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REGULAR ARTICLE

# 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.

**Keywords** Adaptation · Arbuscular mycorrhiza · Monoxenic culture · Salinity · Symbiotic efficiency

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## 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

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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 Ruíz-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 National 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.) Schuessler and Walker 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″E).

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 min at 120 °C. 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 24 °C 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 8 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 −80 °C 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 65 °C for 5 min and then reverse transcribed at 25 °C for 10 min and 42 °C 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 5× 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′-AGATGCTGGCGTAATT GCTGG-3′and Rev, 5′-TGGCGGCACCATATGC AACTG-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 2× 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 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C, 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.

## In vivo experiment

### Soil and biological materials

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) non-inoculated 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, 36°59′34″'N; 3°34′47″W), sieved (5 mm), diluted with quartz-sand (<2 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100 °C 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 25 °C, 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 70 °C for 2 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 2 h 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 × 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 25 °C in 10 ml de-ionized water overnight with continuous shaking at 100 rpm. The samples were then autoclaved at 121 °C for 20 min. Final conductivity was measured after the samples had cooled down to room temperature (Verslues et al. 2006).

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.

## Results

Experiment in vitro

*Fungal development under salt conditions*

After 4 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 (Fig. 1a). The effect of salinity decreasing hyphal length was similar for the two fungal strains. At

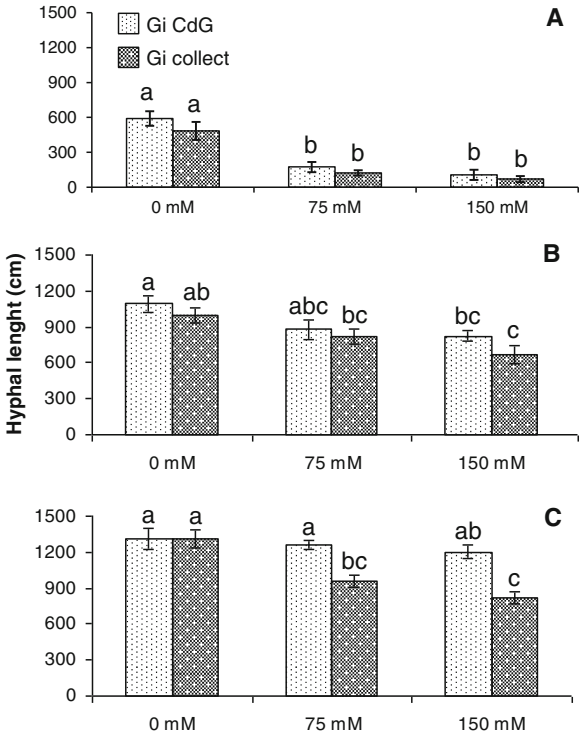


**Fig. 1** Total hyphal length (cm) formed in the hyphal compartment by two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Measurements were done after 4 weeks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

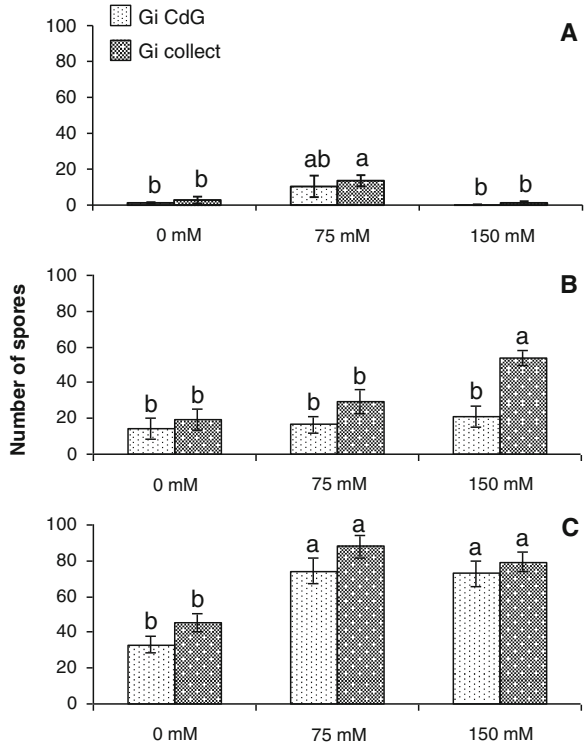


**Fig. 2** Total number of spores formed in the hyphal compartment by two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Measurements were done after 4 weeks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

6 weeks, only 150 mM NaCl decreased significantly the hyphal length of both fungal strains as compared to the control treatment without salt (Fig. 1b). Again, both AM fungal strains showed a similar hyphal length decrease. Finally, after 8 weeks of culture, Gi CdG did not show hyphal length reduction in response to any of the salt levels applied to the medium (Fig. 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 4 weeks after the application of 75 mM NaCl, while under 150 mM NaCl, it showed similar levels than those of the no stress treatment (Fig. 2a). At 6 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 (Fig. 2b). Finally, at 8 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 (Fig. 2c).

After 4 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 (Fig. 3a). At week 6, salinity influenced differently the number of BAS produced by each fungal strain (Fig. 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 8 weeks of fungal culture no significant differences in BAS production were observed among treatments (Fig. 3c).

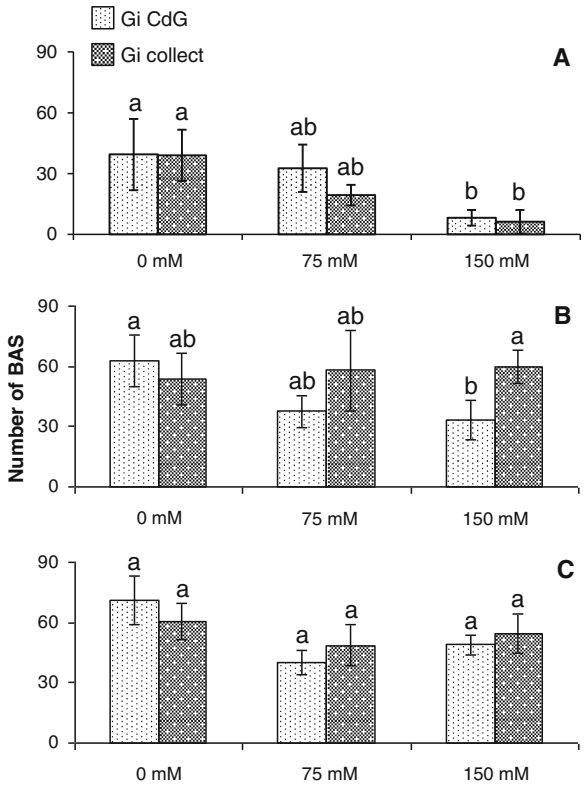


**Fig. 3** Total number of BAS formed in the hyphal compartment by two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Measurements were done after 4 weeks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

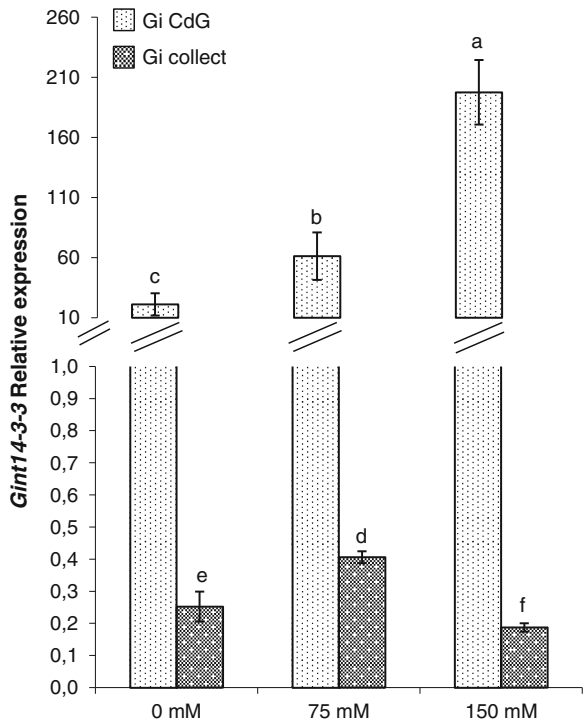


**Fig. 4** Analysis of *Gint14-3-3* gene expression by real time quantitative RT-PCR in two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Means followed by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

*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* (Figs. 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.

*Expression of a SOD-encoding gene*

We analyzed the expression of a SOD encoding gene in the two *G. intraradices* strains (Fig. 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.

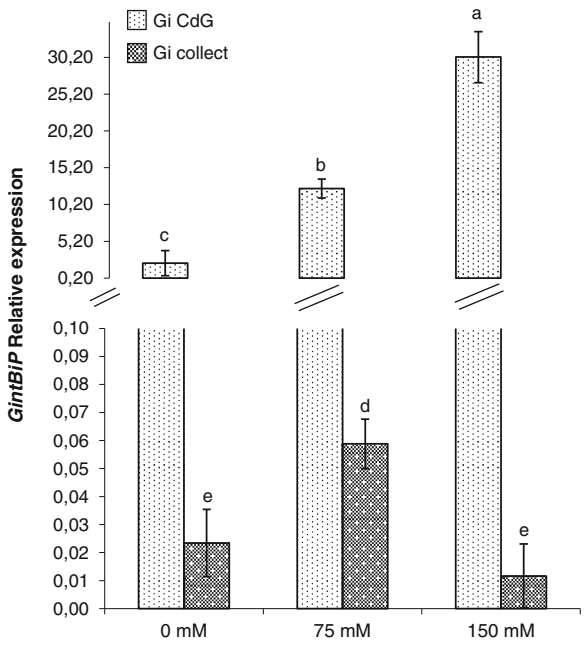


**Fig. 5** Analysis of *GintBiP* gene expression by real time quantitative RT-PCR in two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Means followed by different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

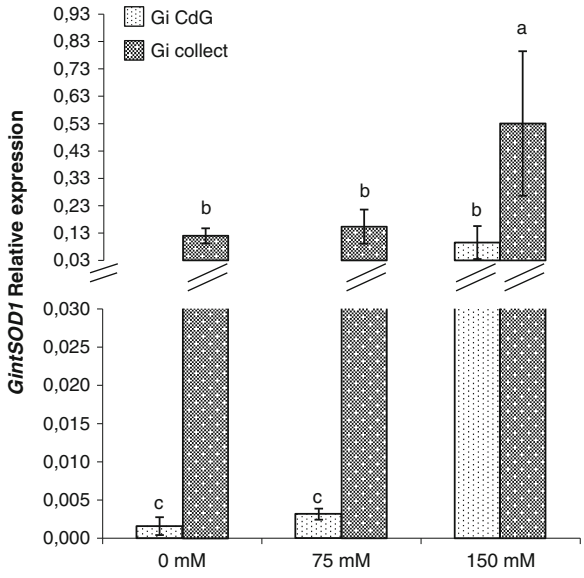


**Fig. 6** Analysis of *GintSOD1* geneexpression by real time quantitative RT-PCR in two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Means followed by different 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 (Fig. 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.

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 (Fig. 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 (Fig. 8b).

*Symbiotic development*

The percentage of mycorrhization was significantly higher in plants inoculated with the Gi collection

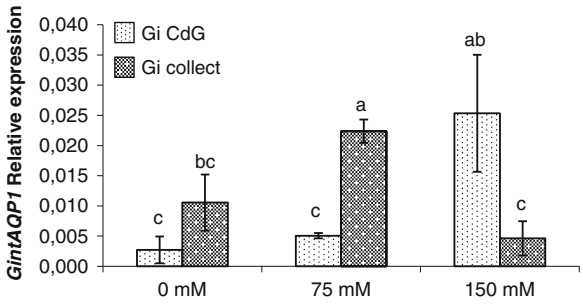


**Fig. 7** Analysis of *GintAQP1* gene expression by real time quantitative RT-PCR in two *G. intraradices* strains grown in monoxenic culture and subjected to 0, 75 or 150 mM NaCl. White bars represents strain Gi CdG and grey bars represent the collection *G. intraradices* strain. Means followed by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

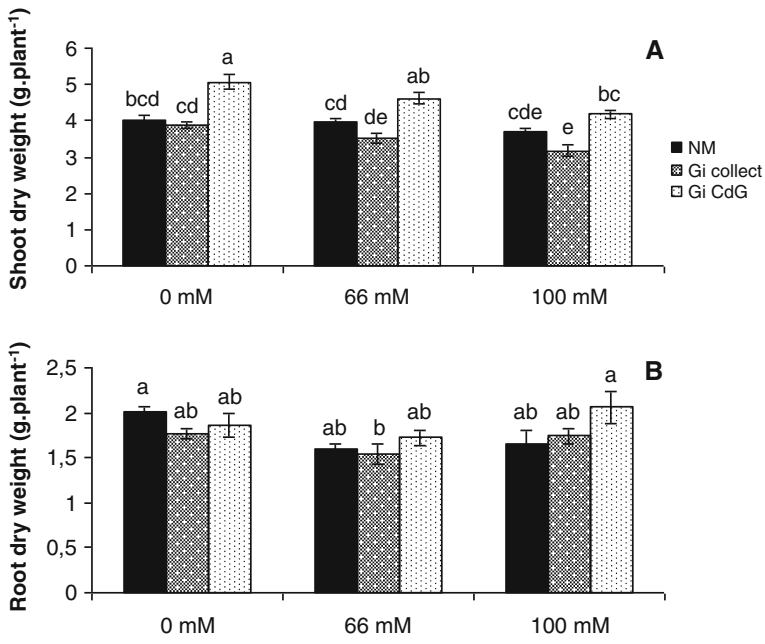


**Fig. 8** Shoot (**a**) and root (**b**) dry weights (g plant$^{-1}$) in maize plants. Black bars represent noninoculated control plants (NM), grey bars represent plants inoculated with the collection *G. intraradices* strain and white bars represent plants inoculated with the native strain Gi CdG.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

strain than in those inoculated with Gi CdG (Fig. 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 (Fig. 9b).

*Relative electrolyte leakage*

The applied salt stress did not significantly increase the relative electrolyte leakage in maize plants from any treatment (Fig. 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,

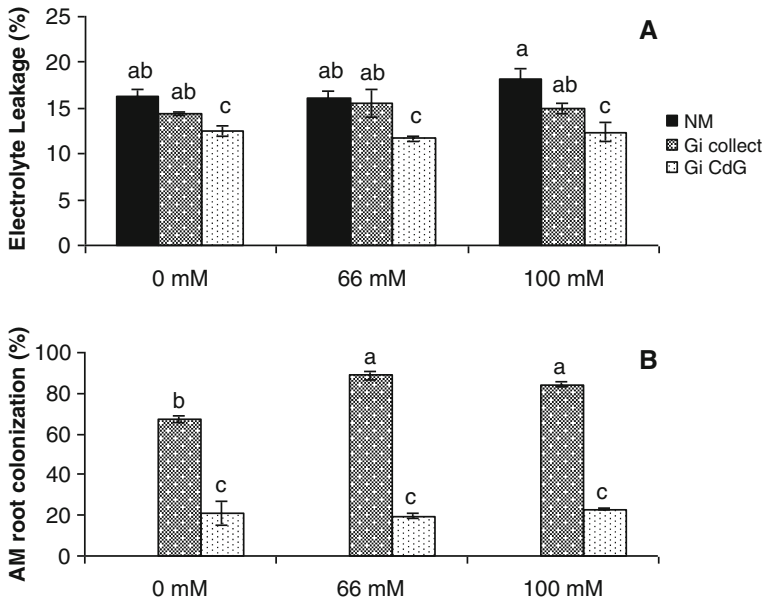


**Fig. 9** Electrolyte leakage (**a**) and percentage of mycorrhizal root length (**b**) in maize plants. Black bars represent noninoculated control plants (NM), grey bars represent plants inoculated with the collection *G. intraradices* strain and white bars represent plants inoculated with the native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

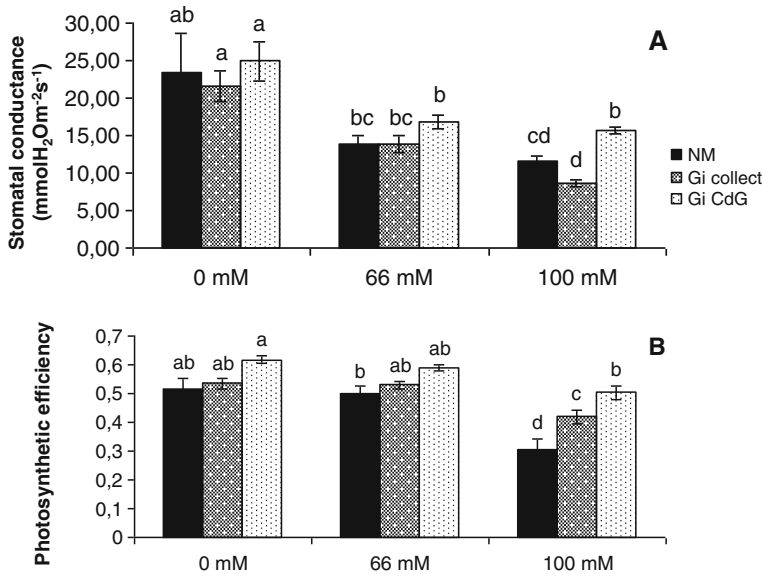


**Fig. 10** Stomatal conductance (mmol $H_2O$ $m^{-2}$ $s^{-1}$) (**a**) and efficiency of photosystem II (**b**) in maize plants. Black bars represent noninoculated control plants (NM), grey bars represent plants inoculated with the collection *G. intraradices* strain and white bars represent plants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

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 (Fig. 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 (Fig. 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.

## 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 (Ruíz-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 1,200 %. 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; He 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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