Plant Science 201–202 (2013) 42–51

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

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

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ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form
12 November 2012
Accepted 26 November 2012
Available online 3 December 2012

Keywords:
Antioxidant system
Arbuscular mycorrhiza
Plant tolerance
Salinity

ABSTRACT

High soil salinity is a serious problem for crop production because most of the cultivated plants are salt sensitive, which is also the case for the globally important crop plant 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 collection AMF species. Thus, we investigated whether native AMF isolated from a dry and saline environment can help maize plants to overcome salt stress better than AMF from a culture 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 decreased 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.

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 the current global climate change scenario one expected threat is the increase in land salinization [3]. Over 6% of the world's land is affected by salinity and its extent is increasing regularly throughout the world [4], causing global agricultural losses equivalent to an estimated US 12 billion a year [5]. Although salinity in soils may occur naturally, inappropriate cultivation practices are also contributing to the salinization of the rhizosphere [6]. Nowadays, high salinity in soils is a very serious problem for crop production because most of the cultivated plants are sensitive to salt stress (glycophytes) [7]. This is the case for maize, which is particularly vulnerable to salinity [8]. Thus land salinization is a major global issue because of its adverse impact on agricultural productivity, sustainability and as a threat for food supply [9]. 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 [10].

Salinity stress leads to secondary oxidative stress in plants [11]. 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) [11]. 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 [12]. This modifies the balance between the formation and removal of ROS species [13]. 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 denaturation of proteins, mutagenesis of DNA and lipid peroxidation [14]. 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 [14]. 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) [15]. 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

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http://dx.doi.org/10.1016/j.plantsci.2012.11.009

plant species [16]. Despite the several mechanisms developed by plants to detoxify the excess of ROS production, most of the cultivated plants are glycophytes and under high salinity in the soil they cannot cope with the extra production of ROS [7].

A number of beneficial soil microorganisms, particularly arbuscular mycorrhizal fungi (AMF) can help plants to cope with abiotic stress conditions [17]. AMF, belonging to the phylum Glomeromycota, are considered the oldest group of organisms living in symbiosis with terrestrial plants [18]. They are fundamental for plant performance, both in natural and agricultural ecosystems and most land plants are colonized by these fungi [19]. AMF are widely found in saline soils [20,21]. 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 loss (reviewed by [12,22]). Among other mechanisms, some studies have demonstrated that AM symbiosis can enhance the activities of antioxidant enzymes, helping plants to alleviate salt stress [23,24]. Most studies have focused in the use of collection species as AMF inoculum [22]. However several studies showed that native AMF can perform better in soils from which they are isolated: agricultural systems [25], polluted soils [26] and semiarid degraded soils [27] among others. According to this, some authors reported that AM symbiosis is more effective with native than with non-native AMF species [28–30]. However, the mechanisms that allow AM plants to have higher salinity tolerance are far from being understood [12]. The isolation and study of AMF isolated from a saline area will contribute to elucidate the ecophysiology of AMF under such stress conditions [26].

The aim of the present study was to investigate whether native AMF isolated from an arid and saline environment can help maize plants to overcome the negative effects of salt stress better than AMF from a culture collection. We also investigated whether protection against oxidative stress is a mechanism by which the native AMF from such environment enhance the tolerance of the host plant to salinity. For that, 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 [31]. The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol–lactic acid–glycerine (PVLG) [32]; a mixture of PVLG and Melzer's reagent [33]; a mixture of lactic acid to water at 1:1; Melzer's reagent; and water [34]. 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. [35]. The species were identified based on its spore morphology as a *Rhizophagus intraradices* [36], *Claroideoglomus etunicatum* [37] and *Septoglomus constrictum* [38].

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 [39]. 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 [40]. 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 (PerkinElmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST program [41].

The BLAST analysis unambiguously placed *R. intraradices* as the closest relative of our *R. intraradices* strain from Cabo de Gata (CdG), with sequence accession number FR750209 [42] having a 99% identity. *S. constrictum* was the closest relative to our *S. constrictum* CdG strain, with sequence accession number FR750212 [42] having a 99% identity. Finally, *C. etunicatum* was the closest relative of our *C. etunicatum* CdG strain, with sequence accession number FR750216.1 [42] 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 *R. intraradices* (Ri collect), reproduced at the collection of the Zaidin Experimental Station (isolate EEZ 58), (3) plants inoculated with the AM fungal strain *R. intraradices* isolated from Cabo de Gata Natural Park (Ri CdG), (4) plants inoculated with the AM fungal strain *S. constrictum* isolated from CdG (Sc CdG) and (5) plants inoculated with the AM fungal strain *C. 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, 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.

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, 36°45′24″N 02°13′17″W), which is an area with serious problems of salinity and affected by desertification. The native AMF strains were isolated in a salt marsh from the rhizosphere of *Astericus maritimus*, so that plants were collected with their intact root systems up to 40 cm soil depth. The electrical conductivity in such rhizospheric soil varied with soil depth, ranging from 3.95 dS m^{-1} at the surface to 7 dS m^{-1} at the deeper soil layer. The AMF isolates were *R. intraradices* (previously named *Glomus intraradices*), *S. constrictum* and *C. etunicatum*. We also used a *R. intraradices* strain from our culture collection (Ri collect, isolate EEZ 58) which came from the Biosystematic Research Center, Ottawa, Canada, and was originally collected in Pont Rouge, Quebec, Canada. It is the model fungus used in many studies dealing

with different topics, including genome sequencing. 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 25 °C, 16/8 light/dark period, and a relative humidity of 50–60%. A photosynthetic photon flux density of 800 μmol 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, according to the amount of substrate in the pots. 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 after salt addition in the soil:sand mixture used as growing substrate were 0.2, 5.1 and 7.4 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.

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 [43]. The extent of mycorrhizal colonization was calculated according to the gridline intersect method [44].

2.7. Shoot biomass production

At harvest (83 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 70 °C 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 [45]. 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 × 1.5 cm as described by Verslues et al. [46]. 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 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 (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, 0.25 N HCl and then incubating the mixture at 100 °C for 30 min [47]. 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 [48]. 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 0.25 N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the reaction mixtures to prevent artificial 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. [49] method, with slight modifications as described previously by Aroca et al. [50]. 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 × *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 45 °C 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 enzyme activities

Enzyme extraction was done as described by Aroca et al. [51] with slight modifications. Five hundred milligrams of leaf or root fresh tissues were homogenized 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 × *g* for 20 min at 4 °C.

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 represents plants inoculated with the collection *Rhizophagus intraradices* strain, Ri CdG represents plants inoculated with the native *Rh. intraradices* CdG strain, Sc CdG represents plants inoculated with the native *Septoglomus claroideum* CdG strain and Ce CdG represents 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).

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

The supernatant was separated and used to determine the activity of antioxidant enzymes. All enzymatic activities were measured in a spectrophotometer Infinite® 200 PRO series (Tecan Trading AG, Switzerland) at 25 °C. Total protein was assayed by the method described by Bradford [52].

Total superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured according to Becana et al. [53]. 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 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.

Catalase (CAT) (EC1.11.1.6) activity was assayed as described by [54]. 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 analyse data from the different AMF treatments within each saline level and the second one to analyse 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 fungi tested, 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 increasing salt concentrations in the soil solution (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).

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).

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).

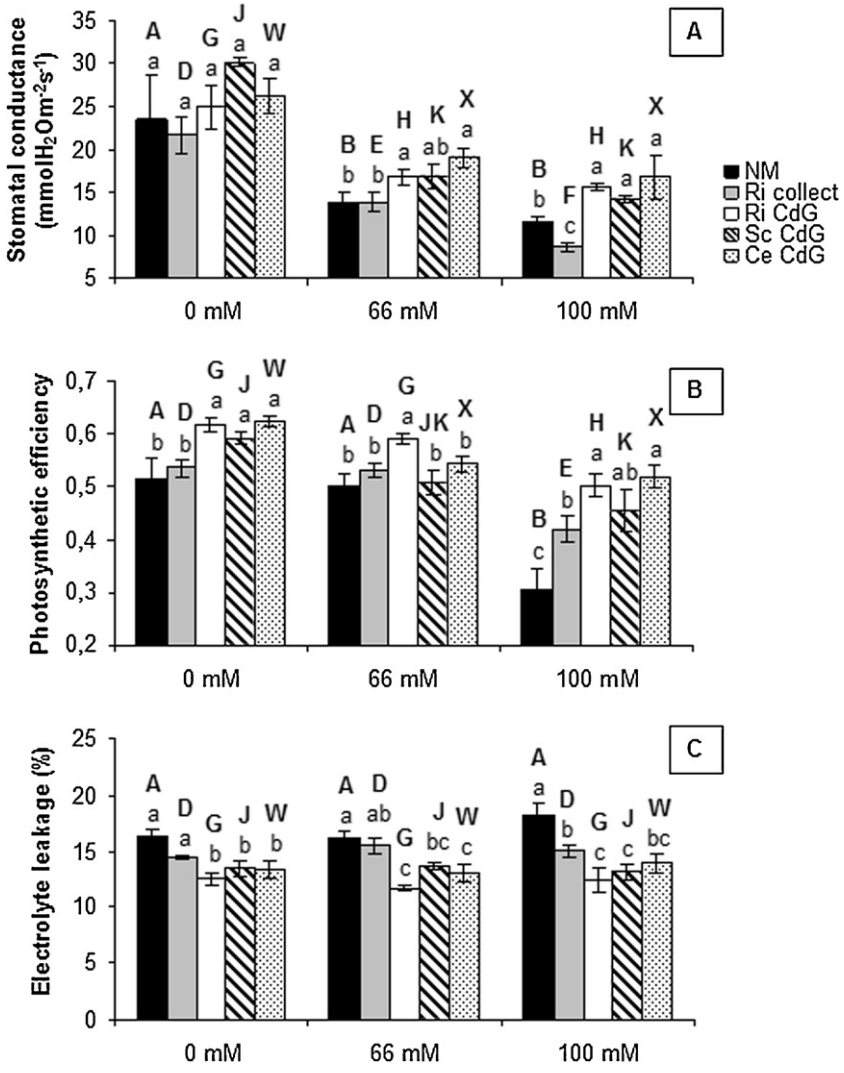

Fig. 1. Stomatal conductance (mmol H_2O m^{-2} s^{-1}) (A), efficiency of photosystem II (B) and electrolyte leakage (C) in maize plants. Black bars represent non-mycorrhizal control plants (NM); grey bars, plants inoculated with the collection *Rhizophagus intraradices* strain (Ri collect); white bars, 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 *C. 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).

3.6. 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 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).

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 analysed 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).

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

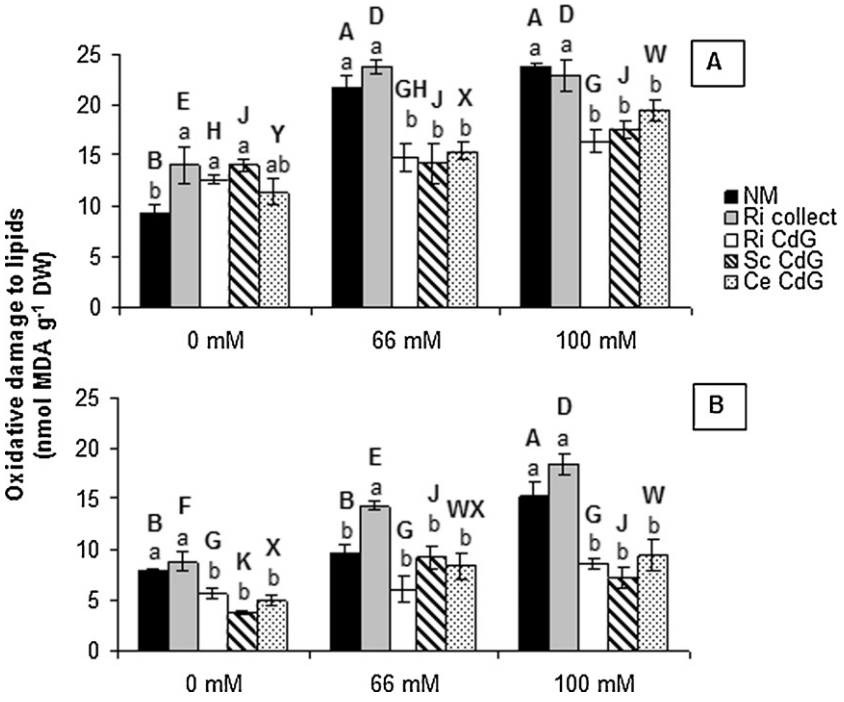

Fig. 2. Shoot (A) and root (B) oxidative damage to lipids in maize plants. See legend for Fig. 1.

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 analysed 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.

4. Discussion

Excessive soil salinity is well known to induce oxidative stress in plants [55]. Previous studies have suggested that tolerance of plants to salt stress is associated with the induction of antioxidant

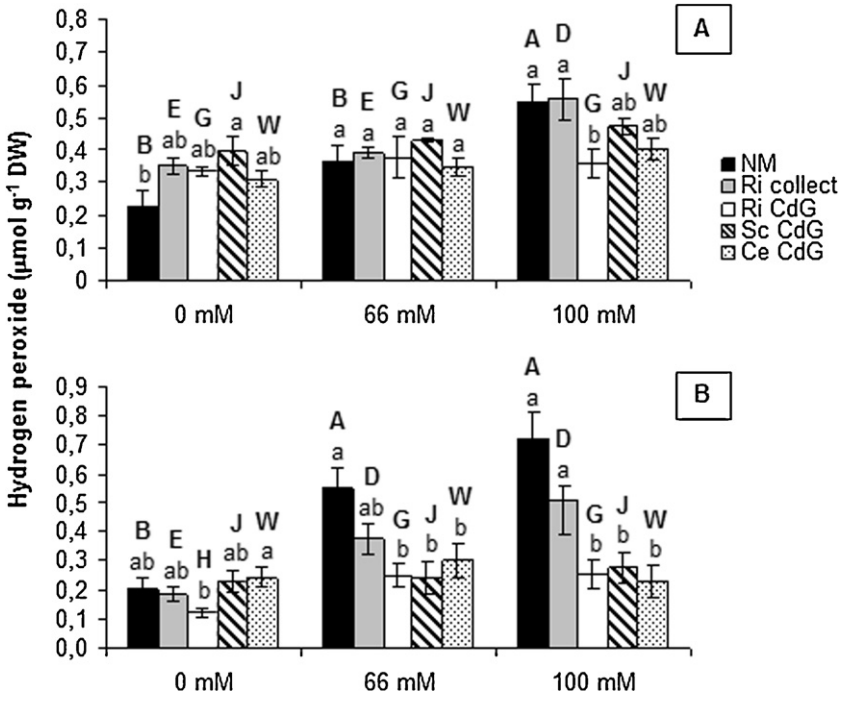

Fig. 3. Shoot (A) and root (B) hydroxide peroxide content in maize plants. See legend for Fig. 1.

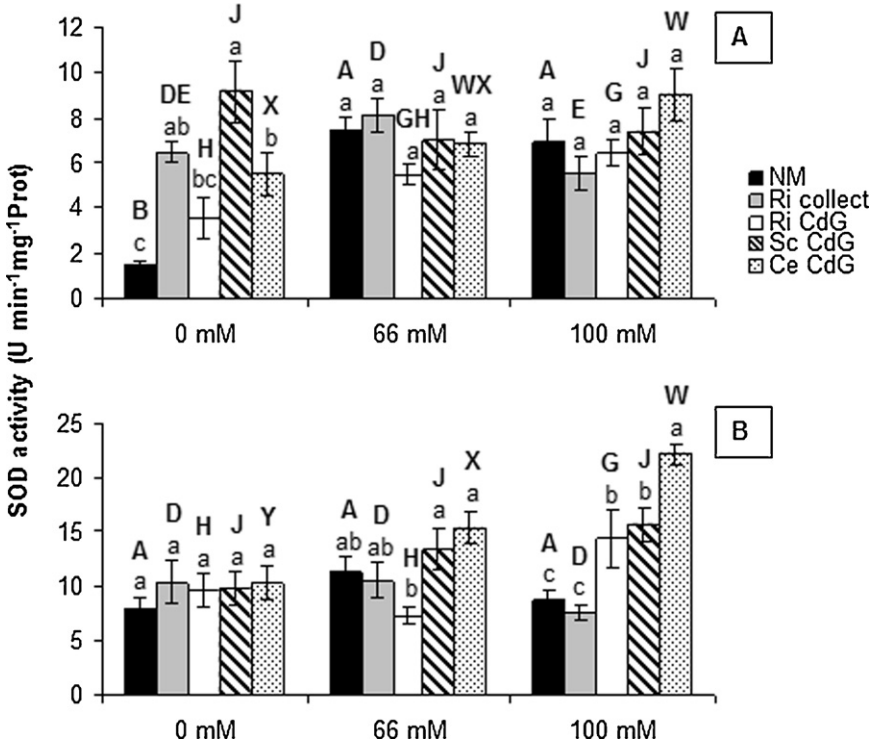

Fig. 4. Shoot (A) and root (B) SOD activity in maize plants. See legend for Fig. 1.

enzymes and reduction of oxidative damage [56,57]. AM symbiosis enhances the activity of antioxidant enzymes, which helps plants to cope with ROS generated by salinity [23,24,58]. Nevertheless the response of the individual enzymes varies with respect to the host plant and the fungal species involved in the association [12]. Results from the present work confirm 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 [59]. In fact, Ri collect had higher colonization rate than the autochthonous AMF but it did not show better symbiotic efficiency.

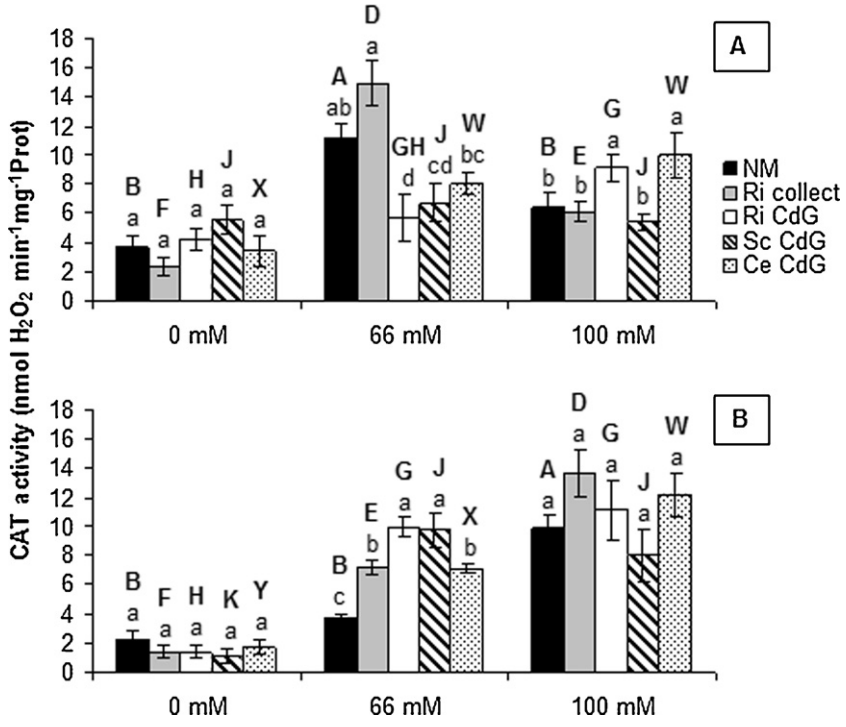

Fig. 5. Shoot (A) and root (B) CAT activity in maize plants. See legend for Fig. 1.

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. [60] and Hajiboland et al. [61] reported a similar tendency, and Querejeta et al. [28] 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 those inoculated with native AMF, 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, for instance for nutrient supply to the host plant, 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 [11]. 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 [61,62]. Our results demonstrate that colonization of maize plants by native AMF lead to a lower accumulation of ROS species. Salt stress negatively affects membrane integrity and results in ion leakage. Thus one of the key processes in salinity tolerance is to prevent lipid peroxidation to maintain the membrane integrity [58,63]. The oxidation of membrane lipids gives a reliable indication of an extra free radical production leading to oxidative stress [64]. 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 [24,61]. These data, together with the lower electrolyte leakage in maize plants inoculated with native AMF from Cabo de Gata, provides evidence that these native AMF are more effective than a fungus from a culture collection in preventing cell membrane damage. This is in accordance to some other works that showed lower lipid peroxidation and membrane permeability in AM plants compared to non-mycorrhizal plants [24,58,63,65]. For instance, Kaya et al. [66] 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 [20,30]. Moreover, Querejeta et al. [28] 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. In the present study we have compared the three native AMF from Cabo de Gata with a single fungal strain from culture collection. Comparison with a higher number of AMF from culture collections would give a more complete idea of the degree of adaptation and effectiveness of the three native AMF from Cabo de Gata. However, the collection fungal strain used in this work is also the model fungus in many studies dealing with different topics in AM symbiosis, including AMF genome sequencing [67]. Thus, it can be considered a representative model AMF strain.

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 [15,68]. 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 SOD is an antioxidant enzyme that plays a role maintaining membrane stability in plant cells [69]. 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 can enhance SOD activity in mycorrhizal plants under salt stress conditions [58,61,70]. 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 native AMF suggests that these fungi 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 [30] 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 [14]. Several authors have reported an enhancement of CAT activity by inoculation with AMF [58,61,71]. However, in our work the effect of AMF on CAT activity under salinity stress was not evident, which is in agreement with other reports indicating that AM symbiosis did not affect CAT activity of salt stressed plants [24,70].

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 salinity 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 native 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 [72] who 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.

Summarizing, this work demonstrates that AMF isolated from saline areas confer higher salinity tolerance to an important crop plant such as maize than a collection AMF strain used as a model fungus. Our results supports that inoculation with these native AMF enhances maize salt tolerance by alleviating the salt induced oxidative stress and membrane damage. The better performance of photosystem II and stomatal conductance of plants inoculated with the three native AMF must have contributed to this effect through reduced ROS generation, as evidenced by the lower accumulation of H_2O_2 in these plants. In agricultural lands with high levels of disturbance, inoculation is beneficial due to low AM fungal inoculum potential [73]. 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.

Acknowledgements

This work was financed by two research projects supported by Junta de Andalucía (Spain). Projects P06-CVI-01876 and P11-CVI-7107. We thank Sonia Molina for technical assistance and Domingo Álvarez (curator of the EEZ germplasm collection), for taking care of the native AMF inocula.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2012.11.009.

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