequently repeated once a week along the experiment. The plates were incubated in the dark at 24ºC for 2 months**.**

Parameters measured

Four, six and eight weeks after inoculation plates were examined under dissecting microscope (Nikon, SMZ 1000, Japan) and AM fungal development was assessed using the method described by Marsh (1971) and modified by Bago and Cano (2005). A transparent 2 mm grid was used to determine the hyphal length, the number of branched absorbing structures (BAS) (Bago et al. 1998a) and the number of spores in three areas of 1 cm^2 per distal compartment of each plate.

RNA extraction from fungal mycelium and synthesis of cDNA

After eight weeks, the mycelium from the distal compartment was isolated. Citric acid monohydrate 10 mM at pH 6 was used to extract the mycelium from the M-C medium. Then it was immersed in liquid nitrogen and stored at -80ºC until RNA was extracted using the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.).

First single-strand cDNA was primed by random hexamers using 100–1,000 ng of DNase-treated RNA. RNA samples were denatured at 65ºC for 5 min and then reverse transcribed at 25ºC for 10 min and 42ºC for 50 min in a final volume of 20 µl containing 10 µl of total RNA, 10 µM random primers (Invitrogen, Carlsbad, CA, USA), 0.5 mM dNTPs, 10 U RNase inhibitor, 4 µl of 5x buffer, 2 µl 0.1 M DTT, and 1 µl of Superscript II Reverse Transcriptase (Invitrogen). The samples were precipitated with 1 (v/v) isopropanol and suspended in 20 µl of water.

Analysis of fungal gene expression

Gene expression analyses were carried out by quantitative reverse transcription (qRT)-PCR using an iCycler iQ apparatus (BioRad, Hercules, CA, U.S.A.). The cDNA samples were standardized to *GintEF*, For (5´-GCTATTTTGATCATTGCCGCC-3´) and Rev (5´-TCATTAAAACGTTCTTCCGACC-3´) (González-Guerrero et al. 2005); and Gint18S rRNA, For, (5′-TGTTAATAAAAATCGGTGCGTTGC-3′) and Rev, (5′-AAAACGCAAATGATCAACCGGAC-3′) (González-Guerrero et al. 2005; Porcel et al. 2006). The same reactions were performed with the specific primers for each of the analyzed genes: *GintSOD1* (For, 5´-GTACTATTACTTTCATTCAGGA-3´ and Rev, 5´-AGTTCATGACCACCTTTACCAA-3´) (González-Guerrero et al. 2005); *Gint14-3-3* (For, 5´-CGCAATCTCCTCTCAGTCGC-3´ and Rev, 5´-GCAATAGCATCATCAAATGC-3´) (Porcel et al. 2006); *GintBiP* (For, 5´-AGATGCTGGCGTAATTGCTGG-3´and Rev, 5´-TGGCGGCACCATATGCAACTG-3´) (Porcel et al. 2007) and *GintAQP1* (For, 5´-AGGACTCGGAGGTAGTGATGC-3´and Rev, 5´-GCCGGATATATCACTCCAAAGC-3´) (Aroca et al. 2009).

Individual real-time RT-PCR reactions were assembled with oligonucleotide primers (0.15 µM each), 10.5 µl of 2x iQSYBR Green Supermix (Bio-Rad; containing 100 mM KCl, 40 mM Tris–HCl pH 8.4, 0.4 Mm dNTPs, 50 U/µl iTaq DNA polymerase, 6 mM MgCl2, 20 nM SYBR Green I, 20 nM fluorescein) plus 1 µl of a 1:10 dilution of each corresponding cDNA in a final volume of 21 µl. The PCR cycling programme consisted of 4 min incubation at 95ºC, followed by 35 cycles of 45 s at 95ºC, 45 s at 60ºC and 45 s at 72ºC, where the fluorescence signal was measured. Experiments were repeated three times, with the threshold cycle (CT) determined in triplicate, using cDNAs originated from three RNAs extracted from three different biological samples, each of them corresponded to a pool of three to five plates. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001). Negative controls without cDNA were used in all PCR reactions.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA) performing first a one-way ANOVA followed by the Tukey test with $P< 0.05$ as the significance cut-off.

***In vivo* experiment**

Soil and biological materials

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) noninoculated control plants, (2) plants inoculated with the

AM fungal strain *G. intraradices* isolated from Cabo de Gata (CdG) and (3) plant inoculated with the model AM fungus *G. intraradices* reproduced at collection of the Zaidin Experimental Station. There were 30 replicates of each inoculation treatment, totalling 90 pots (one plant per pot), so that ten pots of each microbial treatment were grown under nonsaline conditions throughout the entire experiment, while ten pots per treatment were subjected to 66 mM of NaCl and the remaining ten pots per treatment were subjected to 100 mM of NaCl.

Loamy soil was collected from Granada province (Spain, 36º59'34''N; 3º34'47''W), sieved (5 mm), diluted with quartz-sand (<2 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100ºC for 1 h on 3 consecutive days). The original soil had a pH of 8.2 [measured in water 1:5 (w/v)]; 1.5 % organic matter, nutrient concentrations (g kg^{-1}): N, 1.9; P, 1 ($NaHCO_3$-extractable P); K, 6.9. The electrical conductivity of the original soil was 0.5 dS m^{-1}.

Three seeds of maize (*Zea mays*. L) were sown in pots containing 900 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species used were two strains of *Glomus intraradices*, the first one from our culture collection and the second one isolated from Cabo de Gata (Almería, Spain). Appropriate amounts of each inoculum containing about 700 infective propagules (according to the most probable number test), were added to the corresponding pots at sowing time just below maize seeds.

Uninoculated control plants received the same amount of autoclaved mycorrhizal inocula together with a 10 ml aliquot of a filtrate (< 20 μm) of the AM inocula in order to provide a general microbial population free of AM propagules.

Growth conditions

The experiment was carried out under glasshouse conditions with temperatures ranging from 19 to 25ºC, 16/8 light/dark period, and a relative humidity of 70-80%. A photosynthetic photon flux density of 800 μE m^{-2} s^{-1} was measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Water was supplied daily to the entire period of plant growth to avoid any drought effect. Plants were established for 45 days prior to salinization to allow adequate plant growth and symbiotic establishment prior to application of the salt stress. Three concentrations (0, 66, and 100 mM NaCl) of saline solution were reached in the soil substrate by adding appropriate dilutions of a stock 2 M saline solution. The concentration of NaCl in the soil was increased gradually on alternative days to avoid an osmotic shock. It took 8 days, to reach the desired 66 and 100 mM NaCl levels. The electrical conductivities in the soil were 0.25, 6.9 and 9.3 dS

m^{-1} for the salt levels of 0, 66, and 100 mM NaCl, respectively. Plants were maintained under these conditions for additional 30 days.

Parameters measured and statistical analysis

Symbiotic development

The percentage of mycorrhizal root infection in maize plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), as described by Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse 1980).

Biomass production

At harvest (75 days after planting), the shoot and root system were separated and the shoot dry weight (SDW) and root dry weight (RDW) were measured after drying in a forced hot-air oven at 70ºC for two days.

Photosynthetic efficiency

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

Stomatal conductance

Stomatal conductance was measured two hours after light turned on by using a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the third youngest leaf from five different plants from each treatment.

Relative electrolyte leakage

The electrolyte leakage was measured as an index of salt injury to cellular membranes (Verslues et al., 2006). It was calculated on the third leaf of each maize plant from a leaf sample of 3 x 1.5 cm. The initial conductivity was measured with a conductivity metre COND 510 (XS Instruments; OptoLab, Milan, Italy) after subjecting the samples to incubation at 25ºC in 10ml de-ionized water overnight with continuous shaking at 100 rpm. The samples were then autoclaved at 121ºC for 20 min. Final conductivity was measured after the samples had cooled down to room temperature (Verslues et al. 2006).

Results

Experiment *in vitro*

Fungal development under salt conditions

After four week of culture, the hyphal length produced by both AM fungal strains was negatively affected by the presence of 75 and 150 mM NaCl in the medium (Figure 1A). The effect of salinity decreasing hyphal length was similar for the two fungal strains. At six weeks, only 150 mM NaCl decreased significantly the hyphal length of both fungal strains as compared to the control treatment without salt (Figure 1B). Again, both AM fungal strains showed a similar hyphal length decrease. Finally, after eight weeks of culture, Gi CdG did not show hyphal length reduction in response to any of the salt levels applied to the medium (Figure 1C). In contrast, the collection *G. intraradices* strain decreased the hyphal length after addition of 75 mM NaCl (26% of decrease) and 150 mM NaCl (37% of decrease).

The number of spores produced by both fungal strains increased transiently at four weeks after the application of 75 mM NaCl, while under 150 mM NaCl, it showed similar levels than those of the no stress treatment (Figure 2A). At six weeks Gi CdG showed no effect of salt on the number of spores produced, while the collection *G. intraradices* strain increased significantly the number of spores produced after the addition of 150 mM NaCl (Figure 2B). Finally, at eight weeks, both fungal strains enhanced the number of spores produced after the addition of either 75 or 150 mM NaCl, with no significant differences between both strains (Figure 2C).

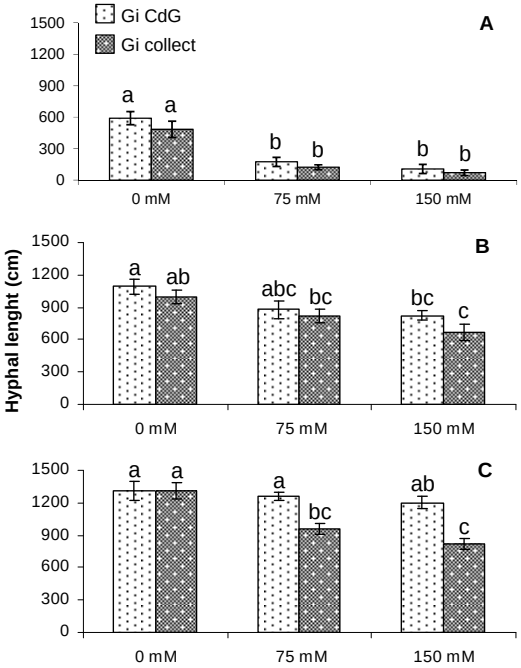

Figure 1. Total hyphal length (cm) formed in the hyphal compartment by two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Measurements were done after 4 weeks (A), 6 weeks (B) or 8 weeks (C) of fungal growth in the medium. Means followed by different letters are significantly different (P<0.05).

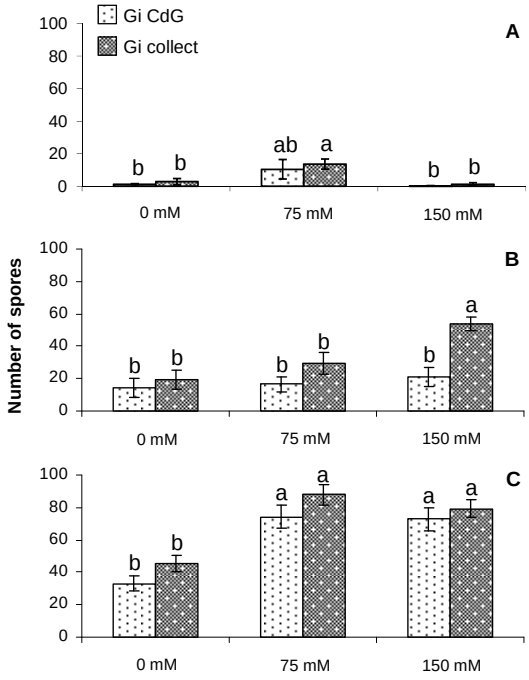

Figure 2. Total number of spores formed in the hyphal compartment by two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Measurements were done after 4 weeks (A), 6 weeks (B) or 8 weeks (C) of fungal growth in the medium. Means followed by different letters are significantly different (P<0.05).

After four weeks of culture, the number of BAS produced was negatively affected by the highest salt level (150 mM) applied, with a similar decrease of this parameter in both fungal strains (Figure 3A). At week 6, salinity influenced differently the number of BAS produced by each fungal strain (Figure 3B). Thus, in Gi CdG, this parameter was decreased by 150 mM NaCl, while the collection *G. intraradices* strain did not show significant changes as a consequence of salt application. However, after eight weeks of fungal culture no significant differences in BAS production were observed among treatments (Figure 3C).

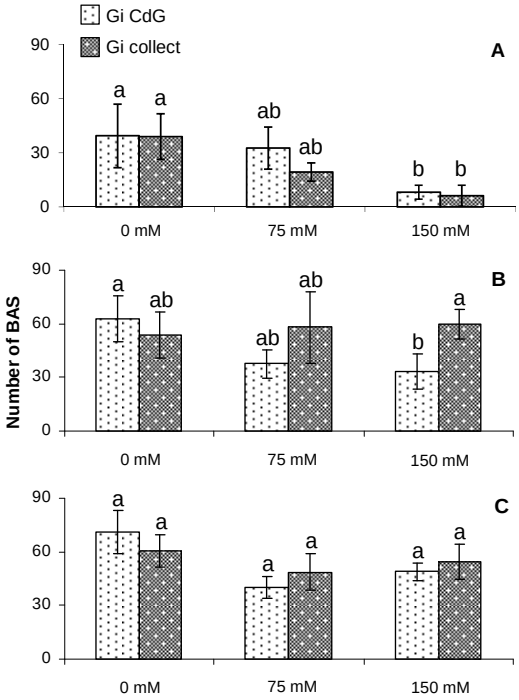

Figure 3. Total number of BAS formed in the hyphal compartment by two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Measurements were done after 4 weeks (A), 6 weeks (B) or 8 weeks (C) of fungal growth in the medium. Means followed by different letters are significantly different ($P<0.05$).

Expression of chaperone-encoding genes

The most remarkable results were those related to the expression of the two chaperone-encoding genes *Gint14-3-3* and *GintBIP* (Figures 4 and 5). The expression of both genes was considerably higher in Gi CdG than in the collection *G. intraradices* strain, even in absence of NaCl in the growing medium (up regulation by 85 fold *Gint14-3-3* and by 96 fold *GintBIP*). The presence of salt in the medium further up regulated the expression of these two genes in Gi CdG, mainly at 150 mM NaCl (up regulation of *Gint14-3-3* by 9 fold as compared to 0 mM NaCl or up regulation of *GintBIP* by 13 fold as compared to 0 mM NaCl). In contrast, in the case of the collection *G. intraradices* strain, only the application of 75 mM NaCl up regulated the

expression of this gene, while the application of 150 mM NaCl down regulated or kept unchanged the expression of *Gint14-3-3* and *GintBIP* genes, respectively.

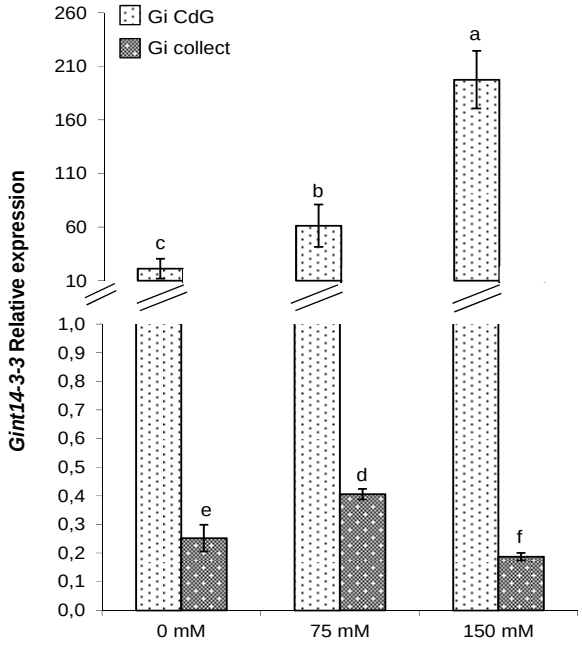

Figure 4. Analysis of *Gint14-3-3* gene expression by real time quantitative RT-PCR in two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Means followed by different letters are significantly different (P<0.05).

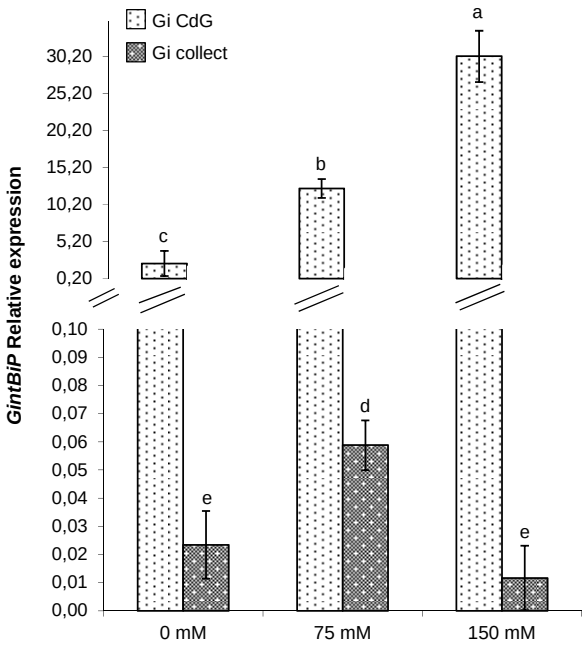

Figure 5. Analysis of *GintBiP* gene expression by real time quantitative RT-PCR in two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Means followed by different letters are significantly different (P<0.05).

Expression of a SOD-encoding gene

We analyzed the expression of a SOD encoding gene in the two *G. intraradices* strains (Figure 6). The results showed that, in contrast to the previous genes, the expression of this gene was higher in the collection *G. intraradices* strain than in Gi CdG, even in absence of salt in the medium (up regulation by 75 fold). The addition of 75 mM of NaCl did not affect the expression of this gene in any of the two fungal strains, while the application of 150 mM NaCl enhanced the expression of this gene both in the collection *G. intraradices* strain (4 fold induction) and in Gi CdG (60 fold induction). In spite of the strong induction of this gene in Gi CdG, the expression level of this gene continued being higher in the collection *G. intraradices* strain.

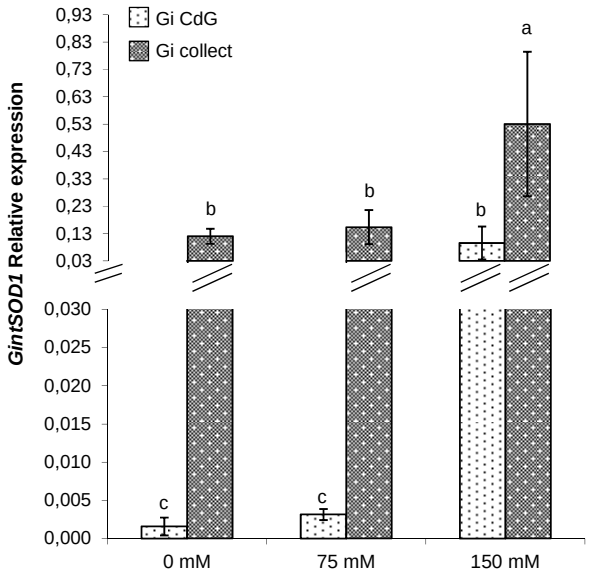

Figure 6. Analysis of *GintSOD1* gene expression by real time quantitative RT-PCR in two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Means followed by different letters are significantly different (P<0.05).

Expression of an aquaporin-encoding gene

The expression of the *GintAQP1* gene in Gi CdG and collection strains increased alter the application of 150 and 75 mM NaCl, respectively. In the collection strain however, addition of 150 mM NaCl to the culture medium caused no significant effect on the expresión of this gene (Figure 7). At 75 mM Na Cl the expression of *GintAQP1* was higher in the collection *G. intraradices* strain than in Gi CdG, while at 150 mM NaCl it was just the opposite.

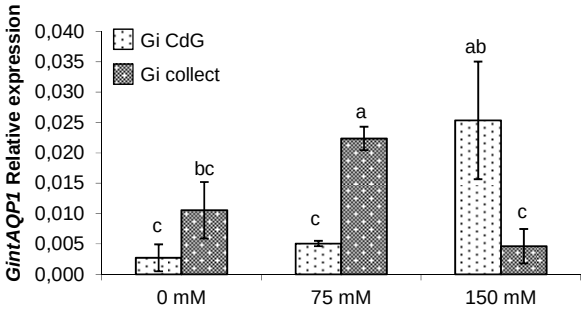

Figure 7. Analysis of *GintAQP1* gene expression by real time quantitative RT-PCR in two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Means followed by different letters are significantly different (P<0.05).

Experiment in vivo

Shoot and root dry weights (SDW and RDW)

Salt stress did not affect significantly SDW in non-AM plants, while both AM treatments reduced SDW at 100 mM NaCl as compared to the non-salt stressed treatment (Figure 8A). In any case, plants inoculated with *G*i CdG exhibited the highest SDW production at all salt levels studied. Indeed, the increases in SDW at 0 and 100 mM NaCl were, respectively, 26 and 17%, as compared to non-AM plants. Plants inoculated with the collection *G. intraradices* strain exhibited similar SDW values than non-AM plants at all salt levels.

The RDW showed no significant changes as a consequence of either salt levels applied or the AM fungal strain inoculated (Figure 8B).

Symbiotic development

The percentage of mycorrhization was significantly higher in plants inoculated with the Gi collection strain than in those inoculated with Gi CdG (Figure 9B). Under the non-saline conditions plants inoculated with the collection *G. intraradices* strain had 67% of mycorrhizal root length. This was significantly lower than at 66 and 100 mM NaCl, where the root infection reached 88% and 84%, respectively. Plants inoculated with Gi CdG reached about 20% mycorrhizal root length, with no significant differences among salt treatments (Figure 9B).

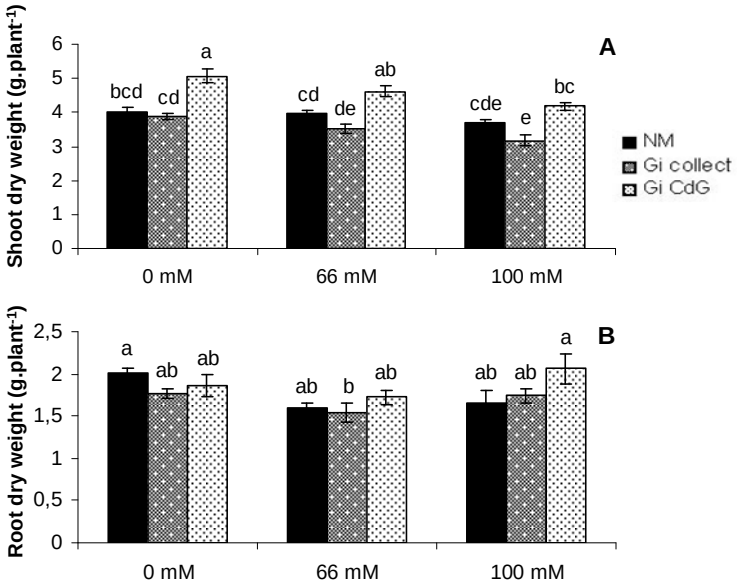

Figure 8. Shoot (A) and root (B) dry weights (g plant^{-1}) in maize plants. Black bars represent noninoculated control plants (NM), grey bars represent plants inoculated with the collection *G. intraradices* strain and white bars represent plants inoculated with the native strain Gi CdG. Plants were subjected to 0, 66 or 100 mM NaCl. Columns with different letters are significantly different (P<0.05).

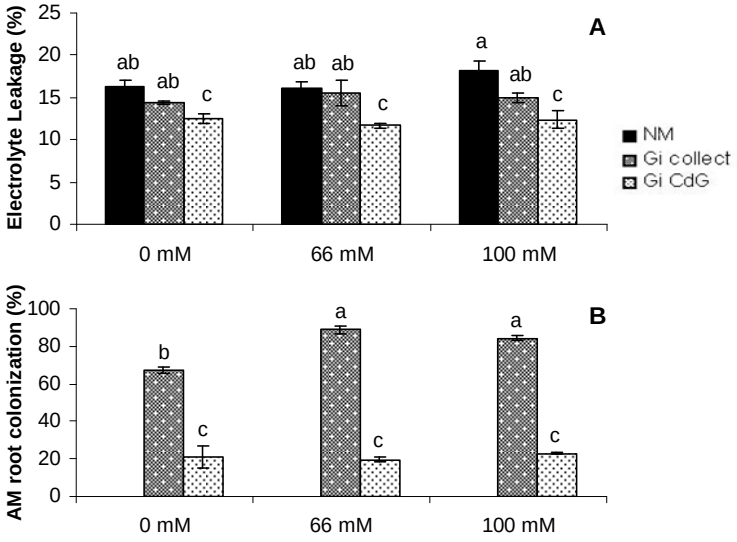

Figure 9. Electrolyte leakage (A) and percentage of mycorrhizal root length (B) in maize plants. Black bars represent noninoculated control plants (NM), grey bars represent plants inoculated with the collection *G. intraradices* strain and white bars represent plants inoculated with the native strain Gi CdG. Plants were subjected to 0, 66 or 100 mM NaCl. Columns with different letters are significantly different (P<0.05).

Relative electrolyte leakage

The applied salt stress did not significantly increase the relative electrolyte leakage in maize plants from any treatment (Figure 9A). Under non saline conditions, plants colonized by Gi CdG exhibited 25% less electrolyte leakage than non-AM plants, while no significant effect was observed in plants inoculated with the collection *G. intraradices* strain. At 66 mM NaCl, results were similar than under non saline conditions. Finally, at 100 mM NaCl both AM fungi decreased electrolyte leakage as compared to non-AM plants. In fact, plants inoculated with the collection *G. intraradices* strain decreased this parameter by 22% and those inoculated with Gi CdG did it by 36%.

Stomatal conductance

Under the non-saline conditions, there were no significant differences among treatments in stomatal conductance (Figure 10A). The application of salt greatly affected this parameter, which decreased in all treatments, even at 66 mM NaCl. However, at 66 mM NaCl no significant differences in stomatal conductance were found among treatments. In contrast, at 100 mM NaCl, Gi CdG exhibited 45% more stomatal conductance than non-AM plants and 82% more than plants inoculated with the collection *G. intraradices* strain.

Photosystem II efficiency

The efficiency of photosystem II was assessed by measuring chlorophyll a fluorescence (Figure 10B). For all treatments, this parameter was only reduced by salinity after the application of 100 mM NaCl. Under non-stressed conditions (0 mM NaCl) and under moderate salinity (66 mM NaCl), the efficiency of photosystem II was similar in AM and non-AM plants. In contrast, significant differences among treatments were found at 100 mM of NaCl. In fact, non-AM plants reduced this parameter by 41% as compared to 0 mM NaCl. In the case of AM treatments, the reduction was 21% and 19% for the collection *G. intraradices* strain and *G*i CdG, respectively. At this salt level both AM treatments exhibited significantly higher efficiency of photosystem II than non-AM plants. Thus, AM plants showed 40% and 66% higher values of this parameter than non AM plants after inoculation with the collection *G. intraradices* strain or with Gi CdG, respectively.

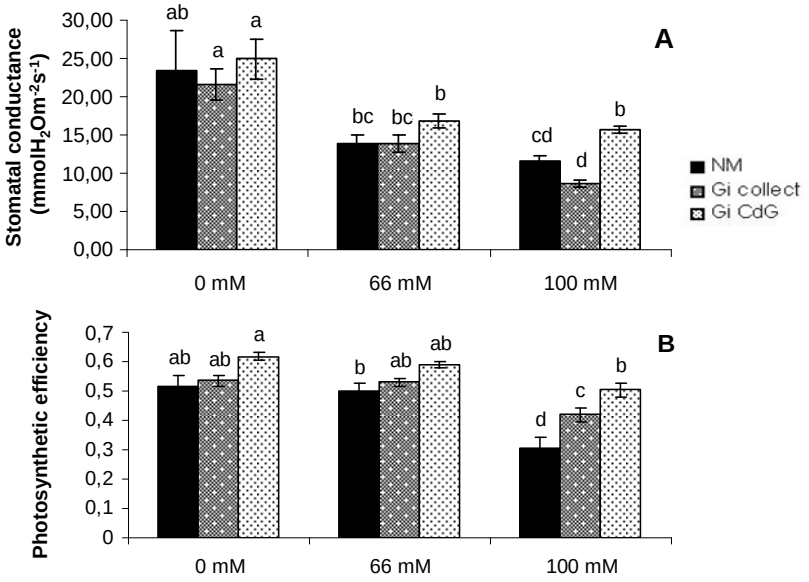

Figure 10. Stomatal conductance (mmol H_2O $m^{-2}s^{-1}$) (A) and efficiency of photosystem II (B) in maize plants. Black bars represent noninoculated control plants (NM), grey bars represent plants inoculated with the collection *G. intraradices* strain and white bars represent plants inoculated with the native strain Gi CdG. Plants were subjected to 0, 66 or 100 mM NaCl. Columns with different letters are significantly different ($P<0.05$).

Discussion

The beneficial effects of different AM fungi on plant growth under saline conditions have been demonstrated in various plant species (Ruíz-Lozano et al. 1996; Feng et al. 2002; Yano-Melo et al. 2003; Cho et al. 2006; Zuccarini and Okurowska 2008; Wu et al. 2010). However, the symbiotic efficiency of AM fungi can vary according to their origin and the growing conditions (Porcel et al. 2012). Indeed, AM fungi can be found under extreme saline conditions, and they can be adapted to these conditions (Wilde et al. 2009). Native AM fungi from areas affected by osmotic stresses may potentially cope with salt stress in a more efficient way than other fungi (Ruiz-Lozano and Azcón 2000; Querejeta et al. 2006). Estrada-Luna and Davies (2008) observed that the association of *Opuntia albicarpa* with a mixture of fungi from Sonoran Desert enhanced the nutrient concentrations in the plant more than ZAC-19 (an experimental biofertilizer) and a *G. intraradices* obtained from a culture collection. Cabo de Gata Natural Park is an arid region of south-east Spain which is subjected to desertification and has important levels of soil salinity. In this work we isolated a *G. intraradices* strain from Cabo de Gata Natural Park and we tested its symbiotic efficiency with maize plants growing under salt stress. Results showed that Gi CdG stimulated the growth of maize plants under two levels of salinity more than the collection *G. intraradices* strain.

Salt stress inhibits plant photosynthetic ability, which leads to a decrease in crop production (Pitman and Läuchli 2002), but several publications report that AM fungi in saline soils can decrease plant yield losses by increasing their photosynthetic capacity (Reviewed by Evelin et al. 2009; Ruiz-Lozano et al. 2012). This agrees with results obtained in this work with maize plants inoculated with Gi CdG. Moreover, plants inoculated with the collection *G. intraradices* strain had higher percentage of root colonization than those inoculated with Gi CdG, stressing that the symbiotic efficiency of *Gi* CdG in terms of plant growth is higher than that of the collection *G. intraradices*. The higher symbiotic efficiency of Gi CdG was also corroborated by the enhanced efficiency of photosystem II and stomatal conductance and the lower electrolyte leakage exhibited by maize plants under the different conditions assayed. This agrees with previous reports that found higher photosynthetic efficiency in leaves of mycorrhizal plants under saline conditions (Sheng et al. 2008; Zuccarini and Okurowska 2008), higher stomatal conductance (Ruíz-Lozano et al. 1996; Jahromi et al. 2008; Sheng et al. 2008) and improved integrity and stability of the cellular membranes (Feng et al. 2002; Garg and Manchanda 2008; Kaya et al. 2009). Stahl and Smith (1984) reported that *Agropyron smithii* colonized with *G. microcarpum* collected from a desert had increased stomatal opening under arid condition than that colonized with *G. microcarpum* collected from a more mesic site. This is an example to show a specific AM fungal strain being more adapted to specific environmental condition.

The presence of salts in the growth medium may induce changes in the length and other morphological properties of the hyphae, thus affecting their symbiotic efficiency and also their infective capacity. Indeed, some studies state that salt inhibits spore germination or other fungal propagules, colonization of the plant roots and sporulation of AM fungi (Juniper and Abbott 2006; Giri et al. 2007; Sheng et al. 2008; Jahromi et al. 2008). In this study, the collection *G. intraradices* strain showed a transient enhancement in the number of spores and BAS structures at 6 weeks after growing, with no significant differences at 8 weeks. This could be regarded as a symptom of stress perception in this fungal strain, because spores are a form of resistance propagules that can survive under adverse conditions and BAS are thought to be associated with the formation of spores (Bago et al. 1998a). In contrast, the hyphal length was reduced in both fungal strains by salt application, but at 8 weeks after growing this decrease was significantly higher in the collection *G. intraradices* than in Gi CdG. As the mycelium is not a form of resistance propagule in AM fungi, a higher hyphal development can be considered in terms of tolerance, being the AM fungus from Cabo de Gata a more tolerant strain than the collection *G. intraradices*. Previous reports have indicated that hyphal networks are a very important source for the rapid initiation of root colonization (McGee et al. 1997; Smith and Read 2008). The maintenance of AM fungi in ecosystems is dependent on the persistence of a potential inoculum in soils (Brundrett 1991). Carvalho et al. (2004) found evidence for potential adaptation of indigenous AM fungi to salt marsh conditions and for the ability of different propagules

of these fungi to colonize new plants and spread the infection through the roots. Brito et al. (2011) also showed that extraradical mycelium of native AM fungi can survive the dry and hot summer in a typical Mediterranean region and initiate colonization of wheat plants at the onset of the growing season; the same may occur with Gi CdG.

To determine the possible causes of the different behaviour and tolerance of both AM fungal strains under saline conditions, we evaluated the effects of salinity on several fungal genes potentially involved in the response to salinity. The induction of genes encoding for chaperones, ROS scavengers, as well as, water channels is important to re-establish cellular homeostasis and membranes stability during stresses (Xiong and Zhu 2002; Bhatnagar-Mathur et al. 2008).

All the genes studied were up-regulated by increasing salinity in the fungus isolated from Cabo de Gata, while the collection *G. intraradices* strain showed only an up-regulation of the *GintSOD1* gene. The overexpression of these genes under saline conditions indicates that they have a role in the response of the fungus against osmotic stress. Indeed, chaperone-like proteins, such as 14-3-3 and BiPs, have been demonstrated to confer tolerance to a variety of stresses in plants, while in fungi the literature is scarce. It has been proposed that BiP overexpression may prevent the cell from sensing osmotic stress-induced variations in ER function by keeping ER basic activities to a normal level under saline conditions (Valente et al. 2009). This is because protein folding in the ER is facilitated by molecular chaperones, which prevent nonproductive intermolecular interactions of folding intermediates and subsequent misaggregation of proteins within the lumen of the ER (Hammond and Helenius 1995). The AM fungal gene *GintBIP*, was studied *in vitro* by Porcel et al. (2007): they added 25% of PEG to the medium and the *GintBIP* gene expression increased by 41%. When the gene was analyzed *in vivo* using maize, soybean and tobacco plants inoculated with *G. intraradices*, the gene showed even higher expression. This was concomitant with improved tolerance to drought (Porcel et al. 2007).

In a previous study, Porcel et al. (2006) found that the addition of PEG to the medium increased *Gint14-3-3* gene expression by 1200%. Expression of the gene was also up-regulated in roots of mycorrhizal maize, lettuce and tobacco but not in soybean where it did not show any change. It was proposed that Gint14-3-3 protein could regulate the activity of plasma membrane H+-ATPases of either the fungus or the host plant, to activate its pumping activity, which is essential to cope with osmotic stress (Palmgren 1998). Indeed, the activity of plasma membrane H+-ATPase is highly regulated by factors that affect the cell physiology, including stress conditions and enhanced ATPase activity is crucial for the protective system that different organisms have developed against external adverse influence (Palmgren 1998). Moreover, as 14-3-3 proteins are found in association with key control enzymes of primary metabolism, its overexpression could rapidly alter metabolic flux in response to signals such as salt stress (Finnie et al. 1999) and they could also regulate the expression of stress-inducible

genes by regulating the activity and or localization of transcription factors (Muslin and Xing 2000).

Like other abiotic stresses, salinity also induces oxidative stress in plants (Hajiboland and Joudmand 2009). In the field of the AM symbiosis, several studies suggested that AM symbiosis helps plants to alleviate salt stress by enhancing the activities of antioxidant enzymes (Alguacil et al. 2003; Zhong Qun et al. 2007; Garg and Manchanda 2009; Talaat and Shawky 2011), but the response of the individual enzymes varies with respect to the host plant and the fungal species involved in the association. From the fungal side, González-Guerrero et al. (2010) described a *GintSOD1* gene encoding a functional protein that scavenges ROS. The up-regulation of *GintSOD1* transcripts in the fungal mycelia treated with paraquat and Cu indicated that the gene product might be involved in the detoxification of the ROS induced by these two external agents. Lanfranco et al. (2005) described and orthologous gene of *Gigaspora margarita*, which may play a pivotal role in the relationship of the fungus with its host plant, as it has been described in the ericoid mycorrhizal fungus *Oidiodendron maius* (Abbà et al. 2009). As far as we know, this is the first study on the effect of salinity on the expression of *GintSOD1*, showing that the gene is up-regulated under saline conditions and providing evidence for a role of *GintSOD1* in the fungal response to the oxidative stress induced by salinity. In any case, the up-regulation of this gene was lower in Gi CdG, suggesting that under salinity the accumulation of ROS by this fungal strain could be lower than by the collection *G. intraradices* strain.

Salinity decreases the water potential of the medium, hampering the uptake of water from the growing medium. Thus, the activity of aquaporins should be important to living organisms in order to cope with the water deficit induced by salt stress. Although it is well known that mycorrhizal mycelium transports water from the soil to the roots, only three reports have studied mycorrhizal fungal aquaporins (Aroca et al. 2009; Dietz et al. 2011; Navarro-Ródenas et al. 2012). Dietz et al. (2011) reported that, in the aquaporin gene family of *Laccaria bicolor*, three out of seven *L. bicolor* membrane intrinsic proteins showed high water permeability and two of them were also found to increase ammonia transport. Navarro-Ródenas et al. (2012) found high levels of water conductivity of TcAQP1 that could be related to the adaptation of *Terfezia claveryi* to semiarid areas because, as it was shown in a previous study, the mycelium of this mycorrhizal fungus exhibited drought tolerance under *in vitro* conditions (Navarro-Ródenas et al. 2012). However, only one study has been done so far on aquaporins from an AM fungus (Aroca et al. 2009). Authors found some evidences supporting the idea that fungal aquaporins could compensate the down regulation of host plant aquaporins caused by osmotic stress. They also found that *GintAQP1* expression was up regulated in the osmotically non-stressed part of the mycelium when the other mycelium part was stressed by NaCl. In the present study we found an up-regulation of *GintAQP1* gene at 75 mM NaCl in the isolate from collection, but not in Gi CdG. In contrast, at the highest salinity level (150 mM NaCl) the up regulation was found only in Gi CdG. Thus, Gi

CdG has the ability to induce the expression of this aquaporin gene when the salt in the medium reaches high levels. The biological significance of the up-regulation of *GintAQP1* gene remains to be elucidated since it was not possible to demonstrate whether the respective aquaporin protein indeed transport water or other substrates (Aroca et al. 2009).

In conclusion, results from this study show that the strain Gi CdG exhibited a higher tolerance to salinity than the collection *G. intraradices* strain and grew and developed better under saline conditions. The present study demonstrates that the Gi CdG strain exhibits a better symbiotic efficiency in an already established symbiosis under conditions of salt stress. These effects could be due to a fungal adaptation to the saline environment where the fungus was isolated. The adaptation to salinity may be related to the significant up-regulation of genes with chaperone activity or genes encoding for aquaporins. The fungus from Cabo de Gata may reduce the production of ROS, which in turns was evidenced by a lower induction of *GintSOD1* gene.

The present study underlines the importance of salt adaptation in AM fungi. Stress tolerance can only be gained through long-term exposure to chronic stress. Thus AM fungi isolated from areas affected by salinity will be a powerful strategy to enhance the tolerance of crops to saline stress conditions or in revegetation programs of degraded areas affected by osmotic environmental constrains.

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CAPÍTULO 4

CHAPTER 4

Arbuscular mycorrhizal fungi native from a Mediterranean saline area enhance maize plants tolerance to salinity through improved ion homeostasis

Submitted to Annals of Botany by Estrada B, Aroca R, Maathuis F.J.M, Barea J.M and Ruiz-Lozano J.M.

Hongos micorrícico arbusculares nativos de áreas salinas Mediterráneas aumentan la tolerancia de plantas de maíz mediante la mejora de la homeostasis iónica

Resumen

La salinidad del suelo restringe seriamente el crecimiento y la productividad vegetal. El maíz es una planta sensible a la sal, donde el Na^+ representa el ión principal que causa toxicidad, ya que puede competir con el K^+ por sitios de unión en la membrana plasmática. La inoculación con micorrizas arbusculares (MA) puede aliviar el estrés salino en las plantas hospedadoras a través de varios mecanismos. Estos pueden incluir la selección de iones durante la absorción de los nutrientes del suelo por parte de los hongos o durante la transferencia a la planta huésped. Se ha propuesto que los beneficios de las MA podrían verse aumentados usando aislados nativos de MA, fisiológica y genéticamente adaptados a las condiciones de estrés de su entorno. En este estudio se investigó si hongos nativos MA aislados del Parque Natural de Cabo de Gata (CdG) (una zona con graves problemas de salinidad y afectada por la desertificación) pueden ayudar a las plantas de maíz a superar los efectos negativos del estrés salino mejor que plantas inoculadas con hongos MA no nativos o que plantas no micorrizadas . Los resultados mostraron que las plantas de maíz inoculadas con dos de los tres hongos nativos MA tuvieron el mayor desarrollo de parte aérea en todos los niveles de salinidad, mientras que la biomasa de las plantas inoculadas con el aislado MA de colección fue similar a las plantas no micorrizadas. Las plantas inoculadas con los tres hongos nativos MA mostraron un aumento significativo de K^+ y una reducción en la acumulación de Na^+ en comparación con las plantas no micorrizadas, coincidente con una mayor relación K^+/Na^+ en sus tejidos. Estos efectos se correlacionaron con la regulación de la expresión de los genes *ZmAKT2*, *ZmSOS1* y *ZmSKOR* en las raíces de las plantas de maíz colonizadas por hongos MA nativos, lo que contribuye a la homeostasis de K^+ y Na^+.

Palabras clave: adaptación, homeostasis iónica, hongos micorrízicos arbusculares nativos, salinidad, tolerancia

Arbuscular mycorrhizal fungi native from a Mediterranean saline area enhance maize plants tolerance to salinity through improved ion homeostasis

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Running title: Improved ion homeostasis in maize plants by salt-adapted AMF

Abstract

Soil salinity seriously restricts plant growth and productivity. Maize is a salt-sensitive plant, where Na^+ represents the major ion causing toxicity because it can compete with K^+ for binding sites at the plasma membrane. Inoculation with arbuscular mycorrhizal fungi (AMF) can alleviate salt stress in several host plants through several mechanisms. These may include ion selection during the fungal uptake of nutrients from the soil or during transfer to the host plant. It has been proposed that AM benefits could be enhanced when native AMF isolates, physiologically and genetically adapted to the stress conditions of their environment, are used. In this study we investigated whether native AMF isolated from Cabo de Gata Natural Park (CdG) (an area with serious problems of salinity and affected by desertification) can help maize plants to overcome the negative effects of salinity stress better than non-AM plants or plants inoculated with non-native AMF. Indeed, results showed that maize plants inoculated with two out the three native AMF had the highest shoot dry biomass at all salinity levels, while biomass in plants inoculated with the collection AM strain was similar to the non-mycorrhizal plants. Plants inoculated with the three native AMF showed significant increase of K^+ and reduced Na^+ accumulation as compared to non-mycorrhizal plants, concomitantly with higher K^+/Na^+ ratios in their tissues. These effects correlated with regulation of *ZmAKT2*, *ZmSOS1* and *ZmSKOR* genes expression in the roots of maize plants colonized by native AMF, contributing to K^+ and Na^+ homeostasis.

Key words: adaptation, ion homeostasis, native arbuscular mycorrhizal fungi, salinity, tolerance

Introduction

Salinity is a major and increasing problem which restricts plant growth and productivity. More than 800 million hectares of land throughout the world are salt affected (including both saline and sodic soils) (FAO, 2005). This is over 6% of the total land area of the world. High amounts of salts in soils are responsible for yield reduction in one third of the global arable land (Lambers, 2003). This is particularly the case in regions with high rates of evaporation, like arid and semiarid areas (Hammer *et al.*, 2011). Most crops are glycophytic and tolerate salinity to a threshold level. Above this level, yield decreases (Khan *et al.*, 2006), since excess of salt inhibits photosynthetic ability and induces physiological drought in plants (Pitman and Läuchli, 2002). Maize (*Zea mays* L.) is classified as a salt-sensitive plant (Maas and Hoffman, 1977). Although maize is originally from Mesoamerica, nowadays it is the third most

important cereal crop and ranks first in countries with developing economies (Mejía, 2003).

Most glycophytes tolerate salinity by restricting the uptake of Na^+ and Cl^- while maintaining uptake of macronutrients such as K^+ or N (Teakle and Tyerman, 2010). Although Cl^- is considered an essential micronutrient for higher plants involved in the regulation of important cellular functions such as enzyme activity, maintenance of membrane potentials, and as a co-factor in photosynthesis and pH gradients (White and Broadley, 2001), it can be toxic to plants at high concentrations (Xu *et al.*, 2000). However for maize, it has been shown that Na^+ (and not Cl^-) represents the major ion causing toxicity related to salinity (Fortmeier and Schubert, 1995) because it can compete with K^+ for binding sites at the plasma membrane. The K^+ ion is essential for protein synthesis, activation of many enzymes and photosynthesis and it plays a central role in osmotic adjustment, turgor maintenance, and in the control of stomata opening (Maathuis and Amtmann, 1999). It has been shown that chloroplast function is impaired when K^+ is displaced by Na^+, leading to uncontrolled water losses (Slabu *et al.*, 2009). Furthermore, adequate K^+ is very important to maintain cytosolic ion homeostasis in Na^+-stressed plants (Zhu, 2003), a function which is disrupted by excessive Na^+ entry (Demidchik and Maathuis, 2007). Accumulation of Na^+ and impairment of K^+ nutrition is a major characteristic of salt stressed plants, the mechanisms of which are only partially understood. However, salt stress often causes reduction in plant tissue K^+ content, and the K^+/Na^+ ratio is considered a useful parameter to assess salt tolerance (Maathuis and Amtmann, 1999; Chen *et al.,* 2007). Another important response of glycophytes to salinity stress, associated with osmoregulation adjustment, is the accumulation of osmotically active organic solutes such as proline and glycine-betaine (Munns, 2005). Proline maintains the osmotic balance and protects enzymes in presence of high cytoplasmic electrolyte concentrations (Greenway and Munns, 1980; Hajlaoui *et al.*, 2010). However, the significance of proline accumulation in osmotic adjustment is still debated and varies according to the species (Lutts *et al.*, 1996; Rodriguez *et al.*, 1997).

Plants can overcome salinity effects by interacting with several beneficial soil microorganisms. Soil microbiota, such as arbuscular mycorrhiza fungi (AMF) live symbiotically associated with the roots of 80% of terrestrial plants (Smith and Read, 2008) and are able to increase plant growth and crop productivity under different environmental stresses (Barea *et al.*, 2012). Several studies have shown that inoculation with AMF can alleviate salt stress (Sannazzaro *et al.*, 2006; Jahromi *et al.*, 2008; Estrada *et al.*, 2012). Improved salt tolerance following mycorrhizal colonization may be the result of a more efficient nutrient uptake (Cantrell and Linderman, 2001), ion balance (Giri *et al.*, 2007), protection of enzyme activities (Rabie and Almadini, 2005), increase in photosynthesis ability (Sheng *et al.*, 2008) and facilitation of water uptake in plants (Aroca *et al.*, 2007).

Mycorrhizal colonization has also been shown to enhance K^+ absorption under saline conditions while preventing Na^+ translocation to shoot tissues (Giri *et al.*, 2007; Sharifi *et al.*, 2007; Talaat and Shawky, 2011). Thus, mycorrhizal plants grown under saline conditions often have a higher K^+:Na^+ ratio (Rabie and Almadini, 2005; Sannazzaro *et al.*, 2006), and a lower shoot Na^+ concentration (Al-Karaki and Hammad, 2001) than nonmycorrhizal plants, preventing the disruption of various enzymatic processes and inhibition of protein synthesis. Mycorrhizal fungi may also act as a first barrier for ion selection during the fungal uptake of nutrients from the soil or during transfer to the plant host. It has been indicated that AMF can selectively take up elements such as K^+ and Ca^{2+}, which act as osmotic equivalents while they avoid uptake of toxic Na^+ (Hammer *et al.*, 2011; Evelin *et al.*, 2012). This suggests that AMF induce a buffering effect on the uptake of Na^+ when the content of Na^+ is within the permissible limit (Evelin *et al.*, 2009; Hammer *et al.*, 2011). Indeed, analyzing the regulation by AMF of plant genes involved in ion homeostasis has been encouraged in a recent review on physiological and molecular perspectives in studies of salt stress alleviation by AMF (Ruiz-Lozano *et al.,* 2012). In this sense, the plasma membrane localised Na^+:H^+ antiporter SOS1 has been shown to fulfil two important roles in plants, restriction of net Na^+ uptake by roots and control of xylem loading for long-distance transport of Na^+ (Shi *et al.,* 2002). Transport of K^+ to the shoot depends on xylem delivery, a process largely controlled by SKOR (Gaymard *et al.,* 1998) and on phloem K^+ recycling (Maathuis, 2007). The molecular mechanism for the latter is unclear but likely to involve the phloem expressed K^+ channel AKT2 (Marten *et al.,* 1999). Thus, these three genes were studied in this work.

AMF can be found under severe saline conditions in nature, both in saline inlands and coasts (Aliasgharzadeh *et al.*, 2001; Yamato *et al.*, 2008) and in salt marshes (Carvalho *et al.*, 2004; Wilde *et al.*, 2009). Moreover, the use of AMF adapted to salinity could be a critical issue for success in recovering saline areas either in natural environments or in agricultural lands affected by salinity. Several studies, describing inoculation strategies used in re-vegetation of degraded ecosystems, showed a higher benefit of native AMF, which appear to be physiologically and genetically adapted to the stress conditions of the target environment, than non-native isolates (Ferrol *et al.*, 2004; Oliveira *et al.*, 2005; Querejeta *et al.*, 2006). This can be extrapolated to salt-stressed soil, thus the use of salinity-adapted AMF ecotypes should be rewarding.

The objectives of this work were: (i) to investigate whether native AMF isolated from a saline environment (Cabo de Gata Natural Park, Almería, Spain, an area with serious problems of salinity and affected by desertification) can help maize plants to overcome the negative effects of salinity stress better than non-AM plants or plants inoculated with non-native AMF. (ii) As the molecular mechanisms involved in the better performance of AM plants under salinity stress are almost completely unknown, and there is little information on the effects of the AM symbiosis on plant ion

transporters, we also analyzed the regulation by these AMF of key plant ion transporters expected to be affected by salinity.

Materials and methods

Identification of the mycorrhizal strains isolated from Cabo de Gata Natural Park

AM fungal spores were separated from the soil samples by a wet sieving process (Sieverding, 1991). The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG) (Koske and Tessier, 1983); a mixture of PVLG and Melzer's reagent (Brundrett *et al.*, 1994); a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain, 1990). For identification of the AMF species, spores were then examined using a compound microscope at up to 400-fold magnification as described for glomeromycotean classification by Oehl *et al.* (2011). The species were identified based on its spore morphology as a *Rhizophagus intraradices* (Schenk and Smith, 1982), *Claroideoglomus etunicatum* (Becker and Gerdemann, 1977) and *Septoglomus constrictum* (Trappe, 1977).

In addition to the morphological identification, a molecular identification was also carried out. For that, spores isolated from the bait cultures of each fungal strain were surface-sterilized with chloramine T (2%) and streptomycin (0.02%) and crushed with a sterile disposable micropestle in 40 µL milli-Q water (Ferrol *et al.*, 2004). A two-step PCR was conducted to amplify the AM fungal DNA from the spores. The first PCR step was performed with the universal eukaryote primers NS1 and NS4 region of the small subunit ribosomal gene and the second with the specific AM fungal primers AML1 and AML2 (Lee *et al.*, 2008). The amplified DNA was purified using the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Helthcare, UK). DNA fragments were sequenced on an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST program (Altschul *et al.*, 1990).

The BLAST analysis unambiguously placed *Rhizophagus intraradices* as the closest relative of our *Rhizophagus intraradices* CdG strain, with sequence accession number FR750209 (Krüger *et al.*, 2012) having a 99% identity. *Septoglomus constrictum* was the closest relative to our *Se. constrictum* CdG strain, with sequence accession number FR750212 (Krüger *et al.*, 2012) having a 99% identity. Finally, *Claroideoglomus etunicatum* was the closest relative of our *Cl. etunicatum* CdG strain, with sequence accession number FR750216.1 (Krüger *et al.*, 2012) having also a 99% identity. The AM fungal strains have been incorporated to the collection of Zaidin Experimental Station, Granada, Spain, under accession numbers EEZ 195, EEZ 196 and EEZ 163, respectively.

Experimental design

The experiment consisted of a randomized complete block design with five inoculation treatments: (1) non-mycorrhizal control plants, (2) plants inoculated with the model AM fungus *Rh. intraradices* (Ri collect), reproduced at collection of the Zaidin Experimental Station, (3) plants inoculated with the AM fungal strain *Rh. intraradices* isolated from Cabo de Gata Natural Park (Ri CdG), (4) plants inoculated with the AM fungal strain *Se. constrictum* isolated from CdG (Sc CdG) and (5) plants inoculated with the AM fungal strain *Cl. etunicatum* isolated from CdG (Ce CdG). There were 30 replicates of each inoculation treatment, totalling 150 pots (one plant per pot), so that ten of each microbial treatment were grown under nonsaline conditions throughout the entire experiment, while ten pots per treatment were subjected to 66 mM of NaCl and the remaining ten pots per treatment were subjected to 100 mM of NaCl.

Soil and biological materials

Loamy soil was collected from Granada province (Spain, 36º59'34''N; 3º34'47''W), sieved (5 mm), diluted with quartz-sand (<2 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100ºC for 1 h on 3 consecutive days). The original soil had a pH of 8.2 [measured in water 1:5 (w/v)]; 1.5 % organic matter, nutrient concentrations (g kg^{-1}): N, 1.9; P, 1 ($NaHCO_3$-extractable P); K, 6.9. The electrical conductivity of the original soil was 0.5 dS m^{-1}.

Three seeds of maize (*Zea mays*. L) were sown in pots containing 900 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

Inoculation treatments

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species used were three strains isolated from Cabo de Gata Natural Park (Almería, Spain): *Rhizophagus intraradices* (previously named *Glomus intraradices*), *Septoglomus constrictum* and *Claroideoglomus etunicatum*. A *Rhizophagus intraradices* strain from our culture collection was also used. Appropriate amounts of each inoculum containing about 700 infective propagules (according to the most probable number test), were added to the corresponding pots at sowing time just below maize seeds. Non-mycorrhizal control plants received the same amount of autoclaved mycorrhizal inocula together with a 10 ml aliquot of a filtrate (< 20 μm) of the AM inocula in order to provide a general microbial population free of AM propagules.

Growth Conditions

The experiment was carried out under glasshouse conditions with temperatures ranging from 19 to 25ºC, 16/8 light/dark period, and a relative humidity of 50-60%. A photosynthetic photon flux density of 800 µE m^{-2} s^{-1} was measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Water was supplied daily to the entire period of plant growth to avoid any drought effect. Plants were established for 45 days prior to salinization to allow adequate plant growth and symbiotic establishment. Three concentrations (0, 66, and 100 mM NaCl) of saline solution were reached in the soil substrate by adding appropriate dilutions of a stock 2 M saline solution. The concentration of NaCl in the soil was increased gradually on alternative days to avoid an osmotic shock. It took 8 days, to reach the desired 66 and 100 mM NaCl levels. Plants were maintained under these conditions for an additional 30 days.

Parameters measured and statistical analysis

Symbiotic development

The percentage of mycorrhizal root infection in maize plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), as described Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

Biomass production

At harvest (75 days after planting), the shoot and root system were separated and the shoot dry weight (SDW) and root dry weight (RDW) was measured after drying in a forced hot-air oven at 70ºC for two days.

Proline content

Free proline was extracted from 0.5 g of fresh leaves and roots (Bligh and Dyer, 1959). The methanolic phase was used for quantification of proline content. Proline was estimated by spectrophotometric analysis at 530 nm of the ninhydrin reaction according to Bates *et al.* (1973).

Determination of Mineral Nutrients

Na^+ and K^+ ions were extracted from 0.05 g of ground leaf and root dry material with 10 ml of deionized water. This extract was diluted, and for analysis, an ICP plasma analyzer (IRIS Intrepid II XDL, Thermo Electron Corporation) was used. Extractions

were made from five different plants of each treatment. Mineral analyses were carried out by the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

Chloride anions were determined from an aqueous extraction from 0.4 g of fresh vegetal material with 10 ml of deionized water following the method of Cataldo *et al.* (1975). The quantification of Cl^- in roots and leaves was made from five different plants of each treatment as described by Diatloff and Rengel (2001).

Gene expression analysis

RNA extraction and synthesis of cDNA

RNA was extracted using the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.) from maize roots samples stored at -80ºC. Single-strand cDNA was primed by random hexamers using 100-1,000 ng of DNase-treated RNA. RNA samples were denatured at 65ºC for 5 min and then reverse transcribed at 25ºC for 10 min and 42ºC for 50 min in a final volume of 20 µl containing 10 µl of total RNA, 10 µM random primers (Invitrogen, Carlsbad, CA, USA), 0.5 mM dNTPs, 10 U RNase inhibitor, 4 µl of 5x buffer, 2 µl 0.1 M DTT, and 1 µl of Superscript II Reverse Transcriptase (Invitrogen). The samples were precipitated with 1 (v/v) isopropanol and suspended in 20 µl of water.

Quantitative PCR

Gene expression analyses were carried out by quantitative reverse transcription (qRT)-PCR using an iCycler iQ apparatus (BioRad, Hercules, CA, U.S.A.). The cDNA samples were standardized to four reference genes: alpha tubulin (gi:450292), elongation factor 1-alpha (EF1-α) (gi:2282583), polyubiquitin (gi:248338) and glyceraldehyde phosphate dehydrogenase (GADPH) (gi:22237). The same reactions were performed with specific primers designed for each of the analyzed genes: *ZmAKT2*, For (5´-CCTCAAGCATCAGGTCGAGA-3´) and Rev (5´-CTCTGTAATCTTCCTGGACG-3´), *ZmSKOR*, For (5´-TCAGATCCAAGATGTCCCAG-3´) and Rev (5´-TTCGTATCCTCTTAACGCAG-3´) *ZmSOS1*, For (5´-GCTTGTCACATACTTCACAG-3´) and Rev (5´-ACTTGTCCACTTCACTACAC-3´). Individual real-time RT-PCR reactions were assembled with oligonucleotide primers (0.15 µM each), 10.5 µl of 2x iQSYBR Green Supermix (Bio-Rad; containing 100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 Mm dNTPs, 50 U/µl iTaq DNA polymerase, 6 mM MgCl2, 20 nM SYBR Green I, 20 nM fluorescein) plus 1 µl of a 1:10 dilution of each corresponding cDNA in a final volume of 21 µl. Experiments were repeated three times, with the threshold cycle (CT) determined in triplicate, using cDNAs that originated from three RNAs extracted from three different biological samples.

The relative levels of transcript were calculated using the Normalization Factor (NF) based on the expression levels of the three best-performing housekeeping genes, in our case polyubiquitin, GADPH and EF1-α. NF was measured using a Visual Basic application for excel (GeNorm) that calculates the gene stability as described by Vandesompele *et al.* (2002). The calculation was done for each cDNA used in the Q-PCR quantification. Expression levels were transformed from Cq values using the PCR efficiencies (Ramakers *et al.*, 2003).

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA) performing first a one-way ANOVA followed by the Tukey test with $P< 0.05$ as the significance cut-off. Two independent statistical analyses were carried out: the first to analyze data from the different AMF treatments within each saline level and the second one to analyze data from each fungal species at increasing salinity.

Results

Symbiotic development

Increasing salinity application enhanced the percentage of AM root colonization in all cases, except in plants colonized by Ri CdG, which exhibited similar colonization rates at all salinity levels (Fig. 1). Within each salinity level, the highest rate of AM root colonization was achieved in plants inoculated with Ri collect (up to 88%). High levels of root colonization were also found in plants inoculated with Ce CdG and Sc CdG (Fig. 1). In contrast, the lowest root colonization was always found in plants colonized by Ri CdG (about 20%).

Plant biomass production

The increase of salt application affected negatively the shoot biomass production in all treatments; although in the case of non-mycorrhizal plants the decrease was not significant (Fig. 2A). In all AM treatments, the decrease was more evident at the highest salt level applied (100 mM NaCl). In contrast, the root biomass production only decreased with salinity in the non-mycorrhizal plants (Fig. 2B). When results were analyzed for the three salt treatments it was clear that Ri CdG and Ce CdG, both enhanced maize shoot biomass as compared to the non-mycorrhizal plants, while Ri collect and Sc CdG did not (Fig. 2A).

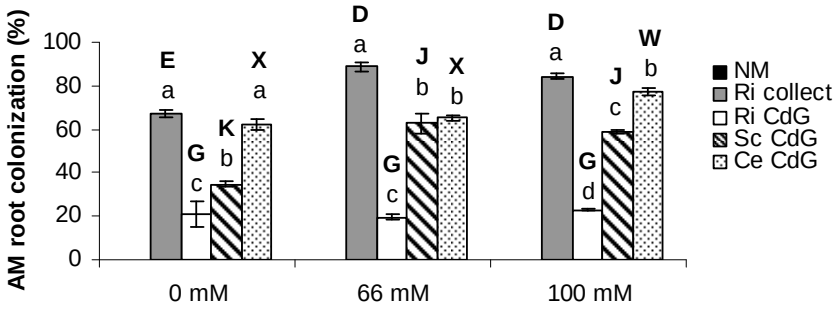

Fig. 1. Percentage of mycorrhizal root length in maize plants. Grey bars represent plants inoculated with the collection *Rhizophagus intraradices* strain (Ri collect); white bars, plants inoculated with the native *Rh. intraradices* CdG strain (Ri CdG); lined bars, plants inoculated with the native *Septoglomus claroideum* CdG strain (Sc CdG) and dotted bars, plants inoculated with the native *Claroideoglomus etunicatum* CdG strain (Ce CdG). Plants were subjected to 0, 66 or 100 mM NaCl. Different letters indicate significant differences ($p < 0.05$) among fungal treatments at each salt level (a, b, c, d) or among salt levels for each AMF treatment: Ri collect (D, E, F), Ri CdG (G, H, I), Sc CdG (J, K, L) or Ce CdG (W, X, Y).

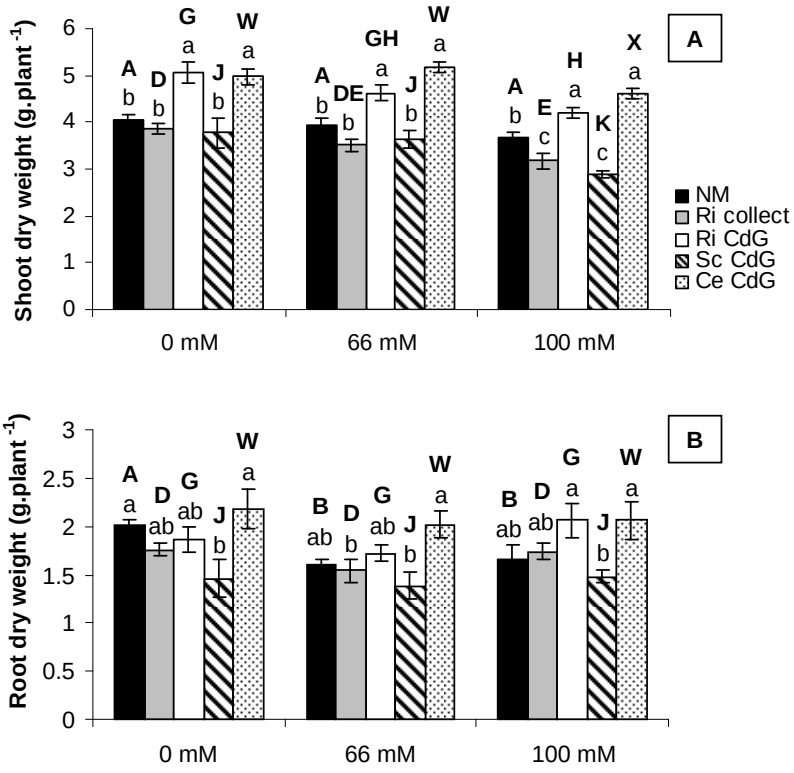

Fig. 2. Shoot (A) and root (B) dry weights (g plant^{-1}) in maize plants. Black bars represent non-mycorrhizal control plants (NM); grey bars, plants inoculated with the collection *Rhizophagus intraradices* strain (Ri collect); white bars, plants inoculated with the native *Rh. intraradices* CdG strain (Ri CdG); lined bars, plants inoculated with the native *Septoglomus claroideum* CdG strain (Sc CdG) and dotted bars, plants inoculated with the native *Claroideoglomus etunicatum* CdG strain (Ce CdG). Plants were subjected to 0, 66 or 100 mM NaCl. Different letters indicate significant differences ($p < 0.05$) among fungal treatments at each salt level (a, b, c, d) or among salt levels for each AMF treatment: NM plants (A, B, C), Ri collect (D, E, F), Ri CdG (G, H, I), Sc CdG (J, K, L) or Ce CdG (W, X, Y).

Accumulation of proline

The accumulation of proline was more pronounced in root than in shoot tissues (Fig. 3A,B) and it increased in the roots with increasing salinity in the growth medium, except for plants inoculated with Sc CdG, where the differences were not significant (Fig. 3B). The highest accumulation of proline occurred in the roots of non-mycorrhizal plants at 100 mM NaCl. It also increased with salinity in roots of plants inoculated with Ri CdG and Ce CdG. In shoots, no significant differences were found either as a consequence of increasing salinity or by the AM fungus inoculated.

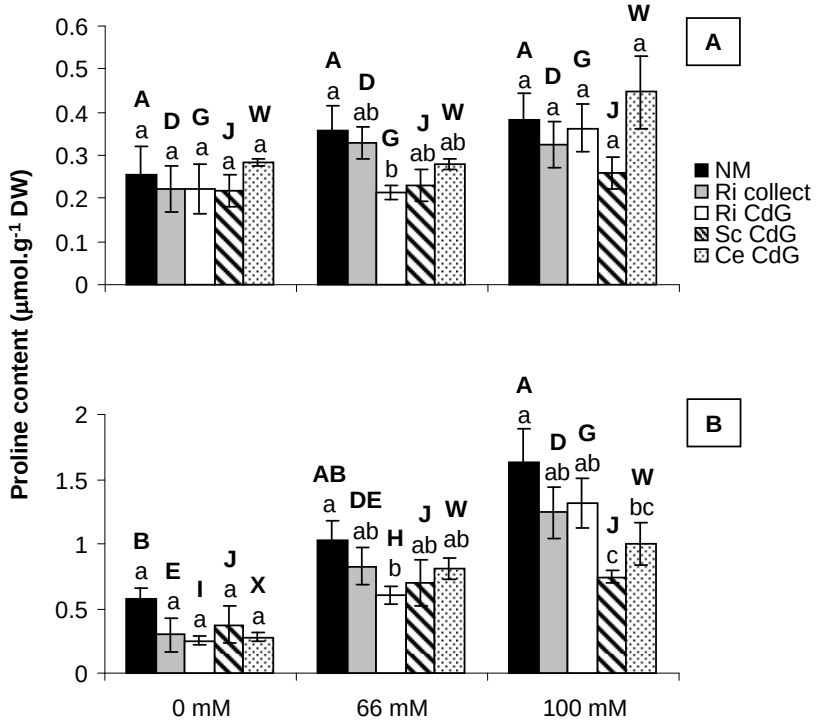

Fig. 3. Shoot (A) and root (B) proline accumulation in maize plants. See legend for Fig. 2.

Accumulation of mineral ions in shoots and roots and K^+/Na^+ ratios

Potassium

The increase of salinity in the growing medium decreased the accumulation of K^+ in the root tissues in all treatments, except in plants inoculated with Ri collect, which had similar K^+ levels at 0 mM NaCl and at 100 mM NaCl (Fig. 4B). In contrast, in shoot tissues, maize accumulated more K^+ at increasing salinity levels (Fig. 4A). This was especially evident in plants inoculated with Sc CdG. At 100 mM NaCl, all the mycorrhizal treatments accumulated more K^+ in roots than the non-mycorrhizal plants (Fig. 4B). At this salt level no significant differences in K^+ accumulation were observed

in roots between AM and non-AM treatments. In shoots, at all salinity levels, the non-mycorrhizal plants and the plants inoculated with Ri collect exhibited always a lower K^+ accumulation than plants inoculated with either of the three native AM fungal strains (Ri CdG, Sc CdG or Ce CdG) (Fig. 4A).

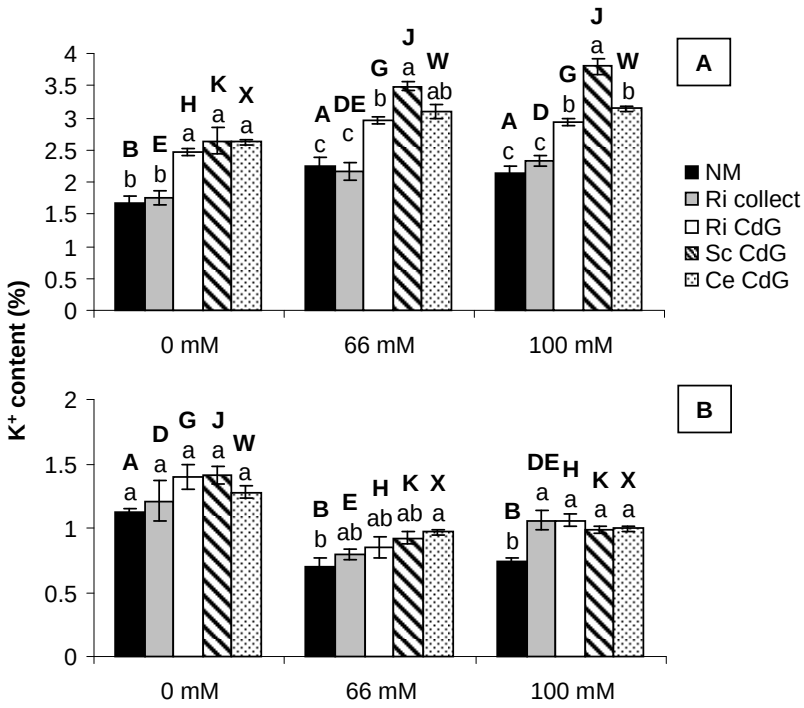

Fig. 4. Shoot (A) and root (B) potassium concentration in maize plants. See legend for Fig. 2.

Sodium

The accumulation of Na^+ in maize plants increased considerably both in shoot and in root tissues when the plants were cultivated under salinity (Fig. 5 A,B). When data were analyzed within each salt level, it was observed that at 0 mM NaCl the three native AMF enhanced the accumulation of Na^+ in root tissues as compared to the non-mycorrhizal plants or those inoculated with Ri collect (Fig. 5B). However, at 66 mM NaCl and 100 mM NaCl no significant differences in Na accumulation in roots were observed among treatments, except that plants inoculated with Ce CdG, reduced significantly this parameter at 100 mM NaCl. In the shoot tissues, it was observed that at 0 mM NaCl the levels of Na^+ were very low in all treatments (Fig. 5A). The accumulation of Na^+ was enhanced at 66 and 100 mM NaCl for all treatments, with non-mycorrhizal plants exhibiting the highest Na^+ accumulation and mycorrhizal plants the lowest, especially those inoculated with Ce CdG (Fig. 5A).

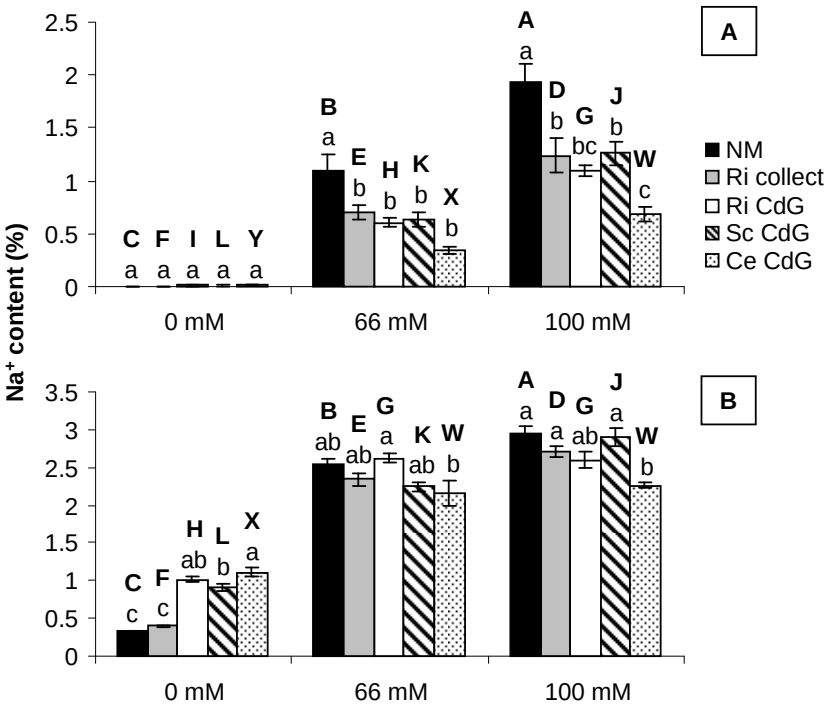

Fig. 5. Shoot (A) and root (B) sodium concentration in maize plants. See legend for Fig. 2.

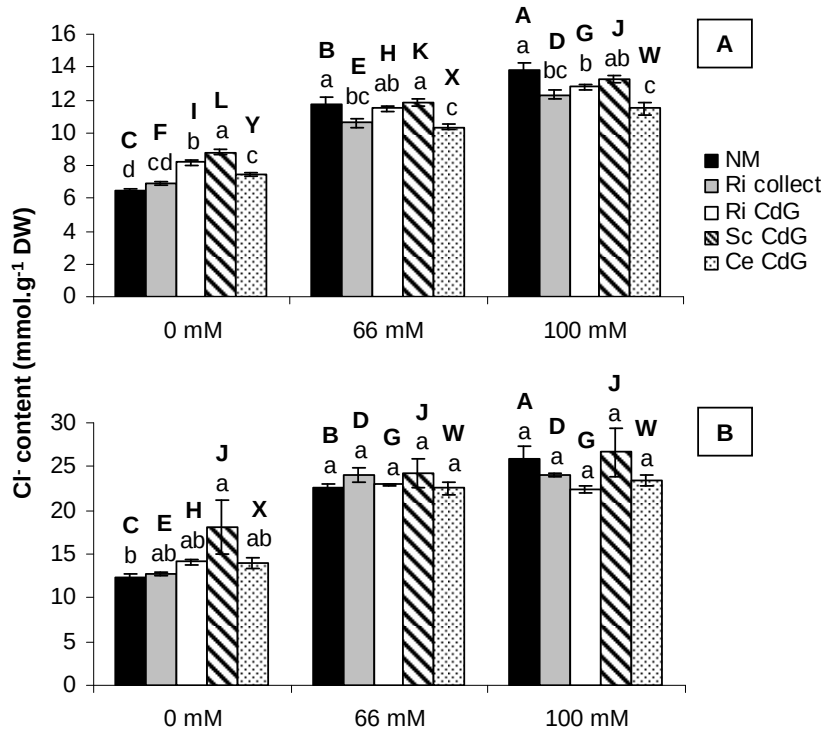

Fig. 6. Shoot (A) and root (B) chloride concentration in maize plants. See legend for Fig. 2.

Chloride

The accumulation of Cl^- increased in both shoot and root tissues with increasing salinity in the growth medium (Fig. 6 A,B). This was more evident in the shoot tissues (Fig. 6A). When data were analyzed within each salt level, it was observed that in roots no remarkable differences in Cl^- accumulation were found among treatments. In shoots, at 0 mM NaCl, plants inoculated with the three native AMF (Ri CdG, Sc CdG and Ce CdG) accumulated more Cl^- than non-mycorrhizal plants or those inoculated with the Ri collect fungus (Fig. 6A). In contrast, when salt was applied to the growth medium, non-mycorrhizal plants always exhibited the highest Cl^- accumulation and no important differences in Cl^- accumulation were observed among fungal treatments. At both saline levels, plants inoculated with Ce CdG showed the lowest accumulation of Cl^-.

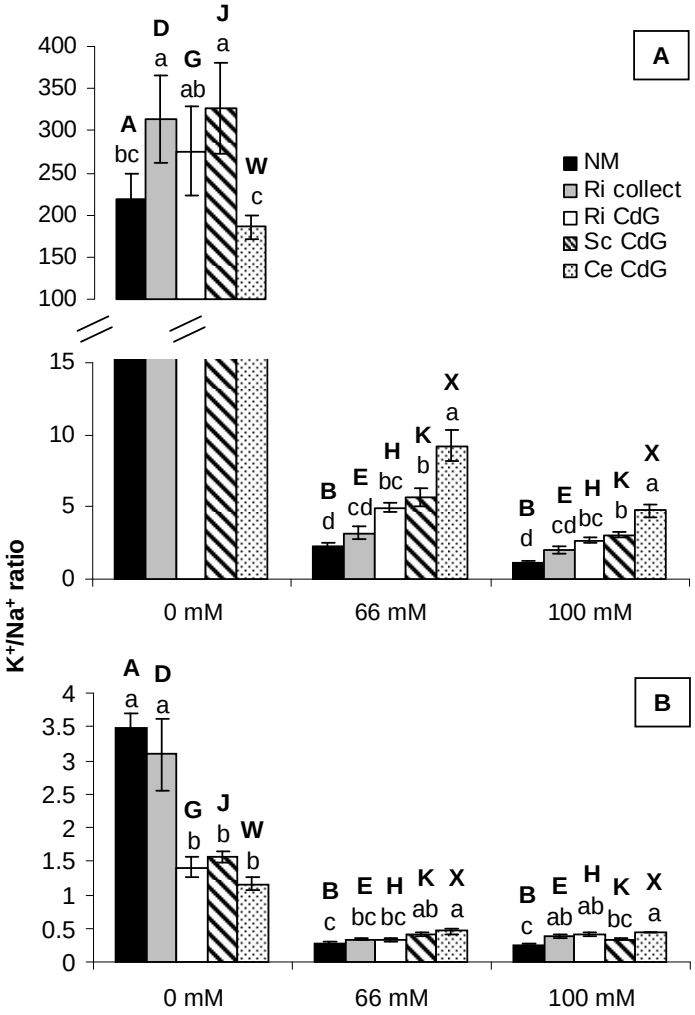

Fig. 7. Shoot (A) and root (B) K^+/Na^+ ratio in maize plants. See legend for Fig. 2.

K^+/Na^+ ratios

The K^+/Na^+ ratio was negatively affected by salinity in both shoots and roots (Fig. 7A,B). However, the effect was more evident in shoot tissues, where the differences between the non-saline treatment and either of the two saline treatments were of two orders of magnitude (Fig. 7A). In roots, the K^+/Na^+ ratio at 0 mM NaCl was lower in plants inoculated with either of the three native AMF as compared to non-mycorrhizal plants or plants inoculated with Ri collect (Fig. 7B). In contrast, when salt was applied non-mycorrhizal plants showed the lowest K^+/Na^+ ratio, especially if compared to roots of plants inoculated with Ce CdG. In the shoots, at both saline levels, the lowest K^+/Na^+ ratios were also found in non-mycorrhizal plants and in plants colonized with the Ri collect strain (Fig. 7A). The three native AMF (Ri CdG, Sc CdG and Ce CdG) showed significantly enhanced K^+/Na^+ ratios in shoots as compared to the non-mycorrhizal plants, especially those colonized by Ce CdG.

Expression of genes encoding for ion transporters

Ion analyses suggest that AMF affect tissue K^+ and Na^+. We therefore tested whether membrane transporters involved in shoot K^+ and Na^+ deposition were affected at the transcript level by AMF colonization.

The expression of the *ZmAKT2* gene was differently affected by increasing salinity in the different fungal treatments (Fig. 8A). In fact, in roots of non-mycorrhizal plants or plants colonized by the Ri collect strain, it decreased its expression at 66 and 100 mM NaCl as compared to 0 mM NaCl. In contrast, the expression of this gene increased steadily with increasing salinity in roots of plants colonized by Sc CdG and Ce CdG. When data were analyzed within each salt level, it was observed that in the absence of salt in the growth medium the expression of *ZmAKT2* was notably higher in roots of non-mycorrhizal plants and plants colonized by the Ri collect strain than in roots of plants colonized by either of the three native AM fungal strains. At 66 mM NaCl, few differences among treatments were observed. Finally, at the highest salt level (100 mM NaCl), the expression of this gene increased notably in roots of plants colonized by Sc CdG and Ce CdG, as compared to the other treatments.

The expression of the *ZmSOS1* gene was negatively affected by the highest salinity level in non-mycorrhizal plants or plants inoculated with Ri collect and Ri CdG (Fig. 8B). In contrast, the application of 100 mM NaCl enhanced considerably the expression of the *ZmSOS1* gene in roots of plants colonized by Ce CdG as compared to 66 mM NaCl or to the non-saline level. When data were analyzed within each salt level, it was observed that in the absence of salinity plants inoculated with Sc CdG or with Ce CdG had a significantly lower expression of *ZmSOS1* than the non-mycorrhizal plants. No significant differences among treatments were observed at 66 mM NaCl, while at

the highest salt level (100 mM NaCl) plants inoculated with Ce CdG exhibited the highest expression of this gene in their roots.

The expression of the *ZmSKOR* gene in roots was little affected by the increasing salinity in non-mycorrhizal plants or in plants inoculated with the Ri collect and Ri CdG strains (Fig. 8C). In contrast, in plants colonized by Sc CdG and Ce CdG, the expression of this gene increased steadily with increasing salinity. When data were analyzed within each salt level, it was observed that in the absence of salinity plants inoculated with Sc CdG or with Ce CdG had a significantly lower expression of *ZmSKOR* than all other treatments. This lower expression was maintained at 66 mM NaCl in roots of plants colonized by Ce CdG, but again, at the highest salt level (100 mM NaCl) plants inoculated with Ce CdG and also plants inoculated with Sc CdG exhibited the highest expression of this gene.

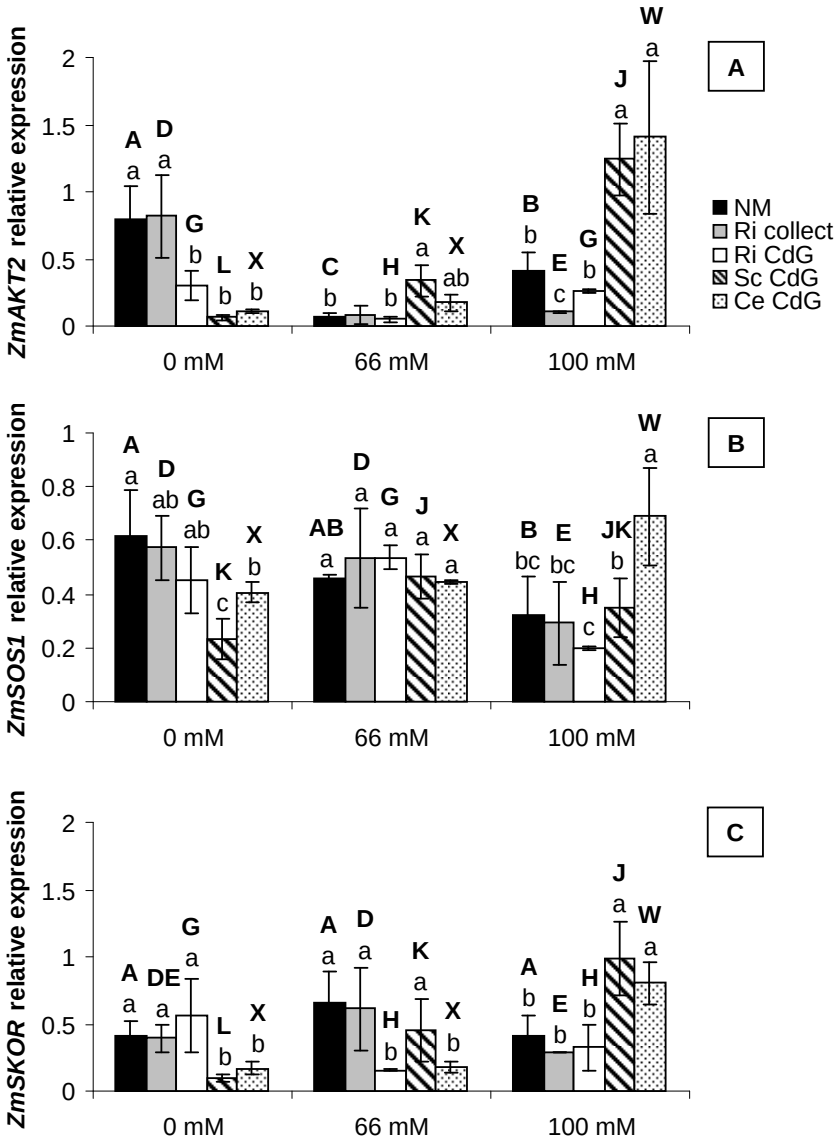

Fig. 8. Analysis of *ZmAKT2* (A), *ZmSOS1* (B) and *ZmSKOR* (C) genes expression by real time quantitative RT-PCR in roots of maize plants from the different treatments. See legend for Fig. 2.

Discussion

Previous studies have demonstrated that maize plants inoculated with AMF grow better than non-mycorrhizal plants under salt stress conditions (Feng *et al.*, 2002; Sheng *et al.*, 2008; Sheng *et al.*, 2011). However these experiments were based on the inoculation of a sole AM fungus, *Glomus mosseae*. Although mycorrhizal symbiosis is usually considered nonspecific, Klironomos (2003) found that there was great variation in growth performance with the same fungal isolate. Therefore, AM colonization would depend on the compatibility of both AMF and host plants. In the present work, we assessed the performance of three AM fungal species isolated from a saline area and compared their effectiveness with a *G. intraradices* (= *Rhizophagus intraradices*) strain belonging to the Zaidin Experimental Station collection. Each species exhibited different mycorrhizal development and symbiotic efficiency under the salt levels assayed.

The results of the present study showed that colonization rates varied among fungal species. Ri collect had the higher rate of root colonization, followed by Ce CdG, Sc CdG and Ri CdG. While most studies reported a decrease in mycorrhizal colonization at increasing salinity levels (Sharifi *et al.*, 2007; Evelin *et al.*, 2012), our study reveals a significant increase in root colonization in three of the AMF strains, Ri collect, Sc CdG and Ce CdG, while Ri CdG had the same colonization rate at all salinity levels. Yamato *et al.* (2008) also found that colonization rates were not reduced in any of the AMF present in coastal vegetation on Okinawa Island. Similar results were reported by Wu *et al.* (2010) in citrus colonized by *Paraglomus occultum* isolated from a saline habitat. These results may be explained in terms of salt-tolerance of the AMF isolates: they can maintain or even increase colonization capacity under saline conditions. On the other hand, Ri collect has been previously described to have a very high rate of colonization (Graham *et al.*, 1996; Ruiz-Lozano *et al.*, 2001), thus it seems not surprising that it maintained or even increased the colonization rate. Nevertheless, under saline conditions the native AMF strains isolated from saline areas maintained a higher symbiotic efficiency with maize plants than the collection strain.

Plant biomass production is an integrative measurement of plant performance under many types of abiotic stress conditions and the symbiotic efficiency of AMF has been measured in terms of plant growth improvement (see reviews by Evelin *et al.*, 2009; Ruiz-Lozano *et al.*, 2012). In our experiment, maize plants inoculated with Ri CdG and Ce CdG had the highest shoot dry biomass at all salinity levels, demonstrating the higher symbiotic efficiency of these native AMF (Oliveira *et al.*, 2005; Querejeta *et al.*, 2006). The growth of maize inoculated with Gi collect was similar to the non-mycorrhizal plants, except at 100 mM NaCl, where it was lower. The latter can be explained due to the high percentage of root colonization by this fungal strain that could demand excessive carbohydrates from the plant. In fact plant growth responses to AMF inoculation can range from parasitic to mutualistic (Klironomos, 2003). Sc CdG had a

similar tendency; previous studies have reported that *G. constrictum* (= *Septoglomus constrictu*m) increased plant dry weight less than other AMF tested (Blaszkowski, 1993; Yu *et al.*, 2010), suggesting a different symbiotic strategy to cope with abiotic stresses rather than a parasitic behaviour. In any case, the positive effect of AM fungal mycorrhization on growth was lower in root tissues than in shoot tissues, which is in agreement with Hajiboland *et al.* (2010).

It has been proposed that mycorrhizal colonization enhances plant salt tolerance by improving photosynthetic ability, water and nutrient uptake, ion balance and osmolite concentration among others (Garg and Manchanda, 2009; Estrada *et al.*, 2012; Ruiz-Lozano *et al.*, 2012). Salt injury can be avoided by maintaining proper osmotic adjustment and ionic homeostasis. Salinity stress induces physiological drought in plants, thus maintaining the water homeostasis is essential to alleviate the impact of salinity on plant growth and crop yield (Dodd and Pérez-Alfocea, 2012). Indeed the extensive hyphal network contributes to water and nutrient uptake because the AM fungus can explore a larger soil volume (Evelin *et al.*, 2012). Another method to maintain a favourable gradient for water flow from soil into the roots is to decrease the plant osmotic potential by active accumulation of organic ions or solutes (Ruiz-Lozano *et al.*, 2012). Proline is a major osmoprotectant osmolyte and in plants colonized by AMF, it has been found to increase more than in non-AM plants at different salinity levels (Sharifi *et al.*, 2007; Talaat and Shawky, 2011). However, reports on the effect of AM symbiosis on proline accumulation are somewhat contradictory and some authors reported that non-MA plants accumulated more proline than AM plants (Rabie and Almadini, 2005; Jahromi *et al.*, 2008; Sheng *et al.*, 2011). Our results did not show significant differences in shoot proline concentration among fungal treatments. In the root, proline content was several times higher than in the shoot and significantly increased with the salinity levels in all treatments, except in Sc CdG. Higher levels of proline in roots could be beneficial as these are the primary sites for water absorption and must maintain osmotic balance between water absorbing root cells and external media (Sharifi *et al.*, 2007). In all, our results suggest that the enhanced salt tolerance in AM maize plants was not due to differences in proline accumulation.

Under salt stress, plants not only accumulate some organic solutes like proline, but also inorganic ions such as potassium to maintain osmotic adjustment (Yang *et al.*, 2009). In salinity conditions, plants increasingly accumulate Na^+ ions which compete with cellular K^+ (Ruiz-Lozano *et al.*, 2012). K^+ functions cannot be replaced by Na^+ ions, thus it is very important to maintain a proper ion homeostasis in terms of K^+/Na^+ ratio (Giri *et al.*, 2007). Our results show a significant increase of K^+ in the leaves of maize plants inoculated with the three native AMF as compared to non-mycorrhizal plants or plants inoculated with the collection fungus. Although in all the treatments Na^+ accumulation increased with salinity, a higher K^+/Na^+ ratio was observed in the plants inoculated with the three native AMF. Several authors have reported a decrease in Na^+ and an increase in K^+ concentrations in AM-inoculated plants (Garg and Manchanda,

2009; Talaat and Shawky, 2011; Evelin *et al.*, 2012). Results are also consistent with Giri *et al.* (2007), who showed higher accumulation of K^+ by mycorrhizal plants in saline soils, thus maintaining a high K^+/Na^+ ratio which influences the ionic balance of the cytoplasm or Na^+ efflux from plants. Recently Hammer *et al*, (2011) demonstrated that *Rh. intraradices* can selectively take up elements such as K^+, Mg^{2+} and Ca^{2+} while avoiding Na^+ uptake. The latter indicates that the AM fungal mycelium might pre-select nutrients for the plants. Moreover, as a significant proportion of elemental nutrient uptake in plants occurs via mycorrhizal fungi, they help to alleviate the effects of the excess of salts in the soil. Our results confirm that *Se. constrictum* also induced a higher K^+/Na^+ ratio compared to non-mycorrhizal plants. In the roots, levels of Na^+ were always higher than in the leaves. It has been proposed that in AM-inoculated pants, Na^+ might be kept inside root cell vacuoles and intraradical fungal hyphae to prevent the allocation of Na^+ to the shoots (Cantrell and Linderman, 2001). Plants inoculated with Ce CdG had the lowest Na^+ concentration at 66 and 100 mM of NaCl, being the most efficient fungus in terms of avoiding Na^+ uptake. Hammer *et al.* (2011) found different concentrations and distributions of Na^+ and Cl^- within the fungal tissue and they hypothesized that AMF exclude Na^+ but include Cl^-. Our results showed that all treatments enhanced Cl^- concentration as salinity in the growing medium increased. Mardukhi *et al.* (2011) proposed that mycorrhizal plants had no control on plant Cl^- uptake. Thus the alleviating effect of AMF on plant growth under salinity stress is more related to Na^+ than Cl^- uptake. Moreover, for maize, Na^+ causes higher ion toxicity than Cl^- (Fortmeier and Schubert, 1995).

The previous results prompted us to hypothesize that AMF may have regulated the expression of plant genes encoding for ion transporters. It is well documented that overexpression of Na^+/H^+ and K^+/H^+ antiporters improve salt tolerance in plants (Zhang *et al.*, 2001; Rodriguez-Rosales *et al.*, 2008). However, scarce information is available on the possible regulation by the AM symbiosis of plant genes involved in ion homeostasis. Until now, only Ouziad *et al.* (2006) have studied the effect of AM symbiosis on the expression of two Na^+/H^+ antiporters in tomato under salt stress conditions, showing no regulation of these genes by the AM symbiosis. Nevertheless, we studied the expression of three genes involved in Na^+ and K^+ transport in order to get some clues on molecular mechanisms involved in the enhanced tolerance of mycorrhizal plants to salinity stress.

The SOS signalling pathway has been pointed out to have a major role maintaining ion homeostasis by regulating Na^+ and K^+ transport at both the plasma membrane and tonoplast (Zhu, 2002, 2003). AKT family contributes to a major potassium acquisition by plants and SKOR transports K^+ to the shoots (Munns, 2005). Based on that, we analyzed the expression of some of these maize genes in the roots of the different treatments. The most important differences among treatments were observed at 100 mM NaCl for the three genes, where plants inoculated with Se CdG or with Ce CdG exhibited enhanced relative expression. In contrast, under non saline

conditions, these plants always showed reduced expression as compared to non-mycorrhizal plants. These results correlate with the higher K^+ and lower Na^+ concentrations found in shoot tissues of maize plants. Thus we suggest that AMF can affect plant ion transport via modification of gene expression. Moreover, different species of AMF differ in the gene transport efficiency and native-AMF have better regulation of these genes, thus enhancing plant salt tolerance.

Summarizing, the tolerance of maize to salt stress was enhanced by the three native AMF more than by the collection one. Based on our results and on existing literature, a major point for salt tolerance in mycorrhizal maize plants is the improvement of plant nutrition and maintenance of ionic homeostasis. We showed selective regulation by AMF of plant ion uptake and accumulation with subsequent effects on the K^+/Na^+ ratio, correlating with plant transporters gene expression. The more effective AMF were *Cl. etunicatum* CdG and *Se. constrictum* CdG. However, also the native *Rh. intraradices* had a better ability to alleviate the inhibitory effect of salt stress than the collection *Rh. intraradices* strain. The characterization of ion transporters of these salt tolerant fungi should be the next step in understanding the molecular mechanisms of salt tolerance acquired by AM symbiosis. The results obtained open important possibilities for sustainable agricultural practices in salinized soils in order to increase crop performance and yield production worldwide.

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CAPÍTULO 5

CHAPTER 5

Native arbuscular mycorrhizal fungi isolated from a saline habitat improved maize antioxidant systems and plant tolerance to salinity

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Hongos formadores de micorrizas arbusculares aislados de hábitat salinos mejoran el sistema antioxidante del maíz y su tolerancia a la salinidad

Resumen

La alta salinidad del suelo es un serio problema para la producción agrícola porque la mayoría de las plantas cultivadas son sensibles sal. Este es el caso de un cultivo tan importante como el maíz. El estrés salino conduce a un estrés oxidativo secundario en plantas y se ha demostrado en varias especies de plantas una correlación entre la capacidad antioxidante y tolerancia a la salinidad. La capacidad antioxidante de la planta puede ser potenciada por los hongos micorrícicos arbusculares (MA) y se ha propuesto que la simbiosis MA es más eficaz con especies nativas que con exóticas. Por lo tanto, se investigó si hongos nativos MA aislados de un ambiente salino pueden ayudar a las plantas de maíz a superar el estrés salino mejor que hongos MA de colección y si la protección frente al estrés oxidativo está implicada en este efecto. Las plantas de maíz inoculadas con los tres hongos nativos MA mostraron una mayor eficiencia del fotosistema II y la conductancia estomática, lo que sin duda contribuyó a reducir la fotorrespiración y la producción de ROS. De hecho, la acumulación de peróxido de hidrógeno, el daño oxidativo a lípidos y la pérdida de electrolitos en la membrana de estas plantas MA fueron significativamente más bajos que en las no micorrizadas o en plantas inoculadas con el hongo MA de colección. La activación de enzimas antioxidantes como la superóxido dismutasa o catalasa también contribuyeron a estos efectos.

Palabras clave: sistema antioxidante, micorriza arbuscular, tolerancia de plantas, salinidad, adaptado a la salinidad

Native arbuscular mycorrhizal fungi isolated from a saline habitat improved maize antioxidant systems and plant tolerance to salinity

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Abstract

High soil salinity is a serious problem for crop production because most of the cultivated plants are salt sensitive. This is the case of a so important crop such as maize. Salinity stress leads to secondary oxidative stress in plants and a correlation between antioxidant capacity and salt tolerance has been demonstrated in several plant species. The plant antioxidant capacity may be enhanced by arbuscular mycorrhizal fungi (AMF) and it has been proposed that AM symbiosis is more effective with native than with exotic AMF species. Thus, we investigated whether native AMF isolated from a saline environment can help maize plants to overcome salt stress better than AMF from collection and whether protection against oxidative stress is involved in such an effect. Maize plants inoculated with three native AMF showed higher efficiency of photosystem II and stomatal conductance, which surely contributed to decrease photorespiration and ROS production. Indeed, the accumulation of hydrogen peroxide, the oxidative damage to lipids and the membrane electrolyte leakage in these AM plants were significantly lower than in non-mycorrhizal plants or in plants inoculated with the collection AMF. The activation of antioxidant enzymes such as superoxide dismutase or catalase also accounted for these effects.

Key words: antioxidant system, arbuscular mycorrhiza, plant tolerance, salinity, salt-adapted

1. Introduction

Maize (*Zea mays* L.) is one of the most important crops both for human and animal consumption. This crop is cultivated on more than 142 million ha of land worldwide and it is estimated to produce around 913 million tonnes of grain in the period 2012/2013 [1], accounting for one third of the total global grain production [2]. In a climate change scenario, one expected threat is the increase in land salinization. Over 6% of the world's land is affected by salinity and its extent is increasing regularly throughout the world [3], causing global agricultural losses equivalent to an estimated US 12 billion a year [4]. Although salinity in soils may occur naturally, inappropriate cultivation practices are also contributing to the salinization of the rhizosphere [5]. Nowadays, high salts in the soils are a very serious problem for crop production because most of the cultivated plants are sensitive to salt stress (glycophytes) [6]. This is the case of maize, which is particularly vulnerable to salinity [7]. Thus land salinization is a major global issue because of its adverse impact on agricultural productivity, sustainability and as a threat for food supply [8]. In arid and semi-arid regions, the issue

is aggravated due to poor soil management practices together with limited rainfall, high evapotranspiration, and high temperature rates [9].

Salinity stress leads to secondary oxidative stress in plants [10]. The latter is produced when pathways are uncoupled in the metabolism of plants and electrons, with high-energy state, are transferred to molecular oxygen to form reactive oxygen species (ROS) [10]. ROS are normally produced at a low level in organelles such as chloroplasts, mitochondria and peroxisomes. However, under salt-stress conditions, their production dramatically increases due to the accumulation of NADPH and ATP that cannot be consumed [11]. This modifies the balance between the formation and removal of ROS species [12]. Singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) can seriously disrupt normal metabolism through denaturalization of proteins, mutation of DNA and lipid peroxidation [13]. Thus, plants need to have appropriate detoxification systems to allow rapid removal of these compounds. They constantly sense the level of ROS and reprogramme their gene expression to respond to changes in their environment [13]. The most common mechanism to detoxify ROS produced during salt-stress response is the induction of ROS-scavenging enzymes, such as superoxide dismutase (SOD) and catalase (CAT) [14]. SOD converts $O_2^{\bullet -}$ to H_2O_2 and then CAT converts H_2O_2 to water and molecular oxygen in peroxisomes. A correlation between antioxidant capacity and NaCl tolerance has been demonstrated in several plant species [15]. Despite the several mechanisms developed by plants to detoxify the excess of ROS production, most of the cultivated plants are glycophytes and under high salts in the soil they cannot cope with the extra production of ROS [6].

Fortunately, a number of beneficial soil microorganisms, particularly arbuscular mycorrhiza fungi (AMF), help plants to cope with abiotic stress conditions [16]. AMF, belonging to the phylum Glomeromycota, are considered the oldest group of organisms living in symbiosis with terrestrial plants [17]. They are fundamental for soil fertility, both in natural and agricultural ecosystems and most land plants are colonized by these fungi [18]. AMF are widely found in saline soils [19-20]. In fact, several authors have demonstrated the beneficial effect of AMF in growth and productivity under salt stress conditions on glycophytes and halophytes, decreasing plant yield loses (reviewed by [11, 21]. Among other mechanisms, some studies have demonstrated that AM symbiosis enhances the activities of antioxidant enzymes, helping plants to alleviate salt stress [22-23]. Most studies have focused in the use of collection species as AMF inoculum [21]. However several studies showed that native AMF can perform better in soils from which they are isolated: agricultural systems [24], polluted soils [25] and semiarid degraded soils [26] among others. According to this, some authors reported that AM symbiosis, is more effective with native than with exotic AMF species [27-29]. However, the mechanisms that allow AM plants to have higher salinity tolerance are far from being understood [11]. The isolation and study of AMF isolated from a saline area

will contribute to elucidate the ecophysiology of AMF under such stress conditions [25].

The aim of the present study was to investigate whether native AMF isolated from a saline environment can help maize plants to overcome the negative effects of salt stress better than AMF from collection and whether protection against oxidative stress is involved in such an effect. Plant physiological and biochemical parameters related to the oxidative status were determined in non-AM maize plants and in maize plants inoculated with different AMF isolates after a salt stress period.

2. Materials and methods

2.1. Identification of the mycorrhizal strains isolated from Cabo de Gata Natural Park

AM fungal spores were separated from the soil samples by a wet sieving process [30]. The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG) [31]; a mixture of PVLG and Melzer's reagent [32]; a mixture of lactic acid to water at 1:1; Melzer's reagent; and water [33]. For identification of the AMF species, spores were then examined using a compound microscope at up to 400-fold magnification as described for glomeromycotean classification by Oehl et al. [34]. The species were identified based on its spore morphology as a *Rhizophagus intraradices* [35], *Claroideoglomus etunicatum* [36] and *Septoglomus constrictum* [37].

In addition to the morphological identification, a molecular identification was also carried out. For that, spores isolated from the bait cultures of each fungal strain were surface-sterilized with chloramine T (2%) and streptomycin (0.02%) and crushed with a sterile disposable micropestle in 40 µL milli-Q water [38]. A two-step PCR was conducted to amplify the AM fungal DNA from the spores. The first PCR step was performed with the universal eukaryote primers NS1 and NS4 region of the small subunit ribosomal gene and the second with the specific AM fungal primers AML1 and AML2 [39]. The amplified DNA was purified using the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Helthcare, UK). DNA fragments were sequenced on an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST program [40].

The BLAST analysis unambiguously placed *Rhizophagus intraradices* as the closest relative of our *Rhizophagus intraradices* CdG strain, with sequence accession number FR750209 [41] having a 99% identity. *Septoglomus constrictum* was the closest relative to our *Se. constrictum* CdG strain, with sequence accession number FR750212 [41] having a 99% identity. Finally, *Claroideoglomus etunicatum* was the closest relative of our *Cl. etunicatum* CdG strain, with sequence accession number FR750216.1 [41] having also a 99% identity. The AM fungal strains have been incorporated to the

collection of Zaidin Experimental Station, Granada, Spain, under accession numbers EEZ 195, EEZ 196 and EEZ 163, respectively.

2.2. Experimental design

The experiment consisted of a randomized complete block design with five inoculation treatments: (1) non-mycorrhizal control plants, (2) plants inoculated with the model AM fungus *Rh. intraradices* (Ri collect), reproduced at the collection of the Zaidin Experimental Station (isolate EEZ01), (3) plants inoculated with the AM fungal strain *Rh. intraradices* isolated from Cabo de Gata Natural Park (Ri CdG), (4) plants inoculated with the AM fungal strain *Se. constrictum* isolated from CdG (Sc CdG) and (5) plants inoculated with the AM fungal strain *Cl. etunicatum* isolated from CdG (Ce CdG). There were 30 replicates of each inoculation treatment, totalling 150 pots (one plant per pot), so that ten of each microbial treatment were grown under non-saline conditions throughout the entire experiment, while ten pots per treatment were subjected to 66 mM of NaCl and the remaining ten pots per treatment were subjected to 100 mM of NaCl.

2.3. Soil and biological materials

Loamy soil was collected from Granada province (Spain, 36º59'34''N; 3º34'47''W), sieved (5 mm), diluted with quartz-sand (<2 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100ºC for 1 h on 3 consecutive days). The original soil had a pH of 8.2 [measured in water 1:5 (w/v)]; 1.5 % organic matter, nutrient concentrations (g kg^{-1}): N, 1.9; P, 1 ($NaHCO_3$-extractable P); K, 6.9. The electrical conductivity of the original soil was 0.5 dS m^{-1}.

Three seeds of maize (*Zea mays*. L) were sown in pots containing 900 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

2.4. Inoculation treatments

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species used were three strains isolated from Cabo de Gata Natural Park (Almería, Spain): *Rhizophagus intraradices* (previously named *Glomus intraradices*), *Septoglomus constrictum* and *Claroideoglomus etunicatum.* A *Rhizophagus intraradices* strain from our culture collection was also used. Appropriate amounts of each inoculum containing about 700 infective propagules (according to the most probable number test), were added to the corresponding pots at sowing time just below maize seeds. Non-mycorrhizal control plants received the same amount of autoclaved mycorrhizal inocula

together with a 10 ml aliquot of a filtrate (< 20 μm) of the AM inocula in order to provide a general microbial population free of AM propagules.

2.5. Growth conditions

The experiment was carried out under glasshouse conditions with temperatures ranging from 19 to 25ºC, 16/8 light/dark period, and a relative humidity of 50-60%. A photosynthetic photon flux density of 800 μE m^{-2} s^{-1} was measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Water was supplied daily to the entire period of plant growth to avoid any drought effect. Plants were established for 45 days prior to salinization to allow adequate plant growth and symbiotic establishment. Three concentrations (0, 66, and 100 mM NaCl) of saline solution were reached in the soil substrate by adding appropriate dilutions of a stock 2 M saline solution. The concentration of NaCl in the soil was increased gradually on alternative days to avoid an osmotic shock. It took 8 days, to reach the desired 66 and 100 mM NaCl levels. Plants were maintained under these conditions for additional 30 days.

2.6. Symbiotic development

The percentage of mycorrhizal root infection in maize plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), as described by Phillips and Hayman [42]. The extent of mycorrhizal colonization was calculated according to the gridline intersect method [43].

2.7. Shoot biomass production

At harvest (75 days after planting), the shoot and root system were separated and the shoot dry weight was measured after drying in a forced hot-air oven at 70ºC for two days.

2.8. Photosynthetic efficiency

The efficiency of photosystem II was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll a fluorescence. FluorPen quantifies the quantum yield of photosystem II as the ratio between the actual fluorescence yield in the light-adapted state (FV‘) and the maximum fluorescence yield in the light-adapted state (FM‘), according to [44]. Measurements were taken in the second youngest leaf of ten different plants of each treatment.

2.9. Stomatal conductance

Stomatal conductance was measured two hours after light turned on by using a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the second youngest leaf from five different plants from each treatment.

2.10. Relative electrolyte leakage

The electrolyte leakage was calculated on the third leaf of each maize plant from a leaf sample of 3 x 1.5 cm as described by Verslues et al. [45]. The initial conductivity (C_0) was measured with a conductivity metre COND 510 (XS Instruments; OptoLab, Milan, Italy) after subjecting the samples to incubation at 25ºC in 10 ml de-ionized water overnight with continuous shaking at 100 rpm. The samples were then autoclaved at 121ºC for 20 min. Final conductivity (C_F) was measured after the samples had cooled down to room temperature. The conductivity of distilled water was also measured and referred as C_w. The percentage of electrolyte leakage was calculated as follows:
$[(C_0 – C_w) / (C_F – C_w)] \times 100$.

2.11. Oxidative damage to lipids

Lipid peroxides were extracted by grinding 500 mg of leaves and roots with 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 min. The chromogen was formed by mixing 200 µl of supernatants with 1 ml of a reaction mixture containing 15% (w/v), trichloroacetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl hydroxytoluene, 0.25N HCl and then incubating the mixture at 100ºC for 30 min [46]. After cooling at room temperature, tubes were centrifuged at 800 × g for 5 min and the supernatant was used for spectrophotometric reading at 532 nm. Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge [47]. The calibration curve was made using MDA in the range of 0.1-10 nmol. A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 0.25N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the reaction mixtures to prevent artifactual formation of TBARS during the acid-heating step of the assay.

2.12. Hydrogen peroxide content

Hydrogen peroxide content in leaves and roots were determined by Patterson et al. [48] method, with slight modifications as described previously by Aroca et al. [49]. Two hundred and fifty milligrams of shoot fresh weight was homogenized in a cold mortar with 5 ml 5% (w/v) TCA containing 0.1 g of activated charcoal and 1% (w/v) PVPP. The homogenate was centrifuged at 18,000 x g for 10 min. The supernatant was filtered through a Millipore filter (0.22 μm). A volume of 1.2 ml of 100 mM potassium phosphate buffer (pH 8.4) and 0.6 ml of the colorimetric reagent were added to 130 μl of the supernatant. The colorimetric reagent was freshly made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4-2 (2-pyridylazo) resorcinol (disodium salt). The samples were incubated at 45ºC for 1 h and the absorbance at 508 nm was recorded. The blanks were made by replacing sample extract by 5% TCA.

2.13. Antioxidant enzymes activities

Enzyme extraction was done as described before by Aroca et al. [50] with slight modifications. Five hundred mg of leaf and root fresh tissues were homogenized separately in a cold mortar with 4 ml of 100 mM phosphate buffer (pH 7.0) containing 0.1 mM DTPA (diethylenetriamine pentaacetic acid; a metal chelating agent) and 40 mg PVPP (polyvinylpolypyrrolidone), which removes phenolics and alkaloids from plant extracts, avoiding interference with spectrophotometric measurements and enhancing enzyme stability. The homogenate was centrifuged at 20,000 x g for 20 min at 4ºC. The supernatant was separated and used to determine the activity of antioxidant enzymes. All enzymatic activities were measured in a spectrophotometer Infinite^{R} 200 PRO series (Tecan Trading AG, Switzerland) at 25 °C. Total protein was assayed by the method described by Bradford [51].

Total superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured according to Becana et al. [52]. One unit of SOD activity (U) was defined as the amount of enzyme which produced a 50% inhibition of nitroblue tetrazolium (NBT) reduction. The reaction mixture (1,9 ml) contained 50 mM phosphate buffer (pH 7.8), 14,3 □M methionine, 82,5 μM NBT, 2,2 μM riboflavin and 100 μl enzyme extract. Riboflavin was added last and tubes were shaken and illuminated with fluorescent light. The reaction was allowed to proceed for 10 min until the colour of the blank shifted to dark violet. Absorbance of the reaction mixture was read at 560 nm.

Catalase (CAT) (EC1.11.1.6) activity was assayed as described by [53]. Consumption of H_2O_2 (extinction coefficient of 39.6 $mM^{-1}cm^{-1}$) at 240 nm for 1 min was monitored. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 5 μl of enzyme extract in a 205 μl volume.

2.14. Statistical Analysis

Statistical analysis was performed using SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA) performing first a one-way ANOVA followed by the Tukey test with $P< 0.05$ as the significance cut-off. Two independent statistical analyses were carried out: the first to analyze data from the different AMF treatments within each saline level and the second one to analyze data from each fungal treatment at increasing salinity.

3. Results

3.1. Symbiotic development and shoot biomass production

Root AM colonization increased by salinity in plants colonized by all the fungus except those colonized by Ri CdG, which showed the lowest values of root colonization at all salinity levels (Table 1). Under both saline conditions (66 and 100 mM NaCl) Ri collect showed the highest rate of root length colonization (Table 1). The other two fungi (Sc CdG and Ce CdG) showed intermediate colonization capacity (Table 1).

The shoot biomass production in all treatments was negatively affected by the increase of salt application. In the case of non-mycorrhizal plants the decrease was not significant (Table 1). In any case, at each saline level, both Ri CdG and Ce CdG, enhanced maize shoot biomass as compared to the non-mycorrhizal plants, while Ri collect and Sc CdG did not (Table 1).

3.2. Stomatal conductance

Under non-saline conditions no differences in stomatal conductance were observed among inoculation treatments (Fig. 1A). Salinity application decreased stomatal conductance in all treatments. However, at both saline levels, plants inoculated with the three native AMF from Cabo de Gata showed higher stomatal conductance than non-mycorrhizal plants or plants colonized by Ri collect (Fig. 1A).

3.3. Photosystem II efficiency

The application of 100 mM NaCl, decreased the efficiency of photosystem II in maize plants as compared to plants cultivated at 0 mM NaCl. Under non-saline conditions, photosynthetic efficiency was increased by inoculation with all the native AMF from Cabo de Gata (Fig. 1B). Growing under 66 mM NaCl did not cause any significant descent in photosynthetic efficiency, except in plants inoculated with Ce

CdG (Fig. 1B). When plants were subjected to 100 mM NaCl all the mycorrhizal plants showed higher photosynthetic efficiency than non-mycorrhizal plants, mainly plants inoculated with Ri CdG and Ce CdG (Fig. 1B).

AMF treatment	NaCl (mM)	AM root colonization (%)	Shoot Dry Weight
	0	0	4.03 ± 0.14 Ab
NM	66	0	3.95 ± 0.12 Ab
	100	0	3.69 ± 0.11 Ab
	0	67 ± 1.9 Ea	3.87 ± 0.10 Db
Ri collect	66	88.7 ± 2.0 Da	3.51 ± 0.14 DEb
	100	84.3 ± 1.1 Da	3.17 ± 0.16 Ec
	0	21 ± 5.8 Gc	5.07 ± 0.22 Ga
Ri CdG	66	19.7 ± 1.5 Gc	4.62 ± 0.17 GHa
	100	23 ± 0.7 Gd	4.19 ± 0.11 Ha
	0	34.7 ± 1.5 Kb	3.78 ± 0.31 Jb
Sc CdG	66	62.7 ± 4.5 Jb	3.64 ± 0.20 Jb
	100	58.7 ± 1.1 Jc	2.90 ± 0.08 Kc
	0	62.3 ± 2.3 Xa	4.97 ± 0.16 Wa
Ce CdG	66	65.3 ± 1.1 Xb	5.17 ± 0.11 Wa
	100	77.3 ± 1.8 Wb	4.62 ± 0.10 Xa

Table 1. Percentage of mycorrhizal root colonization and shoot dry weight (g plant^{-1}) in maize plants. NM represents non-mycorrhizal control plants; Ri collect, plants inoculated with the collection *Rhizophagus intraradices* strain; Ri CdG, plants inoculated with the native *Rh. intraradices* CdG strain; Sc CdG, plants inoculated with the native *Septoglomus claroideum* CdG strain and Ce CdG, plants inoculated with the native *Claroideoglomus etunicatum* CdG strain. Plants were subjected to 0, 66 or 100 mM NaCl. Different letters indicate significant differences ($p < 0.05$) among fungal treatments at each salt level (a, b, c, d) or among salt levels for each AMF treatment: NM plants (A, B, C), Ri collect (D, E, F), Ri CdG (G, H, I), Sc CdG (J, K, L) or Ce CdG (W, X, Y).

3.4. Relative electrolyte leakage

The application of 66 or 100 mM NaCl did not significantly affect the electrolyte leakage of maize plants as compared to plants growing in the absence of salinity (Fig. 1C). However, at each saline level non-mycorrhizal plants had always the highest values of electrolyte leakage, followed by plants inoculated with Ri collect. In contrast, maize plants inoculated with any of the three native AMF always showed significantly lower electrolyte leakage than control non-mycorrhizal plants (Fig. 1C).

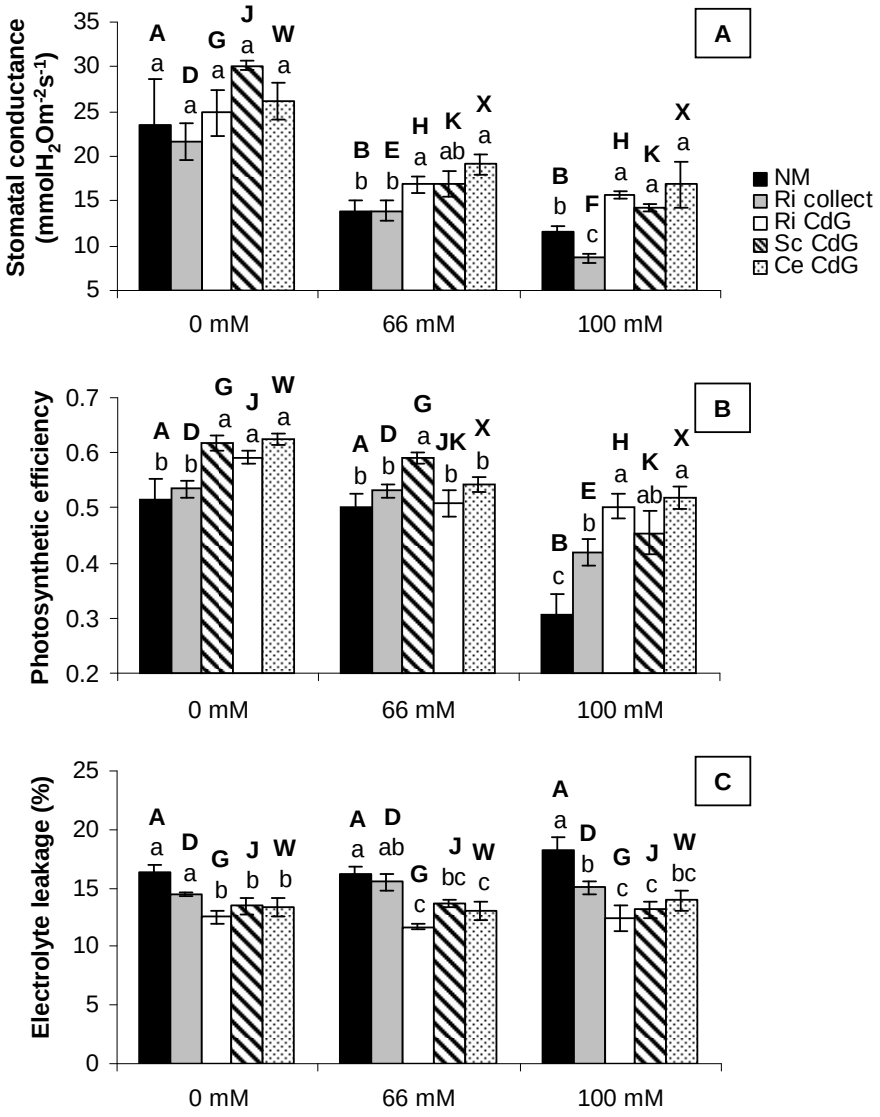

Fig. 1. Stomatal conductance (mmol H_2O $m^{-2}s^{-1}$) (A), efficiency of photosystem II (B) and electrolyte leakage (C) in maize plants. Black bars represent non-mycorrhizal control plants (NM); grey bars, plants inoculated with the collection *Rhizophagus intraradices* strain (Ri collect); white bars, plants inoculated with the native *Rh. intraradices* CdG strain (Ri CdG); lined bars, plants inoculated with the native *Septoglomus claroideum* CdG strain (Sc CdG) and dotted bars, plants inoculated with the native *Claroideoglomus etunicatum* CdG strain (Ce CdG). Plants were subjected to 0, 66 or 100 mM NaCl. Different letters indicate significant differences ($p < 0.05$) among fungal treatments at each salt level (a, b, c, d) or among salt levels for each AMF treatment: NM plants (A, B, C), Ri collect (D, E, F), Ri CdG (G, H, I), Sc CdG (J, K, L) or Ce CdG (W, X, Y).

3.5. Oxidative damage to lipids

The application of 66 or 100 mM NaCl increased lipid peroxidation in leaves of non-mycorrhizal plants and plants inoculated with Ri collect and Ce CdG. Plants inoculated with Ri CdG only enhanced lipid peroxidation at 100 mM NaCl, while plants

inoculated with Sc CdG showed no significant changes in this parameter as a consequence of increasing salinity. When plants were grown at 66 or 100 mM NaCl, the plants inoculated with the three native AMF from Cabo de Gata showed the lowest values of lipid peroxidation in their leaves (Fig. 2A). In roots, the lipid peroxidation also increased with increasing salinity, especially in non-mycorrhizal plants and in plants inoculated with Ri collect at 100 mM NaCl. In contrast, in plants inoculated with any of the three native AMF from Cabo de Gata, the increase in lipid peroxidation due to salinity was less evident. In addition, these plants always exhibited lower lipid peroxidation in roots than non-mycorrhizal plants or those inoculated with Ri collect (Fig. 2B).

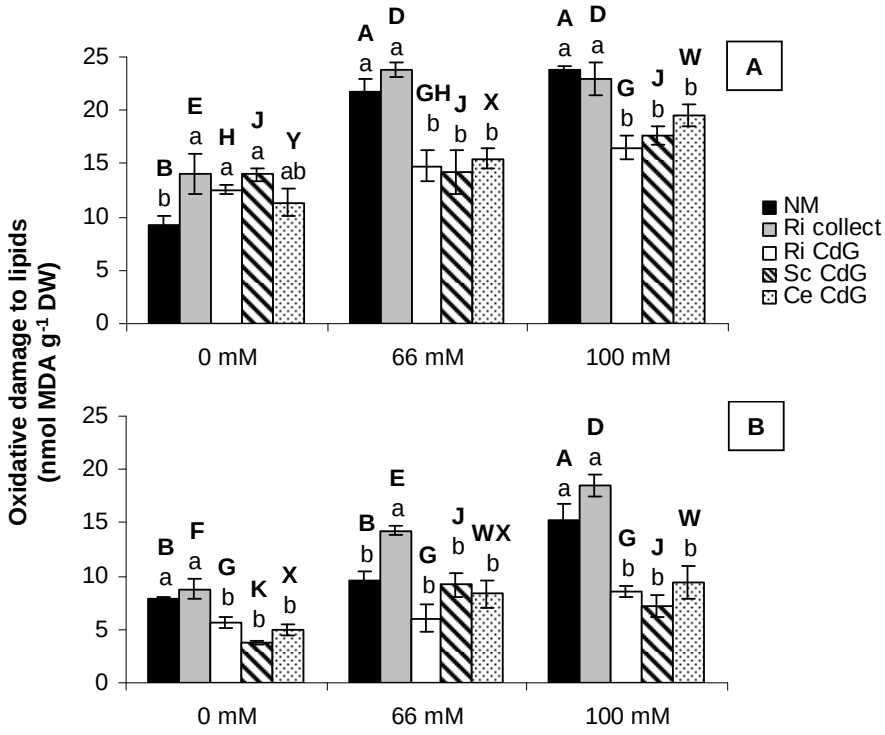

Fig. 2. Shoot (A) and root (B) oxidative damage to lipids in maize plants. See legend for Fig. 1.

3.6. Hydrogen peroxide accumulation

In leaves, the accumulation of hydrogen peroxide due to the increasing salinity only rose in non-mycorrhizal plants and in plants colonized by Ri collect when subjected to 100 mM NaCl (Fig. 3A). At this NaCl concentration the lowest value of hydrogen peroxide was observed in leaves of plants colonized by Ri CdG (Fig. 3A). In roots, the accumulation of hydrogen peroxide increased considerably in non-mycorrhizal plants after exposure to 66 or 100 mM NaCl. Plants inoculated with Ri collect and Ri CdG plants also enhanced this parameter as a consequence of increasing salinity (Fig. 3B). However, at 66 and 100 mM NaCl, the accumulation of hydrogen peroxide was lower in roots of plants colonized by any of the three native AMF from

Cabo de Gata than in the non-mycorrhizal plants or plants colonized by Ri collect (at 100 mM NaCl) (Fig. 3B).

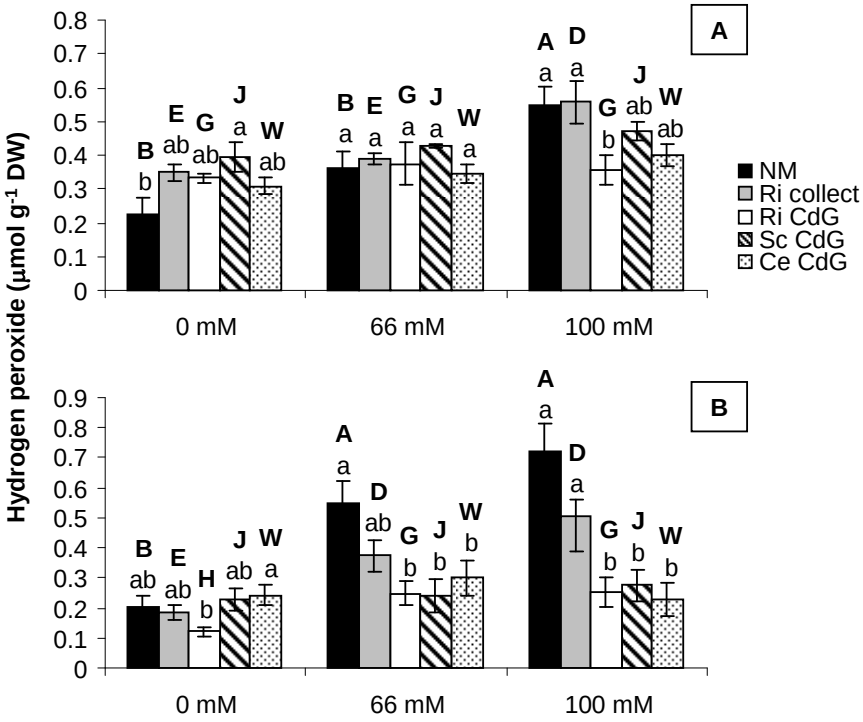

Fig. 3. Shoot (A) and root (B) hydroxide peroxide content in maize plants. See legend for Fig. 1.

3.7. Antioxidant enzyme activity

In non-mycorrhizal plants, leaf SOD activity increased considerably by both salt treatments and it increased only slightly in Ri CdG and Ce CdG plants after application of 100 mM NaCl (Fig. 4A). Plants inoculated with Ri collect or Sc CdG did not alter significantly their leaf SOD activity by any of the salt treatments (Fig. 4A). When data were analyzed within each salt level, no significant differences in leaf SOD activity among inoculation treatments were observed at 66 or 100 mM NaCl, while at 0 mM NaCl, three out the four mycorrhizal treatments exhibited higher SOD activity in their leaves than the non-mycorrhizal plants (Fig. 4A). Root SOD activity increased by salt application only in plants colonized by Ce CdG (at both 66 and 100 mM NaCl) and in plants colonized by Ri CdG (at 100 mM NaCl) (Fig. 4B). Under 100 mM NaCl conditions, plants colonized by Ce CdG showed the highest root SOD activity, followed by plants colonized by Ri CdG and Sc CdG. Non-mycorrhizal plants and those colonized by Ri collect had the lowest values of root SOD activity under the highest salt concentration treatment (Fig. 4B).

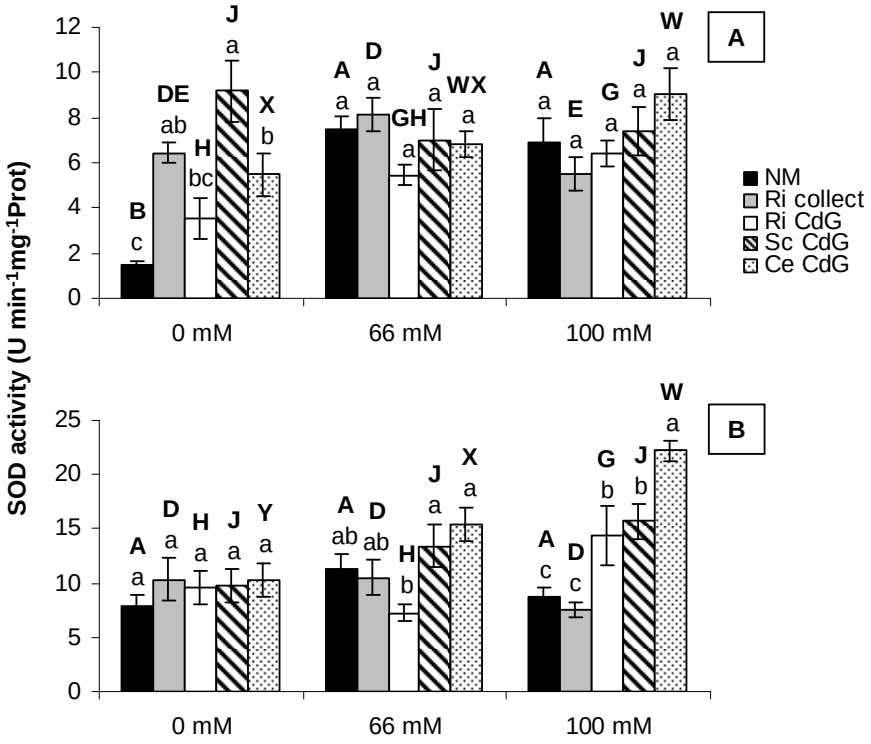

Fig. 4. Shoot (A) and root (B) SOD activity in maize plants. See legend for Fig. 1.

The catalase activity in shoots resulted differently affected by salt application depending on the microbial treatment. Thus, non-mycorrhizal plants and plants inoculated with Ri collect enhanced transiently CAT activity after application of 66 mM NaCl, but they almost recovered this activity to initial values after application of 100 mM NaCl. (Fig. 5A). Plants inoculated with Ri CdG only increased CAT activity when the salt applied reached 100 mM NaCl. No significant effect of salinity on this parameter was observed in plants inoculated with Sc CdG, while plants inoculated with Ce CdG enhanced their shoot CAT activity at both 66 and 100 mM NaCl. In root tissues, CAT activity increased in all treatments after the application of either 66 or 100 mM NaCl. When data were analyzed within each salt level, it was observed that under non-saline conditions CAT activity was unaffected by inoculation treatments in both leaves and roots (Fig. 5A, B). At 66 mM NaCl, all the mycorrhizal plants showed higher CAT activity in their roots than the non-mycorrhizal plants, while in shoots the plants inoculated with the three native AMF exhibited lower CAT activity than those inoculated with Ri collect or the non-mycorrhizal plants. Finally, at 100 mM NaCl, no significant differences in CAT activity were observed in roots, while plants inoculated with Ri CdG and Ce CdG, had the highest CAT activity in shoots.

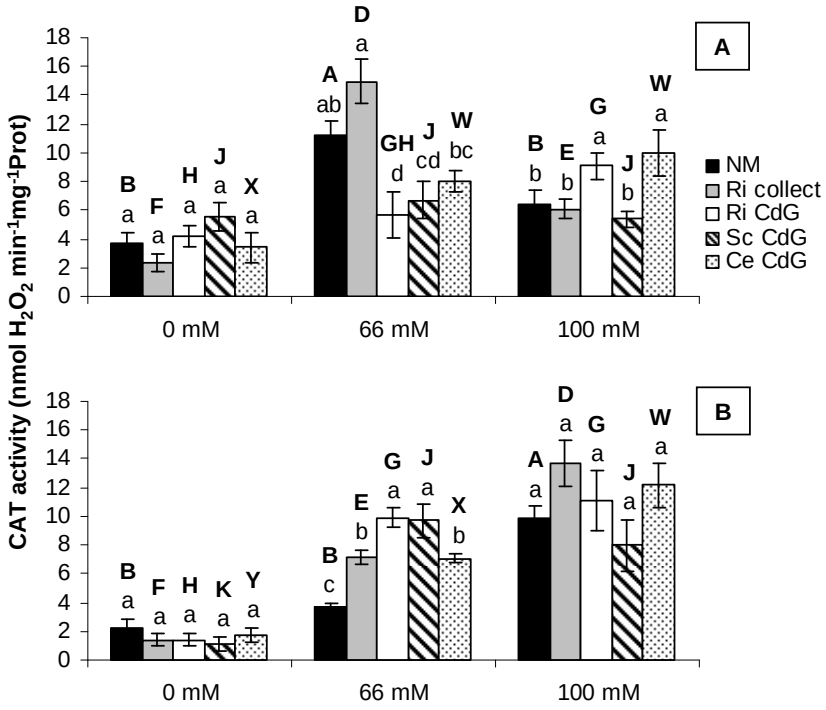

Fig. 5. Shoot (A) and root (B) CAT activity in maize plants. See legend for Fig. 1.

4. Discussion

Excessive soil salinity is well known to induce oxidative stress in plants [54]. Previous studies have suggested that tolerance of plants to salt stress is associated with the induction of antioxidant enzymes and reduction of oxidative damage [55-56]. AM symbiosis enhances the activity of antioxidant enzymes in order to help plants to cope with the reactive oxygen species generated by salinity [22-23, 57]. Nevertheless the response of the individual enzymes varies with respect to the host plant and the fungal species involved in the association [11]. Results from the present work confirms that native AMF reduced the oxidative damage in the host plant, but also that they differed in their response to salinity, in the modulation of the antioxidative capacity of the host plant and in the promotion of plant growth under saline conditions. The degree of root colonization also varied among fungal species, but the literature reflects that there is no threshold value of root colonization for enhancement of plant fitness and this depends on the plant and the fungal species involved [58]. In fact Ri collect had higher colonization rate than the autochthonous AMF but it did not show better symbiotic efficiency.

In this study, AM plants may improve the net assimilation rates by protecting photochemical processes of photosystem II and enhancing stomatal conductance when subjected to salinity stress. Plants inoculated with native AMF exhibited better performance of photosystem II and higher stomatal conductance when subjected to high

salinity level (100 mM NaCl). These results indicate less damage in the photosynthetic machinery and enhanced transpiration rates in maize plants inoculated with native AMF. Sheng et al. [59] and Hajiboland et al. [60] reported a similar tendency, and Querejeta et al. [27] showed that the enhancement of plant stomatal conductance was higher with native AMF than with collection fungi. These two effects may have accounted for the enhanced tolerance of AM plants, particularly native inoculated ones, most likely by enhancing CO_2 fixation and plant growth during salinity stress. In any case, the native Sc CdG also improved these physiological parameters but it did not enhance plant growth, demonstrating a differential ability by each fungus or that an extended growing period was needed to achieve a positive effect on plant growth.

The above mentioned processes could have also contributed to decrease photorespiration and then lead to lower ROS production in AM plants [10]. Indeed, the accumulation of hydrogen peroxide under salinity was considerably lower in roots of plants inoculated with the three native AMF and the oxidative damage to lipids was also lower in shoots and roots of these AM plants. Several studies have reported lower H_2O_2 accumulation in AM plants [60-61]. Our results demonstrate that colonization of maize plants by salinity-adapted AMF lead to a lower accumulation of ROS species. Salt stress injuries the membrane integrity, which produces ion leakage. Thus one of the key processes in salinity tolerance is to prevent lipid peroxidation to maintain the membrane integrity [57, 62]. The oxidation of membrane lipids gives a reliable indication of an extra free radical production leading to oxidative stress [63]. Data from this study showed a lower MDA content in native-AMF inoculated plants. A similar reduction of oxidative damage to lipids by AM symbiosis has been observed in tomato plants subjected to salinity stress [23, 60]. These data, together with the lower electrolyte leakage in maize plants inoculated with native AMF from Cabo de Gata, provides evidence that the presence of native-AM symbiosis can prevent cell membrane injury. This is in accordance to some other works that showed lower lipid peroxidation and membrane permeability in AM plants compared to non-mycorrhizal plants [23, 57, 62, 64]. For instance, Kaya et al. [65] also reported that the electrolyte leakage in leaves of *Capsicum annum* treated with 50 mM and 100 mM NaCl were decreased significantly by AM inoculation. Again, plants inoculated with native AMF Ri CdG and Sc CdG had lower electrolyte leakage than non-mycorrhizal plants or plants inoculated with Ri collect. Several works have shown that AMF isolated from a saline area exhibited significant higher symbiotic efficiency than non-saline AMF [19, 29]. Moreover, Querejeta et al. [27] suggested that modulation of leaf gas exchange parameters in Mediterranean environments by native-adapted AMF is of critical importance for host plant performance in semiarid environments.

As discussed previously, the lower MDA concentrations in AM-inoculated plants may have been due to the better performance of photosystem II and lower generation of ROS in these plants. However, the activation of antioxidant enzymes such as superoxide dismutase (SOD) or catalase (CAT) may have also accounted [14, 66].

Our results showed that AM symbiosis significantly influenced SOD and CAT activities to different extent depending on the AMF species, which might be the result of a complex interaction among AMF, maize and the salinity stress. The reactive oxyradical scavenging enzyme is an antioxidant system that plays a role maintaining cell membrane stability in plant cell [67]. SOD enzyme catalyses the conversion of free $O_2^{\bullet-}$ to O_2 and H_2O_2. In the present work, the effect of salt stress on antioxidant enzyme activity in maize revealed that the increase of SOD activity was higher in roots than in shoots. In the root tissues, at 100 mM NaCl, all native AM-inoculated plants showed significantly higher SOD activity and Ce CdG had the highest value. Several studies reported that AMF enhance SOD activity in mycorrhizal plants under salt stress conditions [57, 60, 68]. The greater SOD activity that we observed in mycorrhizal plants could increase the capacity to scavenge superoxide radicals. Enhancement of SOD activity under salt stress after the inoculation with salinity adapted fungi suggests that native AMF improve the capacity of maize plants to cope with oxidative stress. In a previous work, we provided evidence that the encoding gene for SOD in the fungus Ri CdG was up-regulated under saline conditions. Hence we confirmed a fungal response to the oxidative stress induced by salinity [29] which may have accounted also for the reduced oxidative damage in the inoculated maize plants. Once the free $O_2^{\bullet-}$ is detoxified by SOD activity, there is a need to scavenge hydrogen peroxide which is still toxic and must be eliminated by conversion to H_2O in subsequent reactions. A number of enzymes regulate H_2O_2 intracellular levels in plants, and CAT is among the most important ones [13]. Several authors have reported an enhancement of CAT activity by inoculation with AMF [57, 60, 69]. However, in our work the effect of AMF on CAT activity under salinity stress was little evident, which is in agreement with other reports indicating that AM symbiosis did not affect CAT activity of salt stressed plants [23, 68].

The effects of AM symbiosis on the antioxidant systems observed in this study were more prominent under the highest salt stress level (100 mM NaCl). The latter suggests that under mild salts in the soil, maize antioxidant system could be enough to cope with the stress. However, at higher levels of salt in the soil, AMF, particularly salinity-adapted ones, are important to improve the capacity of maize to grow under such unfavourable conditions. Greater SOD activity in plants inoculated with native AMF compared with non-mycorrhizal or Ri collect plants was associated with lower accumulation of H_2O_2 and less lipid peroxidation, indicating lower oxidative and membrane damage in the native AMF-inoculated plants. Our observations are in agreement with Bartels [70] that proposed both the prevention of oxidative stress and the elimination of ROS as the most effective approaches used by plants to gain tolerance against several abiotic stresses, including salinity.

Our results supports that AM inoculation enhances maize salt tolerance by alleviating the salt induced oxidative stress and membrane damage. To this effect, the better performance of photosystem II and stomatal conductance of plants inoculated with the three native AMF must also have contributed through reduced ROS generation.

This work demonstrates that AMF isolated from saline areas appear to be physiologically adapted to the stress conditions, conferring higher salinity tolerance and improving the growth of an important crop plant such as maize under salinity stress. In agricultural lands with high levels of disturbance, inoculation is beneficial due to low AM fungal inoculum potential [71]. Thus, from this work we highlight the potential use of AMF from saline habitats to make successful inocula to improve crop growth and yield in saline soils.

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CAPÍTULO 6

CHAPTER 6

Importance of native arbuscular mycorrhizal inoculation in the halophyte *Asteriscus maritimus* for successful establishment and growth under saline conditions

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Importancia de la inoculación con hongos nativos formadores de micorrizas arbusculares en la especie halófita *Asteriscus maritimus* para su completo establecimiento y crecimiento bajo condiciones de salinidad.

Resumen

Antecedentes y objetivos:
Los ecosistemas mediterráneos afectados por salinidad se presentan ante un problema creciente de degradación. La restauración biológica de los ecosistemas salinos se podría lograr mediante el uso de especies de plantas halófitas junto con la inoculación de hongos MA adaptados. Una planta de interés para su uso en la restauración de los ambientes salinos, *Asteriscus maritimus* es altamente micotrófica. El objetivo del este estudio fue evaluar la efectividad de hongos MA nativos y alóctonos en el establecimiento y crecimiento de la halófita *A. maritimus* bajo condiciones de salinidad.
Métodos:
Se estudió la efectividad simbióticade cuatro aislados de hongos MA (tres nativos de un suelo salino y uno alóctono de colección) en *A. maritimus* sometido a niveles crecientes de salinidad. Se midieron parámetros fisiológicos de la planta en los que los hongos MA pueden aliviar el efecto negativo del estrés salino, como la conductancia estomática y la eficiencia del fotosistema II, acumulación de compuestos antioxidantes, actividad de enzimas antioxidantes o daño oxidativo a lípidos.
Resultados:
Las plantas de *A. maritimus* mostraron una gran dependencia micorrícica incluso en ausencia de estrés salino. Al nivel más alto de salinidad, las plantas inoculadas con hongos nativos MA tuvieron mayor biomasa aérea, eficiencia del fotosistema II, conductancia estomática, acumulación de glutatión y actividad de varias enzimas antioxidantes que plantas inoculadas con el hongo MA de colección. Además, a este nivel de sal, únicamente 30% de las plantas de *A. maritimus* inoculadas con el hongo MA de colección sobrevivieron, mientras que en las plantas colonizadas por los hongos nativos el porcentaje de supervivencia fue del 100%.
Conclusiones:
El presente estudio muestra la importancia de la inoculación con hongos nativos en establecimiento, supervivencia y crecimiento de *A. maritimus*. El uso de un inóculo adecuado de hongos MA nativos puede ser un elemento crucial para el éxito en la recuperación de áreas salinas degradadas.

Palabras clave: micorrizas arbusculares nativas, ecosistemas mediterráneos, halófitas, restauración, salinidad

Importance of native arbuscular mycorrhizal inoculation in the halophyte *Asteriscus maritimus* for successful establishment and growth under saline conditions

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Abstract

Background and aims:
Salt affected Mediterranean ecosystems have an increasing problem of degradation. The biological restoration of saline habitats could be achieved by using halophyte plant species together with adapted arbuscular mycorrhizal fungi (AMF). An interesting plant to be used in restoration of saline environments, *Asteriscus maritimus,* is highly mycotrophic. The aim of this study was to assess the effectiveness of native and allochthonous AMF to enhance the establishment and growth of the halophyte *A. maritimus* under saline conditions.
Methods:
We studied the symbiotic effectiveness of four AMF strains (three native fungal isolates from a saline soil and one allochthonous, from collection) in *A. maritimus* subjected to increasing salinity stress. We measured plant physiological parameters by which AMF may ameliorate the detrimental effects of salinity stress such as stomatal conductance and efficiency of photosystem II, accumulation of antioxidant compounds, antioxidant enzyme activities or oxidative damage to lipids.
Results:
A. maritimus plants showed a high mycorrhizal dependency, even in absence of salt stress. At the highest level of salinity, plants inoculated with native AMF had higher shoot dry weight, efficiency of photosystem II, stomatal conductance, accumulation of glutathione and activity of certain antioxidant enzymes than those inoculated with the collection AMF. Moreover, at this salt level, only 30% of *A. maritimus* plants inoculated with the collection AMF survived, while with the three native AMF, the rate of survival was 100%.
Conclusions:
Results points out the importance of native AMF inoculation in the establishment, survival and growth of *A. maritimus* plants. Thus, the use of adequate native AMF inocula could be a critical issue for success in recovering saline degraded areas.

Key words: native arbuscular mycorrhiza fungi; Mediterranean ecosystems; halophyte; restoration; salinity.

Introduction

Mediterranean regions are characterized by very limited rainfall, high light irradiance and maximum air temperatures largely above 30ºC in summer, which difficult plant growth (Brito et al. 2011). The latter may provoke the loss of natural plant communities and accelerate the processes of soil degradation and environmental

changes (Alguacil et al. 2011). These conditions make Mediterranean ecosystems fragile and susceptible to degradation and desertification (Ferrol et al. 2004). Thus in Mediterranean areas, global change will involve not only increased in aridity but also significant changes in land use (Valladares 2004). This multiple stress situation is promoting desertification of large areas in Southeast Spain and an alarming increase in salinity. Salinization of soils is a major problem, not only agronomical but also ecological, in particular in arid and semiarid ecosystems, where water is scarce and salts cannot be properly dissolved and they accumulate in the soil (Evelin et al. 2009). High salinity induces ionic toxicity, osmotic stress and leads to secondary oxidative stress in plants (Ding et al. 2010). The excess of reactive oxygen species (ROS) production can damage the structures of enzymes and other macromolecules in plant cells (Mittler 2002). One of the most important tasks to restore the productivity of saline lands is to improve soil conditions, reduce desertification and raise the fertility of soils (Tawfik et al. 2010). Hence in restoration of Mediterranean ecosystems there is a need to look for ecophysiological features that enhance plant performance under conditions that will be exacerbated by climate change, such as salinity (Vallejo et al. 2005).

The below-ground microbial communities, particularly arbuscular mycorrhiza fungi (AMF), are well known to improve soil structure and benefit plant performance by helping in the establishment, enhancing the resistance to environmental stresses and increasing plant nutrient and water uptake (Jeffries and Barea 2012). Mycorrhizal symbiosis is universally distributed among the majority of plants and forms a network of extra-radical mycelium that provides a direct physical link between the plant root and the soil (Smith and Read 2008). Moreover, they affect the diversity and productivity of plants helping in the stability and sustainability of ecosystems (van der Heijden et al. 1998). Although is has been reported that excess of salts in the soil inhibits spore germination and growth of AMF (Juniper and Abbott 2006), several studies found a high diversity of those fungi in saline soils (Yamato et al. 2008; Wilde et al. 2009). Even more, in arid and semiarid Mediterranean regions, AMF play a key ecological role in the functioning of ecosystems (Requena et al. 1996).

On the other hand, halophytes, which constitute about 1% of the world flora, are specialised plants that can tolerate and complete their life cycles under high levels of salts in the soil (Munns and Tester 2008). They are physiologically and biochemically adapted to grow in saline soils (Tawfik et al. 2010). Most of the halophytes in saline sites belong to the *Chenopodiaceae*, *Juncaceae*, *Cyperaceae* or *Brassicaceae* families which are non-or weakly mycotrophic plants. However, associations between AMF and halophytes are also widely formed, including *Aster tripolium*, *Inula crithmoides*, *Plantago maritima*, *Salsola soda* and *Suaeda maritima* (Evelin et al. 2009; Sonjak et al. 2009). For the present study we selected a native halophyte of lands surrounding the Mediterranean Sea, especially Spain, *Asteriscus maritimus* (L.) Less. (Lendínez et al. 2011). Rodríguez et al. (2005) considered this plant as an useful species in revegetation programmes in Mediterranean areas affected by salinity. *A. maritimus* is a member of

the Asteraceae family and known since long time to be highly mycotrophic (Mason 1928). In fact native plants thrive in various abiotically stressed ecosystems thanks to AMF that have co-evolved and are essential for their adaptation to stressed conditions (Rodriguez and Redman 2008). Nevertheless, in arid and semiarid ecosystems there is a low density of AM propagules making difficult the successful reestablishment of native plants (Jeffries et al. 2002). Thus, it is very important the ecophysiological study of autochthonous AMF isolates and the knowledge of their mechanisms of salinity stress adaptation and tolerance (Enkhtuya et al. 2000) in order to select the most adapted and efficient species/strains of AMF to serve as inocula in revegetation programs (Ferrol et al. 2004). Both halophytes and indigenous AMF isolates from saline habitats have developed a variety of modifications to survive in saline environments, such as regulating ionic homeostasis and detoxifying ROS (Zhu 2001; Ruiz-Lozano et al. 2012). The biological restoration of saline ecosystems could be achieved by using halophytes together with inoculation of adapted AMF. However the biochemical mechanisms by which AM-colonized halophytic plants tolerate or reduce salt stress are poorly understood.

In the present work, we studied the symbiotic effectiveness of four AMF strains (three native isolates from a saline soil and one allochthonous, belonging to the EEZ collection) in *Asteriscus maritimus* subjected to salinity stress. The native strains of AMF were successfully isolated from the rizhosphere of *A. maritimus* at Cabo de Gata Natural Park (Almería, SE Spain), which is the most arid ecosystem in Europe (Geiger 1973) with important salinity problems as well. The aim was to assess the effectiveness of AM association among different AM isolates under saline conditions, to enhance the establishment and growth of the halophyte *A. maritimus* for revegetation in salt affected areas. In addition, we tried to elucidate the physiological and biochemical basis of the mechanisms involved in the variability of the symbiotic performance.

Materials and methods

Identification of the mycorrhizal strains isolated from Cabo de Gata Natural Park

AMF spores were separated from the soil samples by a wet sieving process (Sieverding 1991). The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG) (Koske and Tessier 1983); a mixture of PVLG and Melzer's reagent (Brundrett et al. 1994); a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain 1990). For identification of the AMF species, spores were then examined using a compound microscope at up to 400-fold magnification as described for glomeromycotean classification by Oehl et al. (2011). The species were identified based on its spore morphology as a *Rhizophagus intraradices* (Schenk and Smith 1982),

Claroideoglomus etunicatum (Becker and Gerdemann 1977) and *Septoglomus constrictum* (Trappe 1977).

In addition to the morphological identification, a molecular identification was also carried out. For that, spores isolated from the bait cultures of each fungal strain were surface-sterilized with chloramine T (2%) and streptomycin (0.02%) and crushed with a sterile disposable micropestle in 40 µL milli-Q water (Ferrol et al. 2004). A two-step PCR was conducted to amplify the AMF DNA from the spores. The first PCR step was performed with the universal eukaryote primers NS1 and NS4 region of the small subunit ribosomal gene and the second with the specific AM fungal primers AML1 and AML2 (Lee et al. 2008). The amplified DNA was purified using the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Helthcare, UK). DNA fragments were sequenced on an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST program (Altschul et al. 1990).

The BLAST analysis unambiguously placed *Rhizophagus intraradices* as the closest relative of our *Rhizophagus intraradices* CdG strain, with sequence accession number FR750209 (Krüger et al. 2012) having a 99% identity. *Septoglomus constrictum* was the closest relative to our *Se. constrictum* CdG strain, with a sequence accession number FR750212 (Krüger et al. 2012) having a 99% identity. Finally, *Claroideoglomus etunicatum* was the closest relative of our *Cl. etunicatum* CdG strain, with sequence accession number FR750216.1 (Krüger et al. 2012) having also a 99% identity. The AM fungal strains have been incorporated to the collection of Zaidin Experimental Station, Granada, Spain, under accession numbers EEZ 195, EEZ 196 and EEZ 163, respectively.

Experimental design

The experiment consisted of a randomized complete block design with five inoculation treatments: (1) non-mycorrhizal control plants, (2) plants inoculated with the model AM fungus *Rh. intraradices* (Ri collect) reproduced at the collection of the Zaidin Experimental Station (isolated EEZ 01), (3) plants inoculated with the AM fungal strain *Rh. intraradices* isolated from Cabo de Gata Natural Park (Ri CdG), (4) plants inoculated with the AM fungal strain *Se. constrictum* isolated from CdG (Sc CdG) and (5) plants inoculated with the AM fungal strain *Cl. etunicatum* isolated from CdG (Ce CdG). There were 30 replicates of each inoculation treatment, totalling 150 pots (one plant per pot), so that ten of each microbial treatment were grown under nonsaline conditions throughout the entire experiment (only the salinity provided by the soil/sand mixture used), while ten pots per treatment were subjected to 100 mM of NaCl and the remaining ten pots per treatment were subjected to 175 mM of NaCl.

Soil and biological materials

Loamy soil was collected from Cabo de Gata Natural Park (Almería province) (Spain, 36º45´24´´N 02º13´17´´W), sieved (5 mm), diluted with quartz-sand (<2 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100ºC for 1 h on 3 consecutive days). The original soil had a pH of 8.7 [measured in water 1:5 (w/v)]; 0.26 % organic matter, nutrient concentrations (g kg^{-1}): N, 0.3; available P, 47.0 and soil electrical conductivity 3.95 dS m^{-1}.

Seeds of *Asteriscus maritimus*. L were sown on a vermiculite:sand mixture (1:1, v/v) for germination. Two weeks after germination, four seedlings of *A. maritimus* were transplanted per pot containing 900 g of the same soil/sand mixture as described above and thinned to one plant per pot after establishment was successfully done (plants were selected with uniform size for each treatment).

Inoculation treatm

Mycorrhizal inoculum consisted of soil, spores, mycelia and infected root fragments. The AMF species used were three strains isolated from Cabo de Gata Natural Park (Almería, Spain): *Rhizophagus intraradices* (previously named *Glomus intraradices*), *Septoglomus constrictum* and *Claroideoglomus etunicatum* and a *Rhizophagus intraradices* strain from our culture collection. Appropriate amounts of each inoculum containing about 700 infective propagules (according to the most probable number test), were added to the corresponding pots at transplanting time just below *A. maritimus* plantlets. Non-mycorrhizal control plants received the same amount of autoclaved mycorrhizal inocula together with a 10 ml aliquot of a filtrate (< 20 µm) of the four AM inocula in order to provide a general microbial population free of AM propagules.

Growth conditions

The experiment was carried out under glasshouse conditions with temperatures ranging from 19 to 25ºC, 16/8 light/dark period, and a relative humidity of 50-60%. A photosynthetic photon flux density of 800 µE m^{-2} s^{-1} was measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Water was supplied daily to the entire period of plant growth to avoid any drought effect. Plants were established for 6 weeks prior to salinization to allow adequate plant growth and symbiotic establishment. Three concentrations (0, 100, and 175 mM NaCl) of saline solution were reached in the soil substrate by adding appropriate dilutions of a stock 2 M saline solution. The concentration of NaCl in the soil was increased gradually on alternative days to avoid an osmotic shock. It took 6 weeks, to reach the desired 100 and 175 mM NaCl levels. The electrical conductivities in the soil:sand mixture used as growing substrate were 2.2, 8.9

and 13.5 dS m^{-1} for the salt levels of 0, 100, and 175 mM NaCl, respectively. Plants were maintained under these conditions for additional 8 weeks.

Symbiotic development

The percentage of mycorrhizal root infection in *A. maritimus* plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), as described by Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method Giovannetti and Mosse (1980).

Biomass production

At harvest (5 months after planting), the shoot and root system were separated and the shoot dry weight (SDW) and root dry weight (RDW) were measured after drying in a forced hot-air oven at 70ºC for two days. Ten plants per treatment were used (except for plants inoculated with Ri collect and subjected to 175 mM NaCl, where only three plants survived).

Photosynthetic efficiency

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

Stomatal conductance

Stomatal conductance was measured two hours after light turned on by using a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the third youngest leaf from five different plants from each treatment.

Proline content

Free proline was extracted from 0.5 g of fresh leaves and roots (Bligh and Dyer 1959) in five plants per treatment. The methanolic phase was used for quantification of

proline content. Proline was estimated by spectrophotometric analysis at 530 nm of the ninhydrin reaction according to Bates et al. (1973).

Glutathione content

Glutathione content was measured as described by (Smith 1985). Five hundred milligrams of roots and leaves of five plants from each treatment were homogenized in a cold mortar with 5 ml 5% (w/v) sulfosalicylic acid and the homogenate was filtered and centrifuged at 10,000 x g for 10 min. 0.75 ml of supernatant was neutralized by 1.125 ml 0.5 M K-phosphate buffer (pH 7.5). The standard incubation medium was a mixture of: 0.5 ml 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.2 ml 6 mM 5,5´- dithiobis-(2-nitrobenzoic acid), 0.1 ml 2 mM NADPH, and 0.1 ml (1 unit) glutathione reductase. The reaction was initiated by the addition of 0.1 ml of extract or glutathione. The change in absorbance at 412 nm was recorded for 9 min.

Ascorbate content

Ascorbate was assayed photometrically by the reduction of 2,6-dichlorophenolindophenol (DCPIP) as described by Leipner et al. (1997). Two hundred mg of roots and leaves of five plants from each treatment were homogenized in 5 ml ice-cold 2% (w/v) metaphosphoric acid in the presence of 1 g NaCl. The homogenate was filtered through a filter paper. An aliquot of 30 µl of the extract was mixed with 20 µl 45% (w/v) K_2HPO_4. After 15 min incubation at 25ºC, 100 µl 2 M citrate-phosphate buffer (pH 2.3) and 100 µl 0.003% (w/v) DCPIP were added. The absorbance at 524nm was measured immediately. The content of ascorbate was calculated by reference to a standard curve made of ascorbate.

Oxidative damage to lipids

Lipid peroxides were extracted by grinding 500 mg of leaves and roots of five plants from each treatment with 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 min. The chromogen was formed by mixing 200 µl of supernatants with 1 ml of a reaction mixture containing 15% (w/v), trichloroacetic acid, 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl hydroxytoluene, 0.25N HCl and then incubating the mixture at 100ºC for 30 min (Minotti and Aust 1987). After cooling at room temperature, tubes were centrifuged at 800 × g for 5 min and the supernatant was used for spectrophotometric reading at 532 nm. Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge (1989). The calibration curve was made using MDA in the range of 0.1-10

nmol. A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 0.25N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the reaction mixtures to prevent artifactual formation of TBARS during the acid-heating step of the assay.

Antioxidant enzyme activities

Enzyme extraction was done as described before by Aroca et al. (2001) with slight modifications. Five hundred mg of leaf and root fresh tissues were homogenized separately in a cold mortar with 4 ml of 100 mM phosphate buffer (pH 7.0) containing 0.1 mM DTPA (diethylenetriamine pentaacetic acid; a metal chelating agent) and 40 mg PVPP (polyvinylpolypyrrolidone), which removes phenolics and alkaloids from plant extracts, avoiding interference with spectrophotometric measurements and enhancing enzyme stability. The homogenate was centrifuged at 20,000 x g for 20 min at 4ºC. The supernatant was separated and used to determine the activity of antioxidant enzymes. All enzymatic activities were measured in a spectrophotometer Infinite^{R} 200 PRO series (Tecan Trading AG, Switzerland) at 25ºC. Total protein was assayed by the method described by Bradford (1976).

Superoxide dismutase (SOD.) (EC 1.15.1.1) activity was measured according to Becana et al. (1986). The reaction mixture (1,9 ml) contained 50 mM phosphate buffer (pH 7.8), 14,3 µM methionine, 82,5 µM NBT, 2,2 µM riboflavin and 100 µl enzyme extract. Riboflavin was added last and tubes were shaken and illuminated with fluorescent light. The reaction was allowed to proceed for 10 min until the colour of the blank shifted to dark violet. Absorbance of the reaction mixture was read at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme which produced a 50% inhibition of nitroblue tetrazolium (NBT) reduction.

Glutathione reductase (GR) (EC 1.6.4.2), was assayed as described by Schaedle and Bassham (1977) with slight modifications as 200 µl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.3 mM $NADPH_2$, 1 mM oxidized glutathione (GSSG), and 3 mM $MgCl_2$. The oxidation of $NADPH_2$ was recorded at 340 nm for 3 min after adding 10 µl of leaf and root extract.

Ascorbate peroxidase (APX) (EC 1.11.1.11) was assayed as described by Nakano and Asada (1981) with slight modifications as a 200 µl reaction mixture containing 80 mM phosphate (pH 7), 1 mM ascorbate, and 0.5 mM H_2O_2. The oxidation of ascorbate was recorded at 290 nm for 3 min after adding 10 µl of leaf and root extract.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA) performing first a one-way ANOVA followed by the Tukey test with $P< 0.05$ as the significance cut-off. Two independent statistical analyses were carried out: the first to analyze data from the different AMF treatments within each saline level and the second one to analyze data from each fungal species at increasing salinity.

Results

Symbiotic development

All the fungi exhibited similar colonization rates at all salinity levels (Table 1). Within each salinity level, the highest rate of AM root colonization was achieved in plants inoculated with Ri collect (up to 77%). High levels of root colonization were also found in plants inoculated with Ri CdG and Sc CdG (Table 1). In contrast, the lowest root colonization was always found in plants colonized by Ce CdG (about 25%).

Percentage of survival and plant biomass production

Non-mycorrhizal *A. maritimus* plants did not survive under the conditions of this experiment, even in the absence of salinity. At the highest salinity level, only 30% of plants inoculated with Ri collect survived. The rest of the inoculation treatments had 100 % of survival rate (Table 1).

The increase of salt application affected negatively the shoot biomass production in all treatments except in plants inoculated with Ri CdG (Table 1). In plants inoculated with Sc CdG and Ce CdG the decrease was only observed at 100 mM. In plant inoculated with Ri collect the reduction was not significant until 175 mM NaCl was reached (Table 1). However, under the highest saline conditions, shoot dry weight was higher in plants colonized by AMF from Cabo de Gata than in those colonized by Ri collect (Table 1). In contrast, the root biomass production decreased at each salinity level in plants inoculated with Ri both collect and CdG, two of them showing the lowest values of root dry weight at 175 mM NaCl (Table 1). Root dry weight was unaffected in plants inoculated with Ce CdG. Root dry weight was reduced to a half in plants inoculated with Sc CdG at 100 mM NaCl and it was not changed further at 175 mM NaCl, being the highest value at this salt level (Table 1).

Table 1. Percentage of mycorrhizal root colonization, percentage of survival, shoot and root dry weight (g plant^{-1}) in *Asteriscus maritimus* plants. NM represents non-mycorrhizal control plants; Ri collect, plants inoculated with the collection *Rhizophagus intraradices* strain; Ri CdG, plants inoculated with the native *Rh. intraradices* CdG strain; Sc CdG, plants inoculated with the native *Septoglomus claroideum* CdG strain and Ce CdG plants inoculated with the native *Claroideoglomus etunicatum* CdG strain. Plants were subjected to 0, 100 or 175 mM NaCl. Different letters indicate significant differences ($p < 0.05$) among fungal treatments at each salt level (a, b, c, d) or among salt levels for each AMF treatment: Ri collect (A, B, C), Ri CdG (D, E, F), Sc CdG (G, H, I) or Ce CdG (J, K, L).

AMF treatment	NaCl (mM)	AM root colonization (%)	% survival	Shoot dry weight	Root dry weight
NM	0	0	0	0	0
	100	0	0	0	0
	175	0	0	0	0
Ri collect	0	82.0 ± 1.7 Aa	100	0.41 ± 0.040 Abc	0.37 ± 0.041 Ab
	100	77.7 ± 2.2 Aa	100	0.36 ± 0.032 Aa	0.21 ± 0.035 Bb
	175	81.0 ± 1.5 Aa	30	0.22 ± 0.007 Bb	0.11 ± 0.004 Cc
Ri CdG	0	55.3 ± 2.4 Db	100	0.36 ± 0.022 Dc	0.43 ± 0.052 Db
	100	61.7 ± 2.3 Db	100	0.33 ± 0.048 Da	0.28 ± 0.033 Eab
	175	56.7 ± 2.5 Db	100	0.31 ± 0.041 Da	0.15 ± 0.026 Fbc
Sc CdG	0	46.0 ± 3.8 Gb	100	0.58 ± 0.041 Ga	0.61 ± 0.057 Ga
	100	51.7 ± 2.3 Gc	100	0.42 ± 0.025 Ha	0.35 ± 0.043 Ha
	175	47.3 ± 3.0 Gc	100	0.33 ± 0.054 Ha	0.28 ± 0.035 Ha
Ce CdG	0	24.7 ± 2.3 Jc	100	0.50 ± 0.026 Jab	0.33 ± 0.028 Jb
	100	27.3 ± 1.8 Jb	100	0.39 ± 0.031 Ka	0.23 ± 0.025 Jb
	175	28.7 ± 1.5 Jb	100	0.32 ± 0.030 Ka	0.24 ± 0.046 Jab

Photosystem II efficiency

Salinity application did not cause any significant descent in photosynthetic efficiency in *A. maritimus* plants inoculated with the three native AMF from Cabo de Gata (Fig. 1A). Only plants inoculated with Ri collect decreased the efficiency of photosystem II when they were subjected to 175 mM NaCl (Fig. 1A).

Stomatal conductance

Under non saline conditions no significant differences in stomatal conductance were observed among inoculation treatments (Fig. 1B). Salinity application decreased stomatal conductance in all the treatments. However, at 175 mM NaCl, plants inoculated with Ri collect showed lower stomatal conductance than the three native AMF from Cabo de Gata (Fig. 1B).

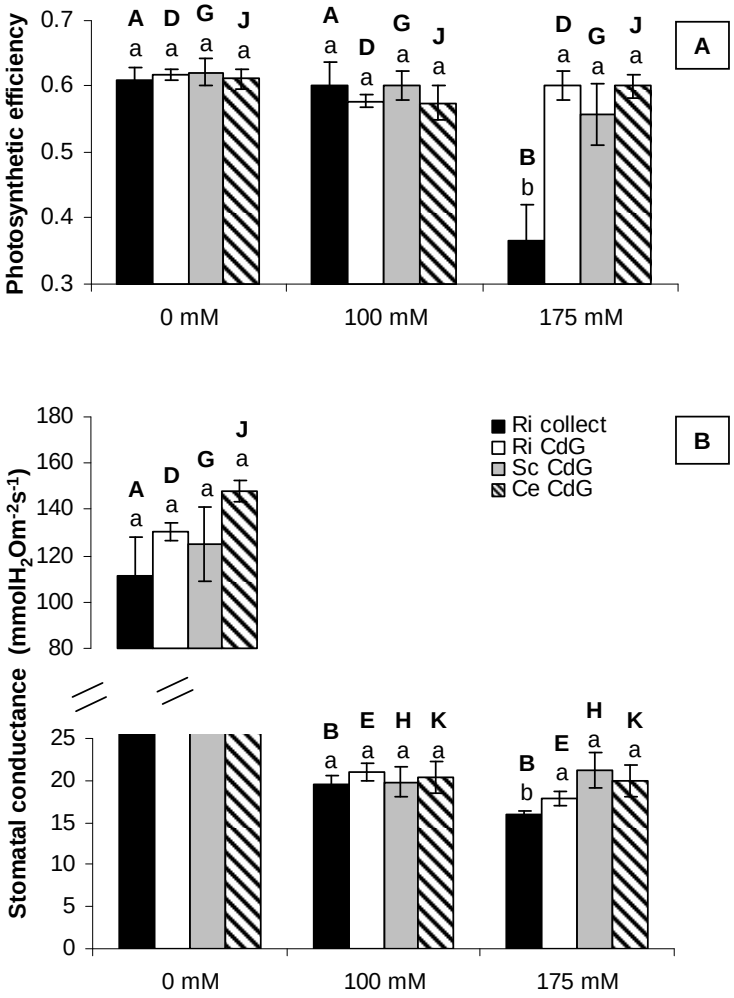

Fig. 1. Efficiency of photosystem II (A) and stomatal conductance (mmol H_2O $m^{-2}s^{-1}$) (B) in *Asteriscus maritimus* plants. Black bars represent plants inoculated with the collection *Rhizophagus intraradices* strain (Ri collect); white bars, plants inoculated with the native *Rh. intraradices* CdG strain (Ri CdG); grey bars, plants inoculated with the native *Septoglomus claroideum* CdG strain (Sc CdG) and lined bars, plants inoculated with the native *Claroideoglomus etunicatum* CdG strain (Ce CdG). Plants were subjected to 0, 100 or 175 mM NaCl. Different letters indicate significant differences ($p < 0.05$) among fungal treatments at each salt level (a, b, c) or among salt levels for each AMF treatment: Ri collect plants (A, B, C), Ri CdG (D, E, F), Sc CdG (G, H, I) or Ce CdG (J, K, L).

Accumulation of proline

The accumulation of proline was more pronounced in shoot than in root tissues (Fig. 2A,B). In leaves proline increased after exposure to salinity in the growth medium. Differences between treatments were only evident at 100 mM NaCl, where plants inoculated by any of the two Ri strains had the highest values (Fig. 2A). In roots only plants inoculated with Ri CdG increased the accumulation of proline with increasing salinity. All the other fungi increased the accumulation of proline at 100 mM and did not change it at 175 mM NaCl (Fig. 2B). *A. maritimus* inoculated with Ri CdG or Ce CdG had the lowest proline value at 100 mM NaCl(Fig. 2B).

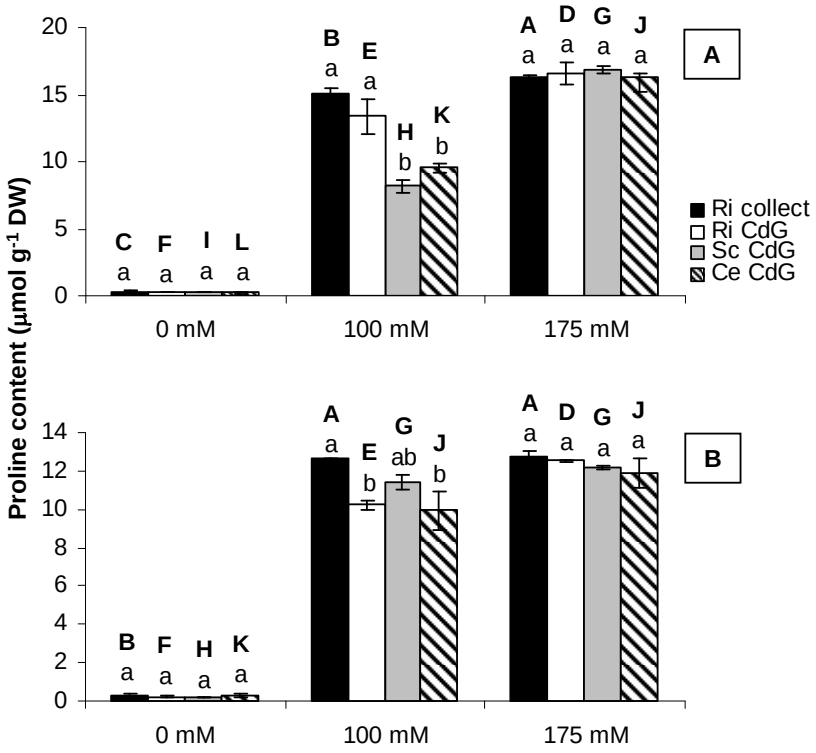

Fig. 2. Shoot (A) and root (B) proline content in *A. maritimus* plants. See legend for Fig. 1.

Glutathione content

The highest level of salinity increased the content of glutathione in leaves of plants inoculated with the three native AMF from Cabo de Gata (Fig. 3A*)*. In contrast, *A. maritimus* inoculated with Ri collect reduced the accumulation of glutathione at 175 mM NaCl, having the lowest content. In roots, only plants inoculated with Sc CdG increased the content of glutathione at 175 mM NaCl, showing the highest content (Fig. 3B).

Ascorbate content

In leaves, the content of ascorbate due to the increasing salinity only rose in Ri CdG-inoculated plants at 100 mM NaCl and in plants colonized by Ri collect when subjected to 175 mM NaCl (Fig. 4A). At 100 mM NaCl plant inoculated with Ce CdG showed the lowest ascorbate content but at the higher level of NaCl no differences were observed. In roots, exposure to 100 or 175 mM NaCl did not significantly affect the ascorbate content in *A. maritimus* plants (Fig. 4B). But again, at 100 mM NaCl treatment, Sc CdG roots showed the highest ascorbate values. At 175 mM NaCl the highest values were found in Ri collect roots instead.

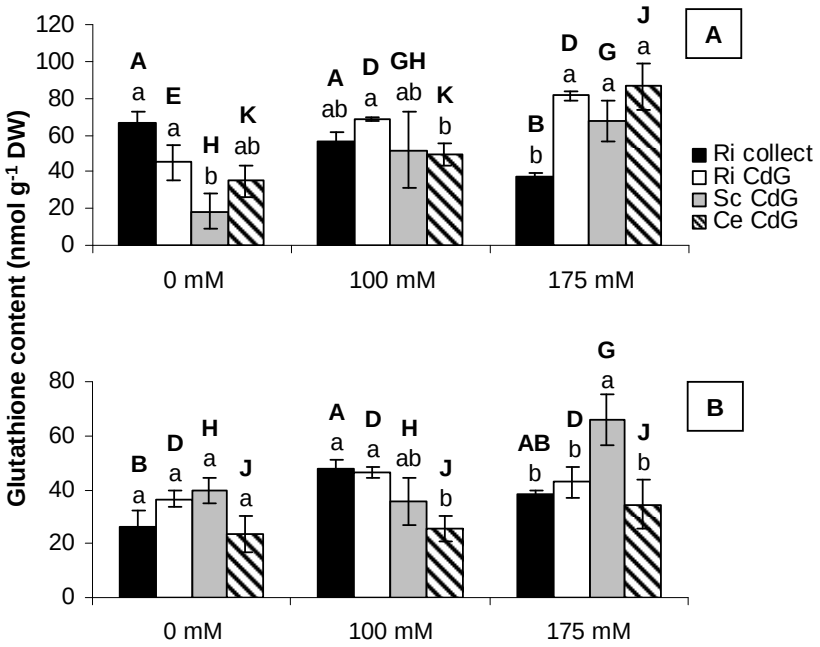

Fig. 3. Shoot (A) and root (B) glutathione content in *A. maritimus* plants. See legend for Fig. 1.

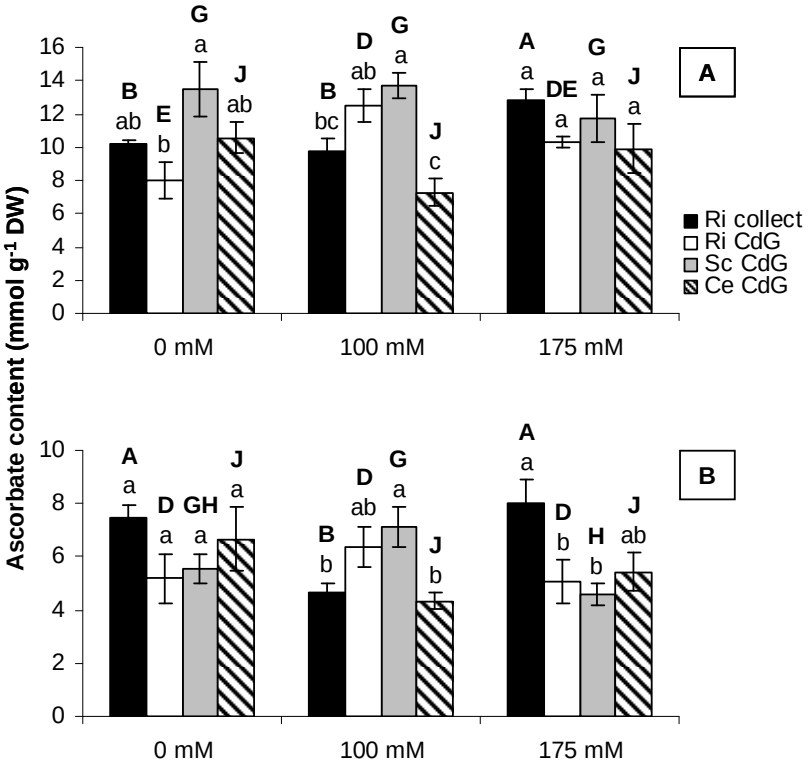

Fig. 4. Shoot (A) and root (B) ascorbate content in *A. maritimus* plants. See legend for Fig. 1.

Oxidative damage to lipids

The application of 100 mM NaCl increased lipid peroxidation in leaves of all plants except those inoculated with Sc CdG (Fig. 5A). When plants were grown at 175 mM NaCl, only inoculation with Sc CdG enhanced lipid peroxidation as compared to 100 mM NaCl. However these plants showed the lowest values of lipid peroxidation in their leaves as compared to the other treatments. In roots, salinity application did not cause any significant increase in lipid peroxidation in plants inoculated with Ri collect, Ri CdG or Ce CdG (Fig. 5B). Plants inoculated with Sc CdG increased root lipid peroxidation with increasing salinity. However, the values were always lower than those of the remaining treatments (Fig. 5B).

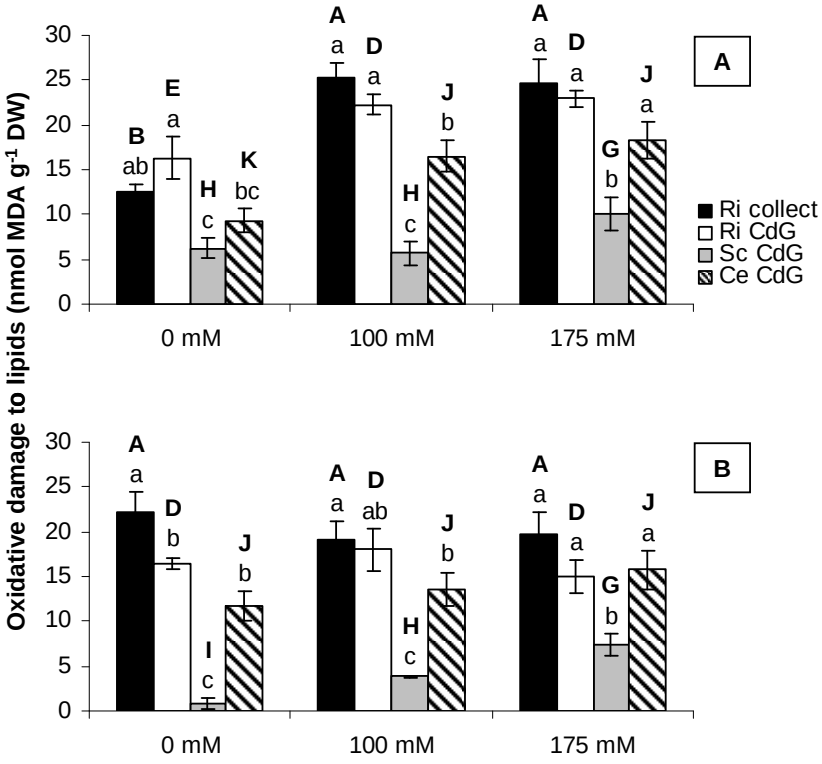

Fig. 5. Shoot (A) and root (B) oxidative damage to lipids in *A. maritimus* plants. See legend for Fig. 1.

Antioxidant enzyme activities

Leaf SOD activity increased considerably by both salt treatments in plants inoculated with Sc CdG and Ce CdG (Fig. 6A). Plants inoculated with Ri CdG did not alter significantly their leaf SOD activity by any of the salt treatments and those inoculated with Ri collect reduced the enzyme activity at 100 mM NaCl (Fig. 6A). When data were analyzed within each salt level, plants colonized by Ce CdG showed

the lowest activity at 0 and 100 mM NaCl, together with Ri collect-inoculated plants (Fig. 6A). At 175 mM NaCl plants inoculated with Sc CdG had the highest SOD activity, followed by those inoculated with Ce CdG. Root SOD activity increased by salt application in plants colonized by Sc CdG (Fig. 6B). Ri CdG inoculated plants showed the lowest SOD activity under non-saline conditions. No significant differences in root SOD activity among inoculation treatments were observed at 100 mM NaCl (Fig. 6B).

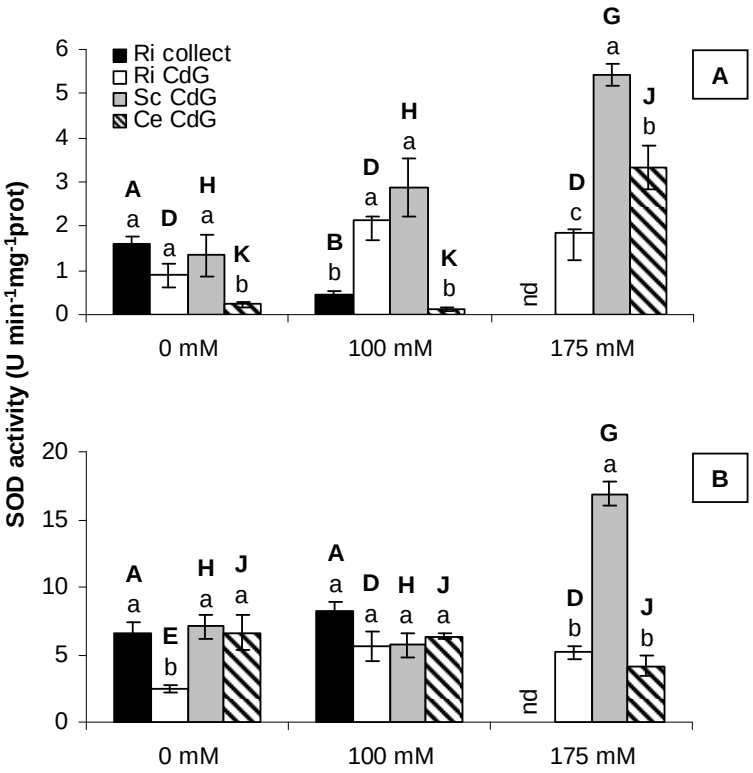

Fig. 6. Shoot (A) and root (B) SOD activity in *A. maritimus* plants. See legend for Fig. 1. (nd means non-determined enzyme activity).

Under non-saline conditions, no differences in leaf GR activity were observed among fungal treatments. The GR activity in leaves was only increased at 100 mM NaCl in plants inoculated with Sc CdG, reaching the highest value. However it did not increase further at 175 mM NaCl (Fig. 7A). *A. maririmus* plants inoculated with Ri CdG and Ce CdG rose their leaf GR activity at 175 mM NaCl, with Ri CdG-inoculated plants having the highest GR activity (Fig. 7A). Plants inoculated with Ri collect did not rise their GR activity. In roots, plants inoculated with Sc CdG enhanced their GR activity with increasing salinity, and those inoculated with Ri CdG also did it at 175 mM NaCl (Fig. 7B).

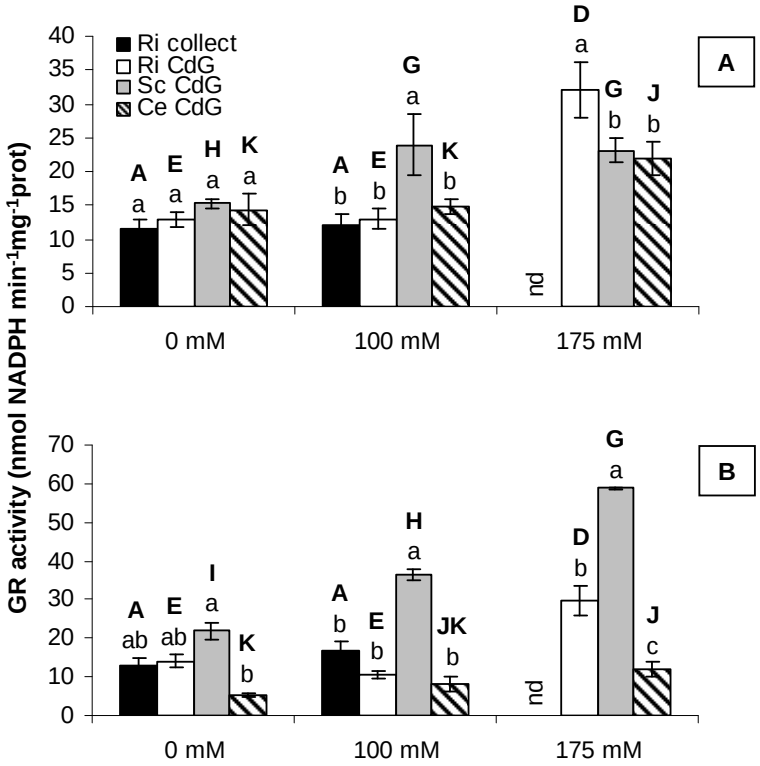

Fig. 7. Shoot (A) and root (B) GR activity in *A. maritimus* plants. See legend for Fig. 1. (nd means non-determined enzyme activity).

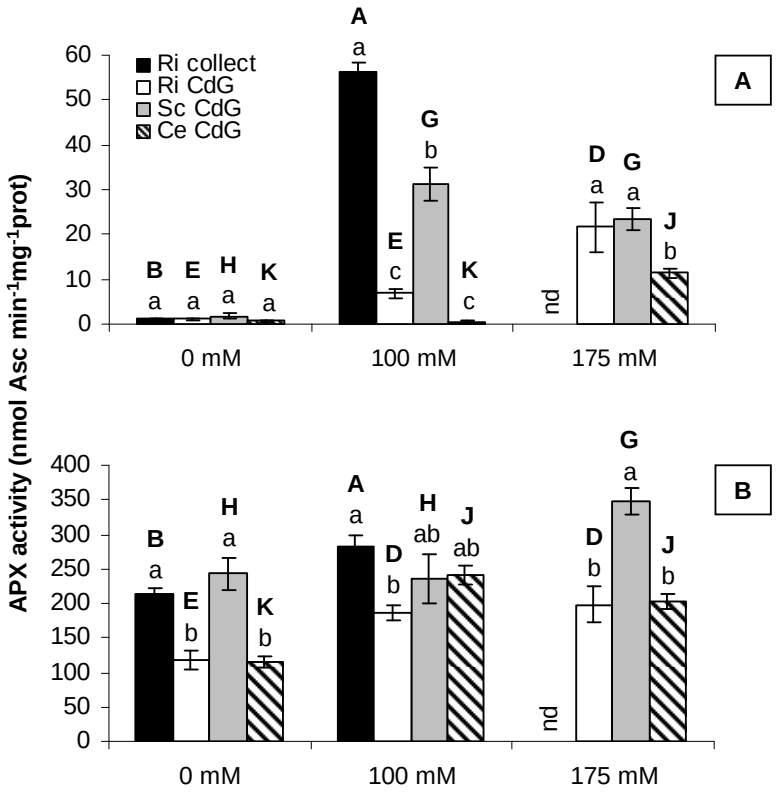

Fig. 8. Shoot (A) and root (B) APX activity in *A. maritimus* plants. See legend for Fig. 1. (nd means non-determined enzyme activity).

Leaf APX activity increased considerably at 100 mM NaCl in plants inoculated with Ri collect, as well as, in plants inoculated with Sc CdG (Fig. 8A). In contrast, plants inoculated with Ri CdG and Ce CdG only increased their leaf APX activity at 175 mM NaCl. In roots, under non-saline conditions, plants inoculated with Ri collect and Sc CdG had the highest levels of APX activity (Fig. 8B). APX activity also increased with salt increase, but the increase was slight at 100 mM NaCl. At 175 mM NaCl plants inoculated with Sc CdG showed the highest root APX activity (Fig. 8B).

Discussion

A. maritimus, a native halophyte plant from Mediterranean areas, showed a high degree of mycotrophy since all non-mycorrhizal plants did not survive, even in absence of an additional NaCl treatment. This evidences the importance of the rhizosphere microbiota, particularly the AMF, in the establishment and growth of this plant species. Herrera et al. (1993) showed that the restoration of a desertified area was most successful using native plant species inoculated with soil microbiota, including AMF. In fact, Klironomos (2003) concluded that for the establishment of a diverse plant community it is important to use locally adapted AMF community, as plant species benefited most from certain AMF species. In fact, at the highest salinity level, only 30% of *A. maritimus* plants inoculated with the collection AMF survived, while with the three native AMF, the rate of survival was 100%, pointing out the importance of inoculation with native-AMF in the survival of this plant species. Thus, any attempt of revegetation of degraded saline Mediterranean areas using *A. maritimus* should consider the mycorrhizal dependency of this plant and the use of adequate inocula. Although there are AMF species which seem to be globally distributed (Öpik et al. 2006), van der Heijden et al. (1998) pointed out that plant species benefited differently from the fungal strains regarding biomass production. Our results showed that plants inoculated with native AMF had higher shoot dry weight than those inoculated with the collection AMF at the highest level of salinity. This is in accordance to Requena et al. (2001) who found that native AMF species were more efficient in promoting plant growth of *Anthyllis cytisoides* than AMF derived from a different habitat. Similar results were obtained in *Pulsatilla* species (Moora et al. 2004), *Conyza bilbaoana* (Oliveira et al. 2005) and *Arnica montana* (Vergeer et al. 2006).

The nature of AMF hampers the progress in mycorrhiza research, especially in the field of applied ecology (Rosendahl 2008; Young 2008). Thus in this work we tried to elucidate differences in symbiotic efficiency among three AMF native from a saline Mediterranean area and a collection AM fungus when associated to *A. maritimus*. Mycorrhizal colonization was not affected by increasing salinity, as some other authors found (Yamato et al. 2008; Wu et al. 2010). Although Ri collect had the highest percentage of root colonization, the native AMF strains maintained a higher symbiotic

efficiency with *A. maritimus,* as well as a 100% of survival rate under 175 mM NaCl. In fact, the high percentage of root colonization by Ri collect could demand excessive carbohydrates from the plant (Klironomos 2003).

In this study, when plants of *A. maritimus* were subjected to high salinity stress (175 mM NaCl), plants inoculated with the three native-AMF exhibited better performance of photosystem II and higher stomatal conductance. Results on efficiency of photosystem II and stomatal conductance indicate that plants inoculated with native-AMF may improve the net assimilation rates by protecting the photosynthetic machinery and enhancing transpiration rates. Some other authors showed a similar tendency (Sheng et al. 2008; Hajiboland et al. 2010) and Estrada et al. (2012) reported that the improvement in photosystem II was higher with native AMF than with collection fungi together with the enhancement of plant stomatal conductance, in agreement with Querejeta et al. (2006). These two effects may have accounted for the enhanced growth of *A. maritimus* during high salinity stress.

In addition to the beneficial effect of native mycorrhizal colonization in *A. maritimus* salt tolerance by improving photosynthetic ability and stomatal conductance; water and nutrient uptake, ion balance and osmolite concentration among others may have also contributed to it (Ruiz-Lozano et al. 2012). It has been previously shown that a native AMF from Cabo de Gata developed better under salinity than a collection fungal strain (Estrada et al., 2012). Thus, the extensive hyphal network of native AMF can explore a larger soil volume, contributing to water and nutrient uptake (Evelin et al. 2012). The maintenance of proper ionic homoeostasis and osmotic adjustment may also prevent salt injury. The synthesis of organic osmolites, especially proline that is normally located in the cytosol, appears to be of importance in osmotic adjustment (Moghaieb et al. 2004). Our results did not show remarkable differences in proline content among fungal treatments, although proline accumulation increased in all treatments with the salinity stress. The latter may be explained as halophytic plants are themselves tolerant to salinity in part because they are able to maintain a high osmotic potential through the accumulation of organic solutes (Bradley and Morris 1991).

Salinity stress leads to secondary oxidative damage, thus the improvement of stress tolerance is often related to enhancement of contents of antioxidant compounds in plants. Due to the toxicity of ROS, plants have developed appropriate detoxification systems to remove these molecules. These systems include, among others, non-enzymatic antioxidants soluble compounds, such as glutathione and ascorbate that are major plant metabolites that regulate essential cell functions and play a key role in antioxidant defence (Tunc-Ozdemir et al. 2009). Glutathione reacts with superoxide radicals, peroxy radicals and singlet oxygen and forms oxidized glutathione and other disulphides (Meyer 2007). The ascorbate is involved in the removal of H_2O_2 by ascorbate peroxidases, which use ascorbate as electron donor, and is closely related to glutathione in the ascorbate-glutathione cycle where glutathione participates in the reduction of oxidized ascorbate (Noctor and Foyer 1998). Our results showed that

glutathione content had the most significant differences among treatments. *A. maritimus* plants inoculated with native AMF increased it shoot glutathione content with increasing salinity. Plants inoculated with Ri collect reduced the content at 175 mM NaCl and at this salinity level, the content of glutathione in shoots was lower compared to native AMF-inoculated plants. In contrast, the ascorbate content did not show a significant trend. From these results we may propose that *A. maritimus* has preference for glutathione as an antioxidant compound compared to ascorbate. The reason for that could be related to the additional physiological functions ascribed to glutathione, such as the induction of enzyme activities and its participation in sulphur metabolism and regulation of gene expression (Foyer et al. 1995).

Another major effect of salinity stress in plants is the loss of membrane integrity due to the oxidation of membrane lipids. Thus the prevention of lipid peroxidation is of crucial importance to maintain membrane integrity (Garg and Manchanda 2009). Our findings showed that in *A. maritimus,* MDA content (a specific product of lipid peroxidation induced by ROS) increased with salinity in shoots but not in roots, with exception of Sc CdG inoculated plants, which always increased the MDA content. Lipid peroxidation is considered an useful indicator of cellular oxidative damage (Li et al. 2012), however it might not be a good indicator in halophytes due to the particular characteristics of these plants. We cannot compare with some other works that observed a reduction of oxidative damage to lipids by AM symbiosis in plants subjected to salt stress (He et al. 2007; Hajiboland et al. 2010) because of the lack of non-mycorrhizal plants. In spite of that, we observed that, at each salt level, plants inoculated with Sc CdG always had the lowest MDA content.

Oxidative damage in plants can be also ameliorated by an efficient antioxidative enzyme system that may confer plants the ability to tolerate salt stress (Mittler 2002). The induction of ROS-scavenging enzymes, such as SOD, GR and APX is the most common mechanism for detoxifying ROS synthesized during stress response (Hajiboland et al. 2010). In halophytes the effects of AMF on the activities of antioxidant enzymes have been rarely studied (Li et al. 2012). Data from this study is difficult to discuss because there are no data with respect of Ri collect inoculated plants at 175 mM NaCl. Despite of all the above mentioned, the activity of SOD in shoots only increased in plants inoculated with Sc CdG and Ce CdG. In roots, plants inoculated with Sc CdG showed the most important increase in SOD activity, having the highest activity both in shoots and in roots. This may have accounted for the lower MDA content in *A. maritimus* plants inoculated with Sc CdG compared to the rest of the treatments.

Although the pattern of GR activity in roots was not relevant, in shoots of *A. maritimus* it was higher in plants inoculated with the three native AMF at the highest level of salinity. The enhanced GR activity in native AMF inoculated plants may have contributed to generate reduced glutathione that plays a pivotal role in the ascorbate-glutathione cycle among others. As for the APX activity, the data showed that it was enhanced by the increase in salinity in all treatments both in shoots and in roots. In

accordance to He et al. (2007), our results indicated that AMF induce APX activity. Nonetheless, native AMF did not enhance the activity more than the Ri collect fungus contrary to the other studied enzymes. It is known that the response of each enzyme differs with respect not only to the fungal species but to the host plant (Roldán et al. 2008; Evelin et al. 2009; Ruiz-Lozano et al. 2012).

Summarizing, the present study showed the elevated degree of mycotrophy of *A. maritimus* plants, especially regarding native AMF from its environment. In addition, we have observed several physiological mechanisms by which native AMF ameliorate the detrimental effects of salinity stress in *A. maritimus* better than an exotic AMF, such as better photosynthetic efficiency or higher levels of glutathione and of certain antioxidant enzyme activities. In degraded semiarid Mediterranean areas, Alguacil et al. (2011) demonstrated the important impact of inoculation with native AM fungi on the growth of shrub species. Moreover, reestablishment of plants species and restoration of specific ecosystems need careful considerations regarding the AMF inocula and the plant species used. Also the introduction of novel AMF species or isolates to the site could result in the out-competition of native fungal strains that could provoke the up-coming or survival of invasive plant species (Schwartz et al. 2006). Thus, inadequate inocula should be avoided due to possible negative effects on plant species and the plant community. Van der Heijden et al. (1998) showed that under particular environmental conditions, different AMF strains had negative or positive effect on the colonized plant, pointing out the importance of the interactions between AMF and plant species. The results in the present work remark the importance of native AMF inoculation in *A. maritimus* establishment, survival and growth for successful restoration of Mediterranean areas, particularly on sites with high salt levels in the soil.

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CONCLUSIONES

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1. Las micorrizas arbusculares se encuentran ampliamente distribuidas en ambientes salinos, como son las dunas y las marismas del Parque Natural de Cabo de Gata. Este estudio ha permitido describir un total de 30 morfotipos de esporas pertenecientes a tres clases, cinco órdenes, nueve familias y trece géneros. A pesar de que la diversidad fue bastante similar en ambos sitios, en la rizosfera de la marisma la densidad de esporas fue seis veces mayor que en la duna.

2. En las dunas del Parque Natural de Cabo de Gata se ha descrito una nueva especie de MA asociada a la rizosfera de *Asteriscus maritimus* y existe la posibilidad de encontrar más especies aún desconocidas.

3. El aislado nativo de Cabo de Gata *Glomus* (= *Rhizophagus*) *intraradices* ha demostrado tener una mayor capacidad de desarrollo en medios salinos que un aislado de colección de la misma especie, indicando mayor adaptación a la salinidad que el hongo de colección. Este efecto puede estar relacionado con la considerable mayor expresión de los genes *GintBIP*, *Gint14-3-3* y *GintAQP1*, en el hongo aislado de Cabo de Gata.

4. En condiciones de salinidad, el aislado nativo de Cabo de Gata *G. intraradices* presenta además una mayor eficiencia simbiótica con plantas de maíz que un aislado de la misma especie de colección, promoviendo un mayor desarrollo de las plantas.

5. El uso de hongos MA nativos aislados de Cabo de Gata incrementó la tolerancia a la salinidad de una planta glicófita de interés agronómico como es el maíz, en mayor medida que un hongo MA de colección. Las plantas inoculadas con los tres hongos nativos mostraron un aumento significativo de la relación K^+/Na^+ en sus tejidos. Este efecto se correlacionó con la regulación de la expresión de los genes *ZmAKT2*, *ZmSOS1* y *ZmSKOR* en las raíces de dichas plantas, contribuyendo a una mejor homeostasis iónica.

6. Las plantas de maíz inoculadas con los tres hongos nativos de Cabo de Gata mostraron mayor eficiencia del fotosistema II y conductancia estomática, lo que habría contribuido a reducir la fotorrespiración y la producción de ROS. Además, los sistemas antioxidantes de estas plantas fueron inducidos en mayor medida que en las plantas no micorrizadas o en las inoculadas con el hongo MA de colección, siendo el daño oxidativo significativamente menor.

7. La planta halófita *Asteriscus maritimus*, de interés en programas de revegetación de ecosistemas salinos degradados, ha demostrado un elevado grado de micotrofía y ser dependiente de hongos MA nativos para su completa supervivencia y desarrollo en condiciones de salinidad elevada.

8. Existen diferencias en los mecanismos por los que los hongos MA nativos de Cabo de Gata incrementan la tolerancia de la planta hospedadora a la salinidad. Esto estaría modulado, igualmente, por las características fisiológicas de la especie de planta utilizada.

CONCLUSIONS

1. Arbuscular mycorrhiza fungi are widely distributed in saline environments, such as dunes and salt marshes of the Natural Park of Cabo de Gata. This study describes a total of 30 spore morphotypes belonging to three classes, five orders, nine families and thirteen genera. Although diversity was similar at both sites, in the rhizosphere of the salt marsh spore density was six times higher than in the dune.

2. A new species of AMF associated with the rhizosphere of *Asteriscus maritimus* was described in the dunes of the Natural Park of Cabo de Gata. There is a possibility to find more still undescribed species.

3. The native isolate from Cabo de Gata *Glomus* (= *Rhizophagus*) *intraradices* has shown to have greater development capacity under saline conditions than an isolate of the same species from collection, indicating higher adaptation to salinity than the fungus from collection. This effect may be related to the significant up-regulation in the expression of the genes *GintBIP*, *Gint14-3-3* and *GintAQP1*, in the fungus isolated from Cabo de Gata.

4. Under saline conditions, the native isolate from Cabo de Gata *G. intraradices* had also higher symbiotic efficiency with maize plants than an isolate of the same species from collection, promoting a greater development of the plants.

5. The use of native AM fungi isolated from Cabo de Gata increased the salt tolerance of a glycophyte plant of agronomic interest, such as maize, more than a fungus from collection. Plants inoculated with the three native fungi showed a significant increase of the K^+/Na^+ ratios in their tissues. This effect was correlated with the regulation of expression of *ZmAKT2*, *ZmSKOR* and *ZmSOS1* genes in the roots of such plants, contributing to improve the ionic homeostasis.

6. Maize plants inoculated with the three native AM fungi from Cabo de Gata showed higher efficiency of photosystem II and stomatal conductance, which should have contributed to reduce photorespiration and ROS production. In addition, the antioxidant systems of these plants were induced to a greater extent than in non-mycorrhizal plants or plants inoculated with the fungus from collection, and they had a significantly lower oxidative damage.

7. The halophyte *Asteriscus maritimus*, a plant of interest in revegetation of saline degraded ecosystems, has shown to have a high degree of micotrophy and to be dependent of native AM fungi for its complete survival and development under high salinity conditions.

8. There are differences in the mechanisms by which native AMF of Cabo de Gata increase host plant tolerance to salinity. This would be also modulated by the particular physiological characteristics of the plant species used.