Mycorrhiza
DOI 10.1007/s00572-013-0542-7

ORIGINAL PAPER

Arbuscular mycorrhizal symbiosis alleviates detrimental effects of saline reclaimed water in lettuce plants

J. Vicente-Sánchez · E. Nicolás · F. Pedrero · J. J. Alarcón · J. F. Maestre-Valero · F. Fernández

Received: 9 September 2013 / Accepted: 12 November 2013

Abstract The present study evaluated the effects of inoculation with arbuscular mycorrhizal fungi (AMF; *Glomus iranicum* var. *tenuihypharum* sp. *nova*) on the physiological performance and production of lettuce plants grown under greenhouse conditions and supplied with reclaimed water (RW; urban-treated wastewater with high electrical conductivity; 4.19 dS m^{-1}). Four treatments, fresh water, fresh water plus AMF inoculation, RW and RW plus AMF inoculation, were applied and their effects, over time, analyzed. Root mycorrhizal colonization, plant biomass, leaf-ion content, stomatal conductance and net photosynthesis were assessed. Overall, our results highlight the significance of the AMF in alleviation of salt stress and their beneficial effects on plant growth and productivity. Inoculated plants increased the ability to acquire N, Ca, and K from both non-saline and saline media. Moreover, mycorrhization significantly reduced Na plant uptake. Under RW conditions, inoculated plants also showed a better performance of physiological parameters such as net photosynthesis, stomatal conductance and water-use efficiency than non-mycorrhizal plants. Additionally, the high concentration of nutrients already dissolved in reclaimed water suggested that adjustments in the calculation of the fertigation should be conducted by farmers. Finally, this experiment has proved that mycorrhization could be a suitable way to induce salt stress resistance in iceberg lettuce crops as plants supplied with reclaimed water satisfied minimum legal commercial size thresholds. Moreover, the maximum values of *Escherichia coli* in the reclaimed water were close to but never exceeded the international thresholds established (Spanish Royal Decree 1620/2007; Italian Decree, 2003) and hence lettuces were apt for sale.

Keywords Arbuscular mycorrhizal symbiosis · Reclaimed water · Salt stress · Biomass production · Gas exchange · Nutrient acquisition

Introduction

Current global population growth, industrial development, sustained increase of living standards and specifically the trend towards irrigated agriculture have produced strong competition for water resources. The shortage of water is especially severe in arid and semiarid regions such as southeast Spain, where farmers are usually the most affected (Maestre-Valero et al. 2013). This suggests an urgent need to explore new alternative water resources such as reclaimed water to cope with crop water requirements. In southeast Spain, this non-conventional water resource is of great interest for irrigation purposes since apart from the treated water volume having increased in recent years (ESAMUR 2013), it brings important agronomic benefits for crops; reclaimed water usually contains a great concentration of nutrients such as N, P, and K that produce a direct benefit for crops and also allows the accumulation of organic matter (Pedrero et al. 2013).

In contrast, a significant drawback is that reclaimed water has high salt concentrations. ESAMUR (2013) reported that in the Murcia region, in the southeast of Spain, at least 93 % of reclaimed water has an electrical conductivity (EC) greater than 2 dS m^{-1} and about 37 % has EC values greater than 3 dS m^{-1}. Salt, not being altered by the water purification

J. Vicente-Sánchez · F. Fernández (✉)
SYMBORG.SL, Campus de Espinardo 7. Edificio CEEIM, CP 30100 Murcia, Spain
e-mail: felixfernandez@symborg.com

E. Nicolás · F. Pedrero · J. J. Alarcón · J. F. Maestre-Valero
CEBAS-CSIC, Campus de Espinardo, CP 30100 Murcia, Spain

Published online: 28 November 2013

process, remains dissolved in the treated water and its use for irrigation affects the physical and chemical properties of the soil by increasing compaction and limiting the water holding capacity, especially under intensive production systems (Mounzer et al. 2013).

Moreover, salinity is a major constraint for irrigation as water with high salt concentration reduces crop yield once the salinity exceeds a crop-specific threshold (Ayers and Westcot 1985). Evelin et al. (2012) indicated that plants growing in saline soils are subjected to various physiological stresses that induce nutrient imbalance, damage cell organelles, and disrupt photosynthesis and respiration. Allen et al. (1998) reported that for lettuce, a horticultural crop moderately sensitive to salinity, yield was reduced by 12 % with each increment of 1 dS m^{-1} above the threshold level of the electric conductivity of the extract soil saturation (set at 1.3–1.7 dS m^{-1}).

Bearing in mind the above mentioned unfavorable saline conditions of reclaimed water, it is crucial to assess and develop inexpensive and environmentally friendly strategies to overcome salt stress that allow crops to achieve an appropriate commercial size, minimizing losses. In recent years, establishing mutually beneficial associations between the plant and the microorganisms in the rhizosphere seems to be an appropriate technique (Aroca et al. 2013). One of the most well-studied beneficial plant–microorganism associations is that established with certain soil fungi known as arbuscular mycorrhizal fungi (AMF) (Smith and Read 2008). AMF are ubiquitous in terrestrial ecosystems and it has been widely accepted that they play important roles in (1) maintaining structure and functions of ecosystems (Smith and Read 1997); (2) increasing the absorption of water and nutrients from the soil, the photosynthetic capacity, and improving water status of plants (Sánchez-Blanco et al. 2004); and (3) positively affecting plant growth and providing tolerance against biotic and abiotic stresses (Pozo and Azcón-Aguilar 2007).

Additionally, AMF are known to exist in saline environments (Yamato et al. 2008). There is evidence that salinity not only negatively affects the host plant but also the AMF as it can reduce the colonization capacity, the spore germination and the growth of fungal hyphae (Jahromi et al. 2008). However, it has also been demonstrated that AMF symbiosis can alleviate the negative effects induced by soil salinity (Evelin et al. 2009). This positive resistance to soil salinity has been observed in a variety of host plants such as tomato (Al-Karaki 2000), maize (Feng et al. 2002), and lettuce (Jahromi et al. 2008).

The present study evaluates for iceberg lettuce plants (*Lactuca sativa* L.) the effects of root colonization by a salt-adapted AMF on the tolerance to salt stress induced by reclaimed wastewater in terms of commercial size, biomass production (fresh and dry weight, shoot diameter, and number of leaves), nutrient acquisition (macro/micro nutrients) and gas exchange parameters (net photosynthesis and stomatal conductance).

Materials and methods

Water sources and experimental design

This study was conducted from October to December 2011 at the experimental farm of the Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC) in Murcia, Spain (38°07′ 18″ N; 1°13′ 15″ W).

Water used in the experiment was taken from two different sources. One source was the Tajo–Segura transfer channel (electrical conductivity (EC)≈1 dS m^{-1}) which supplies a large part of the water used in Murcia for both human consumption and irrigation practices. The other source was from a sewage treatment plant located near the experiment (Campotéjar, Murcia), where a tertiary purification treatment gave reclaimed water with high EC (≈4.19 dS m^{-1}). Table 1 shows the physical, chemical and microbiological parameters analyzed at the beginning of the experiment for the two different water sources used in this study.

The experiment consisted of a randomized complete block design where the variables evaluated were the two different water sources and inoculation or not with the AMF. Hence the following four treatments were performed: (1) transferred water without inoculation (transferred water; TW), (2) transferred water and inoculation (TW-AMF), (3) reclaimed water

Table 1 Physical, chemical and microbiological analysis for both water sources at the beginning of the experiment

	Reclaimed water	Transferred water
Physical–chemical		
pH	7.67±0.03	7.97±0.02
EC (dS m^{-1})	4.19±0.14	0.87±0.09
Total dissolved solids (ppm)	1,785.00±1.32	383.00±0.90
Turbidity (NTU)	9.40±2.26	2.10±0.64
NO_3^- (ppm)	18.05±0.80	1.68±0.22
$PO4^{---}$ (ppm)	1.62±0.04	0.22±0.01
SO_4^{--} (ppm)	816.80±4.95	220.12±0.80
K^+ (ppm)	48.27±1.25	3.39±0.08
Ca^{++} (ppm)	186.35±1.67	94.21±2.41
Mg^{++} (ppm)	148.80±2.51	41.87±1.43
Na^+(ppm)	662.30±4.4	52.07±1.9
B (ppm)	1.08±0.06	0.09±0.01
Cl^- (ppm)	757±2.01	69.50±1.54
Microbiological		
Fecal coliforms (CFU/100 ml)	1,315±23	34±2
Escherichia coli (CFU/100 ml)	93±5	3±1

without inoculation (RW) and (4) reclaimed water and inoculation (RW-AMF).

Eighteen replicates of each of these treatments were used totaling 72 pots (one plant per pot). Throughout the experimental period, two samplings of lettuce plants, where the shoot was separated from the root system, were carried out to perform the analyses. Firstly, three individual plants of each treatment (12 in total) were collected 30 days after transplanting and finally, another set at 90 days.

Growing conditions and irrigation

The experiment was carried out under controlled conditions in a greenhouse equipped with a cooling system with relative humidity of 55–65 %, day/night temperatures of 23–26/11–15 °C, 10/14 h light/dark period, and a maximum photosynthetic photon flux density of 1500 μmol m^{-2} s^{-1}, measured with an external light meter (LI-Quantum Q-40211, LI-COR Inc., Lincoln, NE, USA) during gas exchange measurements.

Lettuce seedlings (*L. sativa* L.) of variety capitata and type iceberg were transplanted into pots (diameter=25 cm), placing one plant per pot. Iceberg lettuces were selected because (1) it is a variety widely consumed due to its high acceptation by society and (2) it also presents many problems of root growth and hence the use of mycorrhizae is fully justified. Lettuces were planted in the fall as it is considered as the first and most valuable planting cycle for lettuce. Pots were filled with a non-sterilized substrate composed of soil (clay loam texture) and washed sand, mixed in the ratio 3:1 *v*/*v*. It had a pH of 7.8, an electrical conductivity of the soil saturation extract of 2.32 dS m^{-1} and a sodium absorption rate (SAR) of 0.94. The substrate was not sterilized to simulate a scenario of compatibility and competition between the native and the indigenous AMF as real as possible.

Lettuce plants were irrigated daily by drip irrigation during the whole growing period (Gallardo et al. 1996). The volume of water applied was that required to bring the substrate to field capacity and was controlled according to the drainage of the pots. A drip emitter with a nominal flow of 2 L h^{-1} supplied the water to each pot. In addition, fertilizer (1, 000 kg ha^{-1}) with a composition of 7 % total N (5 % N ammonia and 2 % N urea), 10 % phosphorus (4 % soluble in water), 6 % potassium, 25 % sulfur and 2 % magnesium was applied through the drip irrigation system during the whole growing period.

AMF inoculation

The AMF strain used was *Glomus iranicum* var. *tenuihypharum* sp. *nova*. It was obtained by isolating the fungi under extreme saline soil conditions (Solonetz Gley, saline soil type according to FAO soil classification). The multiplication of the strain was performed as proposed by Fernández and Juárez (2011). The inoculum (150 spores g^{-1} and 25 mg g^{-1} of extramatrical mycelium) was supplied through the irrigation system (injection pump regulated at 25 L h^{-1}) in a dose of 3 kg ha^{-1} 5 days after transplantation. The effectiveness of the inoculum (100 infective propagules per gram) was eventually evaluated according to the most probable number test.

Measurements

Symbiotic development

The percentage of mycorrhizal colonization was determined following the gridline intersect method (Giovanetti and Mosse 1980) after clearing washed roots in 10 % KOH and staining with 0.05 % trypan blue in lactic acid (*v*/*v*) (Phillips and Hayman 1970).

Biomass production

Growth of lettuce (shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW) and shoot diameter (SD)) were analyzed at each sampling according to (1) the Regulation Regulation No 771/2009 of the European Union Commission, (2) the indications proposed by Ryder (1979), and (3) by means of personal communication with landholders. Dry weights (DW) were measured after drying in a forced hot-air oven at 80 °C until a constant weight was reached (approximately for 48 h). In addition, the commercial size, measured as the number of lettuces of homogeneous size that can be adequately introduced in a box of 49 cm×39 cm, was determined (Martínez-López 2010). Table 2 shows the relation between the commercial size and the minimum and maximum SFW, SDW, and SD.

Table 2 Relation between commercial size and minimum and maximum SFW, SDW and SD values

Commercial size[a]	Min. SFW (g)	Max. SFW (g)	Min. SDW (g)	Max. SDW (g)	Min. SD (cm)	Max. SD (cm)
13	350	395	39.0	44.0	14.5	15.3
14	300	345	33.5	38.5	13.6	14.4
15	250	295	28.0	33.0	12.8	13.5
16	200	245	22.0	27.5	11.9	12.7
17	150	195	16.5	21.5	11.0	11.8

Commercial size values intended for sale under the premise of first category according to legal requirements up to 16

[a] Regulation No 771/2009 of the European Union Commission, Ryder (1979), Martínez-López (2010) and personal communication with landholders

Regarding only the shoot part, the mycorrhizal dependency (MD), or response to mycorrhizal colonization, was calculated by using the following formula provided by Kumar et al. (2010): MD (%)=(DW of mycorrhizal plant−DW of non-inoculated plant)/(DW of mycorrhizal plant)×100.

Leaf-ion content and chlorophyll

To perform the analyses, leaves located in the medium–inner part of the lettuce head (approximately the fourth or fifth leaf starting at the outside of lettuce) were selected. For mineral analyses, samples (0.2 g of dry matter) of the three plants selected per treatment were dry-ashed, homogenized in a mill, and introduced in teflon tubes. Then, they were attacked with 4 ml HNO_3 PA-ISO 69 % and 1 ml H_2O_2 33 % and digested in a microwave (Milestone, Ethos one). Then, an inductively coupled plasma (ICP-ICAP 6500 DUO Thermo, England) was employed for macronutrients (N, P, K, Ca, and Mg), micronutrients (Mn, Fe, and Zn) and phytotoxic elements (Na and B) determination at 30 and 90 days after transplanting. Ion concentration was compared with the macro- and micronutrient ranges determined by Diagnosis and Recommendation Integrated System (DRIS; Hartz et al. 2007; Sanchez et al. 1991).

Additionally, a proxy of chlorophyll was determined with a portable meter (SPAD-502, Konica Minolta). This instrument makes instantaneous and non-destructive chlorophyll measurements known as relative chlorophyll index (SPAD units).

Gas exchange measurements

Net photosynthesis (A) and stomatal conductance (g_s) were measured at solar noon by using a portable photosynthesis device equipped with a leaf chamber fluorometer (LI-6400-40, LI-COR Inc., Lincoln, NE, USA). In this case, measurements were carried out at 30 and 75 days to avoid the lettuce reach a round and dense head that could hinder the measure. Additionally, the water-use efficiency (WUE), computed as the ratio A/g_s, was determined.

Statistical analyses

Data were subjected to two-way analysis of variance (ANOVA) using the software SPSS Statistics v. 21 for Windows and with (1) AMF inoculation, (2) TW or RW, and (3) AMF inoculation+TW or RW interaction as sources of variation. Tukey's range test, at 95 % confidence level was also applied. Percentage values of root colonization were arcsine [square root(X)] transformed before statistical analysis.

Results

Root mycorrhizal colonization

Figure 1 shows the percentage of AMF root colonization in lettuce plants for both irrigation treatments (transferred and reclaimed water) at 30 and 90 days after planting.

As the substrate used to fill the experimental pots was not previously sterilized, a native AMF proliferation was detected in non-inoculated plants (TW=23.3 % and RW=19.5 % treatments at 90 days after planting).

Results also indicated that the type of irrigation water used, transferred or reclaimed water, did not significantly affect the percentage of AMF colonization at any sampling date (Fig. 1). By contrast and to a certain extent expected, inoculated plants in TW-AMF and RW-AMF treatments presented higher rates of colonization than non-inoculated plants. In this latter case, the mycorrhizal colonization rate increased significantly up to 65.2 % and up to 75.5 % at 30 days of planting, and up to 98.0 % and up to 89.3 % at 90 days after planting respectively.

Biomass growth

Thirty days after transplantation, irrigation with RW did not produce any negative effect on the lettuce growth. At this stage, inoculation did not significantly improve the plants development either. Additionally, analyses did not show significant differences in the ratio SDW/RDW in any treatment. However, MD was increased twofold (from 8.9 to 16.5 %) which represented a rise of 85.4 % for RW-AMF compared to TW-AMF treatment (Table 3).

At the end of the experiment, high salinity concentration of RW (≈4.19 dS m^{-1}) adversely affected the lettuce growth. In non-inoculated plants, irrigation with RW led to a significant

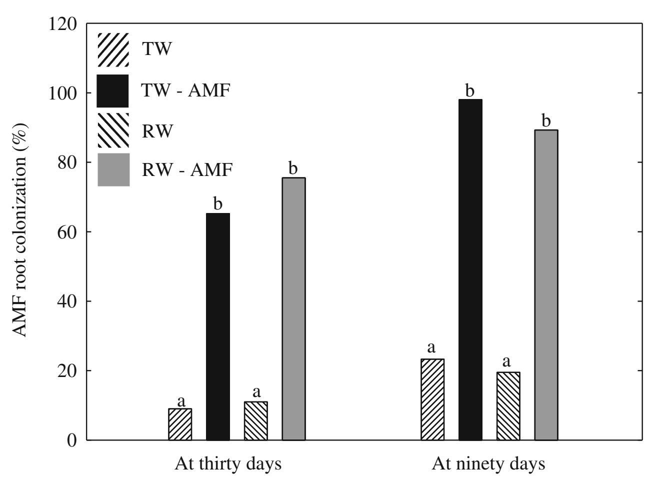

Fig. 1 Percentage of AMF root colonization in lettuce plants (*L. sativa* L.) for the four treatments performed in this experiment at 30 and 90 days after planting. *Data not sharing a letter* in common differ significantly according to Tukey's range test at 95 % confidence level

Table 3 Influence of experimental treatments on lettuce plants growth and mycorrhizal dependence. Data with different letters differ significantly ($P \leq 0.05$) as determined by Tukey's range test

			TW	TW-AMF	MD (%)	RW	RW-AMF	MD (%)
Days after transplanting	At 30 days	SFW (g)	60.12a	65.5a	–	52.13a	63.5a	–
		SDW (g)	5.92a	6.5a	8.9a	5.26a	6.30a	16.5b
		RFW (g)	14.88a	16.38a	–	13.98a	16.38a	–
		RDW (g)	1.56a	1.68a	–	1.32a	1.64a	–
		SDW/RDW	3.79a	3.87a	–	3.98a	3.84a	–
		No leaves	11.35a	11.61a	–	11.29a	12.28a	–
	At 90 days	SFW (g)	352.0c	399.2d	–	189.9a	237.8b	–
		SDW (g)	38.23c	44.4d	13.9a	21.8a	26.33b	17.2b
		RFW (g)	66.5a	82.3b	–	70.2a	103.5c	–
		RDW (g)	7.43a	9.73b	–	7.89a	11.67c	–
		SDW/RDW	5.14b	4.56b	–	2.76a	2.56a	–
		SD (cm)	15.24c	15.39c		11.11a	13.4b	
		No leaves	15.8b	17.7b	–	13.9a	14.3a	–

reduction of SD (from 15.24 cm to 11.11 cm) and yield of 46 % compared to plants supplied with TW (SFW=352 g and SFW=189.9 g for TW and RW treatments respectively; Table 3). However, in this non-inoculated plants case, no significant reductions of RFW and RDW were detected. As expected, SFW, SDW, and SD were significantly higher in TW-AMF treatment; inoculated plants supplied with TW (SFW=399.2 g, SDW=44.4 g and SD=15.39 cm; Table 3). At this stage, increases on SFW of 13.4 and 25.2 % were observed in TW-AMF and RW-AMF with respect of TW and RW respectively. In the case of SDW, those values were 16.3 and 20.8 %. Inoculation also increased RDW by 31.0 % and 47.9 % for TW-AMF and RW-AMF respectively. The highest ratio SDW/RDW values were observed for TW and TW-AMF treatments. Lastly, the MD at the end of the experiment was about 13.9 % and 17.2 % for TW-AMF and RW-AMF respectively which represented an increase of 23.7 % (Table 3).

Leaf-ion content

Tables 4 and 5 present the leaf-ion content (macro- and micronutrients) of lettuce plants at 30 and 90 days after planting for the four experimental treatments, respectively.

Macronutrients

Results from leaf-ion analyses at 30 days indicated that N concentration was somewhat dependent on the type of water used for irrigation but not on the inoculation (Table 4). Highest N concentration values were found in plants supplied with transferred water (TW=3.94 % and TW-AMF=4.03 %). However, analyses at the end of the experiment (90 days after planting) showed that the use of saline RW did not affect the N concentration in leaves but the inoculation with AMF led to a significant increase regardless the type of water applied. RW-AMF treatment presented the highest N concentration (4.33 %) whereas the minimum concentration was observed in the RW treatment (3.58 %) (Table 5).

For the whole experimental period, P concentration was similar for all treatments, except for the TW-AMF treatment where slightly higher P concentrations were detected (0.32 and 0.30 % at 30 and 90 days, respectively).

K concentration was not affected by the type of water used for irrigation but it was by the inoculation with the AMF. Inoculated treatments presented higher K concentrations than non-inoculated treatments (RW-AMF=6.12 % and TW-AMF=6.32 % at 90 days) (Table 5).

Table 4 Concentration of macronutrients (N, P, K, Ca, and Mg) and micronutrients (Mn, Fe, and Zn) on lettuce leaves at 30 days after planting for the four experimental treatments. Data with different letters differ significantly ($P \leq 0.05$) as determined by Tukey's range test

At 30 days	Macronutrients (%)					Micronutrients (ppm)		
	N	P	K	Ca	Mg	Mn	Fe	Zn
TW	3.94b	0.24a	5.65a	1.90a	0.52a	124.8a	238.4a	36.6ab
TW-AMF	4.03b	0.32b	6.57b	3.01b	0.56a	133.4a	470.0c	43.0b
RW	3.58a	0.27a	5.65a	1.38a	0.52a	132.3a	191.3a	30.8a
RW-AMF	3.83ab	0.27a	6.10b	1.80a	0.53a	165.8b	317.3b	36.5ab

Table 5 Concentration of macronutrients (N, P, K, Ca, and Mg) and micronutrients (Mn, Fe, and Zn) on lettuce leaves at 90 days after planting for the four experimental treatments. Also shown are the optimum macro- and micronutrients ranges calculated for lettuce iceberg by Diagnosis and Recommendation Integrated System (DRIS) proposed by Hartz et al. (2007). Data with different letters differ significantly ($P \leq 0.05$) as determined by Tukey's range test

At 90 days	Macronutrients (%)					Micronutrients (ppm)		
	N	P	K	Ca	Mg	Mn	Fe	Zn
TW	3.72a	0.16a	5.69a	2.02ab	0.50a	116.1a	237.6b	31.4a
TW-AMF	4.05b	0.30b	6.32b	4.25c	0.54a	138.1b	608.7d	44.3b
RW	3.58a	0.17a	5.67a	1.62a	0.50a	130.8b	189.5a	28.5a
RW-AMF	4.33c	0.18a	6.12b	3.05b	0.51a	171.8c	567.3c	43.5b
Optimum ranges	3.3–4.8	0.35–0.75	2.9–7.8	0.6–1.1	0.25–0.45	45–74	115–257	25–73

At the end of the experiment, colonization by the AMF also increased Ca concentration (Table 5). TW-AMF treatment presented the highest Ca concentration (4.25 %) whereas the lowest concentration was observed in the RW treatment (1.62 %). This higher value for Ca was already detected at 30 days in TW-AMF treatment (Table 4).

Overall, at the end of the experiment, inoculation of plants supplied with RW increased the concentration of macronutrients, except P and Mg that did not show significant differences. In inoculated plants supplied with transferred water, all treatments showed a significant increase in the concentration of macronutrients, except Mg that remained similar.

Micronutrients

At 30 days, Mn and Fe were not dependent on the type of water used in the irrigation but inoculation increased the content of these nutrients, except for Mn in TW-AMF. In the case of Zn, significant differences between treatments were not detected.

At the end of the experiment, most striking increases were achieved for Fe where concentrations of 608.7 and 567.3 ppm for TW-AMF and RW-AMF were reached. Additionally, significant differences appeared for Zn in inoculated treatments (TW-AMF=44.28 ppm and RW-AMF=43.46 ppm).

To a certain extent, both macro- and micronutrients were within the ranges computed by DRIS (Hartz et al. 2007) (Table 5), except Ca, Mn, and Fe that showed higher values than the optimum ranges proposed and P that was slightly lower (except in TW-AMF).

Phytotoxic elements and chlorophyll

Analyses at 30 days indicated that phytotoxic elements (Na and B) mainly depended on the type of water used (Fig. 2). Plants supplied with RW reached higher Na and B concentrations than plants supplied with TW. In this sense, the highest chlorophyll values were found in non-saline treatments; SPAD values for TW=35.94 and TW-AMF=37.83. Colonization by the AMF did not significantly increase the content of these elements although chlorophyll values were significantly increased.

At the end of the experiment, mycorrhizal effects were only significant for Na in plants supplied with RW where its concentration was significantly reduced. However, chlorophyll significantly increased in inoculated treatments regardless the

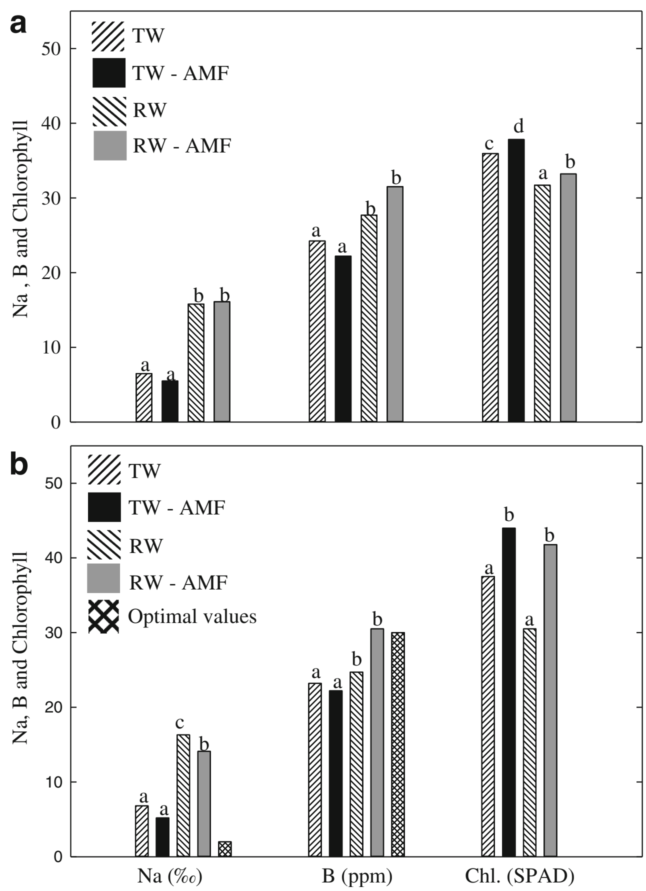

Fig. 2 Concentration of phytotoxic elements (Na and B) and chlorophyll on lettuce leaves at 30 (**a**) and 90 (**b**) days after planting for the four experimental treatments. The optimal values for Na and B determined for iceberg lettuce are also shown. *Data with different letters* differ significantly ($P \leq 0.05$) as determined by Tukey's range test

type of water supplied (SPAD values for TW-AMF=43.97 and RW-AMF=41.76). Phytotoxic elements were also within the ranges computed by DRIS (Sanchez et al. 1991; Hartz et al. 2007) (Fig. 2).

Gas exchange measurements

Overall, A and g_s were dependent on the type of water used for irrigation as plants supplied with non-saline RW reached higher A and g_s values (Fig. 3). At 30 days, inoculation did not increase A and g_s values. In addition, WUE, computed as the ratio A/g_s, was dependent on the type of water used for irrigation and also on the effect of the inoculation with the AMF (except for TW-AMF). At this stage, a significant increase on the WUE value of 28.7 % was detected between RW and RW-AMF treatments (Fig. 3).

At 75 days, inoculation increased A values but it did not affect g_s. Maximum A and g_s values were significantly found for TW-AMF treatment (A=10.29 μmol m^{-2} s^{-1} and g_s= 180 mmol m^{-2} s^{-1}). As expected, the highest WUE value was detected in plants inoculated and supplied with RW (WUE=68.9 μmol CO_2 mol H_2O^{-1}). For the rest of treatments, significant differences were not found.

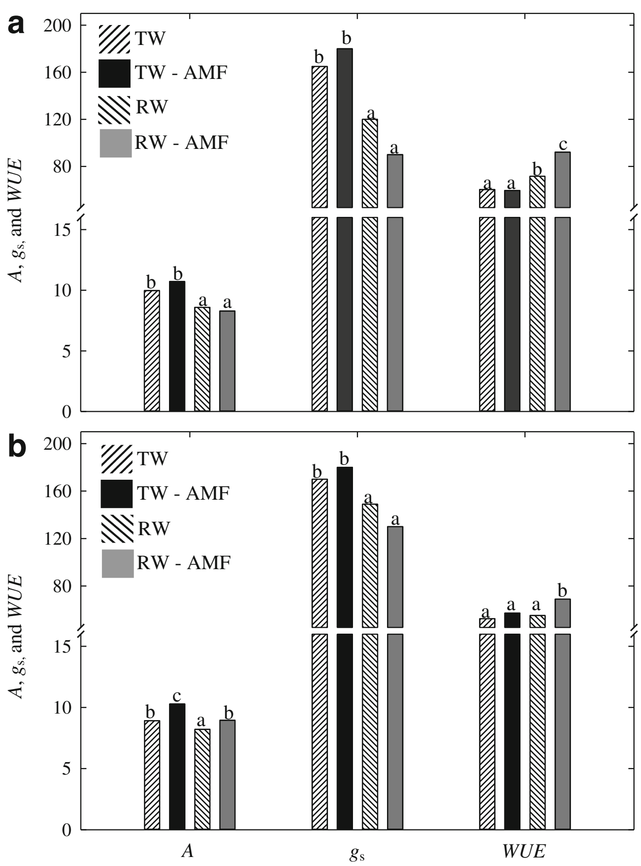

Fig. 3 Photosynthesis (A, micromoles per square meter per second), stomatal conductance (g_s, millimoles per square meter per second) and water-use efficiency (WUE=A/g_s, μmol CO_2 mol H_2O^{-1}) at 30 (**a**) and 75 (**b**) days after planting for the four experimental treatments. *Data with different letters* differ significantly ($P \leq 0.05$) as determined by Tukey's range test

Discussion

High salinity such as the one detected in RW in our study represents a significant drawback for agriculture as it has always been associated with a significant reduction of foliar growth and is widely documented in literature (Allen et al. 1998).

As expected under the unfavorable saline conditions in our study, lettuce plants supplied with RW suffered a significant setback. In fact, lettuce plants only supplied with RW did not fulfill the minimum legal commercial size thresholds and therefore they could not be destined for sale. In contrast, all lettuce plants supplied with TW, which did not suffer from salt stress, satisfied the commercial legal requirements, reaching a suitable commercial size.

Currently, it is fully accepted that AMF symbiosis is a key component in helping plants to cope with adverse environmental conditions such as salt stress (Evelin et al. 2009). Although irrigation water with high EC can reduce the colonization capacity, the spore germination and the growth of fungal hyphae (Jahromi et al. 2008), the fact remains that AMF are able to colonize saline soils. Our results evidence that the AMF symbiosis was well established on lettuce roots regardless of the type of water used (Porras-Soriano et al. 2009) and the colonization rates were in close agreement with those observed in other studies (Ruíz-Lozano and Azcón 1996a; Aroca et al. 2013). It is likely that the isolation of the AMF in an extreme saline medium favored the symbiotic association in the RW-AMF treatment (Wang et al. 1994). In addition, the beneficial effects of different AMF on plant growth and development under saline conditions have been shown in a number of plant species (Feng et al. 2002; Jahromi et al. 2008).

In our lettuce plants inoculated with the AMF and supplied with RW, plants reached a suitable marketable size. In addition, maximum values of *E. coli* measured in RW were close to but never exceeded the international thresholds established (Spanish Royal Decree 1620/2007; Italian Decree, 2003), probably as a result of the tertiary treatment performed. These results are very important for Mediterranean regions where it is increasingly common to use reclaimed water with high EC levels.

Increases of SDW observed in plants inoculated in both RW-AMF and TW-AMF treatments, endorsed the highest degree of MD observed in those plants (Baslam et al. 2011a). Mycorrhizal symbiosis between the AMF and the lettuce root might have favored the absorption of water and nutrients (Navarro et al. 2011) increasing SFW and SD. Maximum values of root development found in the RW-AMF treatment indicated that stressed plants probably tended to develop the root system in order to improve hydraulic conductivity (Kapoor et al. 2008) and take more water from the soil.

The higher N concentration detected in RW-AMF treatment could be mainly attributed to: (1) the high NO_3^- concentration in the RW, (2) a higher N concentration in leaves as a result of smaller lettuce shoots or (3) an increase in the nutrient absorption rate which could improve the nutritional status of the lettuce plants as a result of a more developed root system by effect of the mycorrhizal symbiosis (Marjanović and Nehls 2008). Our results showed that in TW and RW treatments, significant differences in N concentration were not found whereas they were detected in SFW. Moreover, as the type of water used had no significant effects on the final N concentration, that greater N uptake should be attributed to the AMF symbiosis. AMF can actually increase the utilization of different forms of N by plants (Hodge et al. 2001) and have been shown to take up N directly and to transfer it to the host root (Johansen et al. 1996).

Evelin et al. (2009) suggested that colonization of roots with AMF often improves the P nutrition of host plants growing with sparingly soluble P forms. In addition, Hirrel and Gerdemann (1980) indicated that the increase of P uptake by plants inoculated with AMF seems to be one of the key factors responsible for increased salt tolerance. With regard to P in this research, we found that the mycorrhizal effect was only patent in TW-AMF treatment. In addition, a homogenous reduction (except for TW-AMF treatment) of P concentration between analyses at 30 and 90 days regardless the treatment has been observed.

Irrigation with RW could increase the Na concentration in the soil if suitable washing requirements are not applied. When Na or salt concentration in the soil is high, plants tend to take up more Na resulting in decreased K uptake. K plays an important role in stomatal movements, protein synthesis and in the response to changes in leaf water status (Evelin et al. 2012). In our study, the increase in K and the reduction in Na uptakes in the mycorrhizal treatments helped balance the ratio K/Na which was very beneficial for the development of the plant under saline conditions (Giri et al. 2007). Moreover, Na reduction in inoculated plants under saline conditions might allow the host to prevent cellular accumulation of toxic Na concentration (Sharifi et al. 2007).

Ca increases detected in mycorrhizal plants agreed with those observed by Cantrell and Linderman (2001). Those increases were likely to facilitate higher K/Na selectivity leading to salt adaptation (Cramer et al. 1985) and to enhance colonization and sporulation of AMF (Jarstfer et al. 1998).

Mn was somewhat dependent on the type of water used but inoculation increased its concentration on leaves. Similar increases of Mn in mycorrhizal plants were also previously detected by Sylvia et al. (1993) in soybean (cv. Centennial) and sorghum (cv. Funk G-522DR). Mycorrhizal plants also enhanced uptake of relatively immobile metal micronutrients such as Fe and Zn (Baslam et al. 2011b). Overall, these increases may indirectly have a positive impact on subsequent resistance to drought and salinity of the host plants.

Ca, Mg, Mn, and Fe were slightly higher and P was slightly lower than the optimum ranges calculated by DRIS and proposed by Hartz et al. (2007). Na was also slightly higher than the mean optimum value calculated by DRIS (Sanchez et al. 1991). However, there is research that manifests a great variability of those optimum ranges. For instance, in the case of Ca, Mg, and Mn, Jones et al. (1991) reported different optimum ranges (Ca, 1.5–3 %; Mg, 0.36–0.50 %; and Mn, 25–250 ppm). In the case of P, Hochmuth et al. (1991) indicated that P values range between 0.25–0.50 %. Bearing in mind all these assumptions, all macro- and micronutrients were deemed to be within the optimal nutritional level ranges. Phytotoxic elements, Na and B, did not exceed the threshold of toxicity in leaf.

Higher chlorophyll content (SPAD units) was detected in inoculated plants regardless the type of water used and was in agreement with the results shown in other studies (Zuccarini 2007). This suggests that salt interferes less with chlorophyll synthesis in mycorrhizal than in non-mycorrhizal plants (Redondo-Gomez et al. 2010). In addition, at the end of the experiment, inoculated plants under RW conditions (RW-AMF treatment) reached levels of chlorophyll content even higher than those of non-stressed plants (TW treatment), showing that in this respect, mycorrhization is capable of fully counterbalancing such stress (Zuccarini 2007). Van den Driessche (1991) detected a close relationship between some nutrients (N, Mg, and Fe) and chlorophyll and reported that an increase in those nutrients could lead to a stimulation of the synthesis of chlorophyll and hence the photosynthetic capacity. In this sense, the higher concentration of N and Fe measured in mycorrhizal plants could be to a certain extent related to the increase of A detected (Querejeta et al. 2003).

Stability of transpiration rates (g_s) and increased levels of A associated with mycorrhizal plants significantly increased WUE (Jahromi et al. 2008). These effects were most significant in inoculated plants irrigated with RW.

These three positive effects, A, g_s, and WUE, may also have accounted for the enhanced plant growth of colonized plants, most probably by enhancing CO_2 fixation under salt stress (Aroca et al. 2013).

In conclusion, it has been shown that the AMF symbiosis has reached a high degree of root colonization in lettuce plants, in spite of using RW with high salinity concentration for irrigation. AMF alleviate the negative effects of salt stress in lettuce plants, allowing plants to grow better under these unfavorable conditions. In fact, except for plants only supplied with RW, all lettuce heads satisfactorily reached a marketable size and therefore they could have been viable for sale.

Additionally, the levels of most of macro- and micronutrients appeared in higher concentrations which mean that cultivation of mycorrhizal lettuce would allow improving the intake of those compounds without requiring an increase in consumption (Baslam et al. 2011a). The maximum values of *E. coli* in the reclaimed water were close to but never exceeded the international thresholds established (Spanish Royal Decree 1620/2007; Italian Decree, 2003).

Moreover, many growers use a standard fertilization program that is often applied to all their fields, regardless the quality of the water used. As a result of the high concentration of nutrients already dissolved in RW compared with those of transferred water used in this study, and noting that the final concentration of macro- and micronutrients in the leaves was similar in all treatments, adjustments in the calculation of the fertigation should be conducted in order to reduce the cost of unnecessary nutrients, prevent soil contamination and minimize the economic costs.

Overall, the results could confirm the potential of the AMF tested in protecting lettuce iceberg plants against unfavorable environmental conditions and could justify such symbiosis in sustainable agriculture in arid and semiarid areas. However, it is very difficult to generalize, as it has been previously stressed (Baslam et al. 2011b), it must be always borne in mind that lettuce plants depend on several factors, including the fungal species associated with the plant, the source of P nutrition applied to lettuces, the variety or cultivar of lettuce and environmental factors such as the water regime.

Acknowledgments The authors acknowledge the Ministerio de Economía y Competitividad for the project CICYT (AGL2010-17553), the FEDER (Fondo Europeo de Desarrollo Regional) and the European Commission under the EFP7 project SIRRIMED (FP7-KBBE-2009-3-245159) for the financial support of this study. The authors also thank the efforts of the technician associate Bayona Gambín J.M.

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