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Protective effects of *Glomus iranicum* var. *tenuihypharum* on soil and *Viburnum tinus* plants irrigated with treated wastewater under field conditions

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Abstract Currently, irrigation using recycled water is increasing, especially in semiarid environments, but a potential problem of using reclaimed wastewater is its elevated salt levels. The application of arbuscular mycorrhizal fungi (AMF) could be a suitable option to mitigate the negative effects produced by the salinity. In this work, the combined effect of *Glomus iranicum* var. *tenuihypharum* and two types of water: Control, C, with EC <0.9 dS m^{-1} and reclaimed water (wastewater previously treated in a sewage treatment plant) with EC 4 dS m^{-1} during a first saline period (11 weeks) and with EC 6 dS m^{-1} during a second saline period (25 weeks), was evaluated for laurustinus (*Viburnum tinus*) plants under field conditions. This plant is a popular shrub very used for gardening. Chemical properties of soil as well as physiological behavior, leaf nutrition, and esthetic value of plants were evaluated. Due to the high salinity from wastewater at 6 dS m^{-1}, laurustinus plants decreased their stem water potential values and, to a lesser extent, the stomatal conductance. Also, the visual quality of the plants was diminished. The inoculated AMF satisfactorily colonized the laurustinus roots and enhanced the structure of the soil by increasing the glomalin and carbon contents. Furthermore, *G. iranicum* var. *tenuihypharum* inoculation decreased Na and Cl content, stimulated flowering and improved the stem water potential of the plants irrigated with both types of reclaimed water. The AMF also had a positive effect as a consequence of stimulation of plant physiological parameters, such as the stem water potential and stomatal conductance. Effective AMF associations that avoid excessive salinity could provide wastewater reuse options, especially when the plants grow in soils.

Keywords Reclaimed water · Salinity · *Glomus iranicum* · Water relations · Soil structure · Glomalin content

Introduction

The Mediterranean area, both in the agricultural and urban sectors, needs to improve its efficiency of water use. Recycled water produced from treated wastewater can be used in many non-potable applications and can help to reduce the overall demand for fresh water (Anderson 2005; Qadir et al. 2007). Such reclaimed water usually contains high concentrations of nutrients such as N, P, and K that produce a direct benefit for crops and also allow the accumulation of organic matter (Pedrero et al. 2013). However, depending upon its source and treatment, reclaimed water may have high salt content, heavy metals, or pathogenic organisms. Salts normally appear in many types of wastewater although they are more frequent in waste generated by industry, where they are very difficult to eliminate in the treatment process. In general, as salinity increases in the treated wastewater used for irrigation, the probability for cropping problems increases, which may have measurable adverse effect on plants, soils, and systems. The major difference in evaluating the suitability of waters for irrigation of agricultural crops and waters for irrigating

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landscape plants is that the former is based on harvested crop yield, while the latter is based on esthetic quality or appearance (Fox et al. 2005; Cassaniti et al. 2012). In either case, it is important to select plants that are able to tolerate salt stress in order to allow the use of low quality water without damaging the development of plants. Salt tolerance varies considerably among the different genotypes of ornamentals used in landscaping. Apart from plant characteristics, the degree and duration of salt stress as well as soil composition also need to be taken into consideration as they can influence the severity of plant damage by saline irrigation water (Chaves et al. 2009; Cassaniti et al. 2012). In fact, the characteristics of soil such as structure, physicochemical properties, biological activity, possible organic and inorganic amendments, and possible presence of pollutants (heavy metals and others) may influence the growth and survival of plants in the soil (Clemente et al. 2005) and, as a consequence, the response of the plant to salinity.

Many researchers have shown that arbuscular mycorrhizal fungi (AMF) have a positive influence on cultivation systems (Hamel and Plenchette 2007). They not only improve plant growth through increased nutrient uptake, but they have also "non-nutritional" effects in stabilizing soil aggregates, in preventing erosion, and in alleviating negative effects induced by salinity (Evelin et al. 2009; Dudhane et al. 2011; Talaat and Shawky 2011; Çekiç et al. 2012). Several mechanisms can be involved in AMF enhancement of salt tolerance in host plants, such as increased nutrient acquisition, maintenance of the K/Na ratio, biochemical changes, physiological changes like water status and photosynthetic efficiency, and molecular or structural changes (Sheng et al. 2008; Evelin et al. 2009; Hajiboland et al. 2010; Abdel-Fattah and Asrar 2012; Çekiç et al. 2012).

Although salinity may reduce AMF colonization capacity and hyphal growth (Jahromi et al. 2008), the success of AMF development depends on the soil characteristics, the AMF isolate introduced, the selected plant species and their interactions, as well as the degree, duration, and type of stress. Some authors have demonstrated that AMF inoculation can alleviate the negative effects produced by saline irrigation in ornamental and wild plants (Giri and Mukerji 2004; Giri et al. 2007; Kumar et al. 2010; Navarro et al. 2012). In fact, some AMF are more resistant to salinity than others, as is the case for the fungus used in the present study, *Glomus iranicum* var. *tenuihypharum*, which was isolated from extreme saline soil conditions and previously shown to alleviate salt stress in lettuce (Vicente-Sánchez et al. 2013).

Viburnum tinus L. (laurustinus) is a perennial shrub, autochthonous to the Iberian Peninsula, which is used for biodiversity conservation and has a great economic and ecological importance. According to Bañón et al. (2012), laurustinus plants are moderately sensitive to salinity at 6 dS m^{-2} while according to Fox et al. (2005), this species can withstand 2.5–3 dS m^{-1} without losing its esthetic value. Also, in a previous experiment, where the effect of reclaimed water on pot-grown laurustinus plants was evaluated in greenhouse conditions, the esthetic value and growth decreased as consequence of salinity (Gómez-Bellot et al. 2013).

The present study was aimed at checking the effectiveness of *Glomus iranicum* var. *tenuihypharum* in protecting laurustinus plants against salinity of reclaimed wastewater under field conditions. For this, the combined effect was evaluated of AMF inoculation and two kinds of reclaimed water (RW) with different electrical conductivity on the ion acquisition, water status, gas exchange and esthetic value of *V. tinus* plants, as well as the effect of RW on mycorrhiza development and soil properties.

Materials and methods

Plant material and experimental conditions

The experiment was performed using 1-year-old laurustinus plants (n=80) at the experimental farm of CEBAS-CSIC in Santomera (Murcia, Spain) (38°06′ N, 1°02′ W, elevation 110 m) and plots of approximately 1 × 1 m. The soil, classified as Lithic xeric haploxeroll, is stony (33 %, *w*/*w*) and shallow, with a clay–loam texture (Abrisqueta et al. 2013). Analytical data showed a high lime content, low organic matter content, and low cationic exchange capacity (Table 1).

Every 15 min, climatic data were recorded by an automatic weather station located next to the experimental plots. The average maximum and minimum values of air temperature and RH were 24 and 13 °C, and 84 and 35 %, respectively, during the experimental period (Table 1). The annual reference evapotranspiration (ETo), determined by the FAO, Penman-Monteith equation (Allen et al. 1998), had a maximum of 194.98 mm in July.

In April 2012, 10–15-cm-high laurustinus plants were collected from a nursery and transplanted into the experimental plots. The soil was initially amended with 2 g L^{-1} of Osmocote Plus (14:13:13 N, P, K plus microelements), and every 3–4 months, during the acclimation period, a Hoagland

Table 1 Soil characteristics and climatic data throughout the experiment

Soil characteristics		Climatic parameters	
pH	7.92	T_{max} (°C)	24
EC (dS m^{-1})	0.41	T_{min} (°C)	13
D_b (g cm^{-3})	1.56	RH_{max} (%)	85
OM (%)	0.34	RH_{min} (%)	35
$CaCO_2$ (%)	56.00	Annual Eto (mm)	1091
CEC (meq 100 g^{-1})	14.66	Total rainfall (mm)	185

solution (standard nutrient solution) was supplied through a drip irrigation system.

On March 8 2013, half of the plots for each irrigation treatment were inoculated (3 kg ha^{-1}) with *G. iranicum* var. *tenuihypharum* (mixture of spores, mycorrhizal root fragments, and rhizospheric soil), isolated from an extremely saline soil (Solonetz Gley, saline soil type according to FAO soil classification). Inoculum of the AMF was multiplied as proposed by Fernández and Juárez (2011) and was applied through the drip irrigation. The remaining plants were not inoculated.

On April 5 2013, after 1 year of acclimation, the first saline period started with two irrigation treatments, consisting of control (EC <0.9 dS m^{-1}) and reclaimed water RW1 (EC, 4 dS m^{-1}) from a sewage treatment plant located in Campotejar (Murcia, Spain). The wastewater treatment plant applies a conventional activated sludge process followed by tertiary treatment with ultraviolet light, thus eliminating the bacterial load. Therefore, there were four treatments in total: control and RW treatments with and without AMF inoculation.

On June 20 2013, 15 weeks after AMF inoculation, due to hardly any differences being observed in the physiological behavior of plants, a second irrigation period was started by replacing RW1 at 4 dS m^{-1} with a new reclaimed water treatment, RW2 (EC, 6 dS m^{-1}) from another sewage treatment plant located in Mazarrón (Murcia, Spain). The water treatments in the second period therefore consisted of the same control water (EC <0.9 dS m-1) and a reclaimed water, RW2 (EC, 6 dS m^{-1}). The RW2 irrigation period ended on December 18 2013, 40 weeks after AMF inoculation.

Plants were irrigated so that the stem water potential of the control plants did not fall below −0.9 MPa. The quantity of water applied to maintain this potential depended on the season, climatic conditions, and plant development. Irrigation was carried out twice a day using a drip irrigation system with one lateral pipe per plant row and one emitter (each delivering 3 l h^{-1}) per plant. The quantity of water applied was controlled every week using in-line water meters. The volumetric water content (θv) of the top 20 cm of the soil profile was measured every week for seven plants per treatment throughout the experiment, by time domain reflectrometry (TDR) (Model 1502C, Tektronix Inc., Beaverton, OR) as described by Moreno et al. (1996).

Water analyses

The inorganic solute content, pH, and EC of the irrigation waters were assessed in May and June 2013 (at the beginning of the first and second irrigation period, respectively). Samples were collected in glass bottles and stored at 5 °C before being processed for chemical analyses. The pH was measured with a Cryson-507 pH-meter (Crisom Instruments S.A. Barcelona, Spain); EC was determined using the multirange equipment Cryson-HI8734 (Crisom Instruments S.A. Barcelona, Spain); the concentrations of macronutrients (Na, K, P, Ca, and Mg) and micronutrients such as B were determined by inductively coupled plasma optical emission spectrometer (ICP-ICAP 6500 DUO Thermo, England), and chloride was analysed by ion chromatography with a Metrohm Chromatograph (Switzerland).

Mycorrhizal colonization and enzymatic activity

At the end of the first and second irrigation periods, young root samples with surrounding rhizosphere soil were collected at a depth of 20–25 cm to assess symbiotic development. Three root samples per treatment were used with at least three replicates per root. The percentage mycorrhizal root colonization was estimated following the gridline intersect method (Giovannetti and Mosse 1980) under a microscope (×100 magnification), after clearing washed roots in 10 % KOH for 10 min at 90 °C, then in HCl_2N for 10 min and staining with 0.05 % Trypan blue dissolved in lactic acid (*v*/*v*) (Phillips and Hayman 1970). The AMF enzymatic activity was also estimated at the end of the second saline period in the same three young root samples, by staining for succinate dehydrogenase (SDH) (Smith and Gianinazzi-Pearson 1990) and fungal alkaline phosphatase (ALP) (Tisserant et al. 1993) activities, in order to measure living or functional mycorrhiza.

To determine the SDH activity, root samples were incubated at room temperature overnight in a nitro blue tetrazolium (NBT)-succinate solution (MacDonald and Lewis 1978), consisting of 5 ml NBT (4 mg ml^{-1}), 5 ml Tris–HCl buffer (0.2 mol L^{-1}; pH 7.4), 2 ml sodium succinate (2.5 mol L^{-1}), 2 ml $MgCl_2$ (5 mmol L^{-1}), and 6 ml distilled water. To determine the ALP activity, root samples were incubated at room temperature overnight in 20 ml naphthyl acid phosphate (1 mg ml^{-1}), 20 ml Fast Blue RR salt (1 mg ml^{-1}), 18 ml Tris-citric acid (0.05 mol L^{-1}; pH 9.2), 1 ml $MgCl_2$ (0.5 mg ml^{-1}), and 1 ml MnCl2 (0.8 mg ml^{-1}). After incubation, roots were cleared in a sodium hypochlorite solution (containing 3 and 1 % active chlorine for SDH and ALP, respectively) during 5 min and washed with distilled water. The percentage of enzymatic active mycorrhiza (SDH and ALP) was estimated following the gridline intersect method (Giovannetti and Mosse 1980) under a microscope (×100 magnification).

Measurement of glomalin and mineral content in soil

Three soil samples per treatment were collected at the end of the first and second irrigation periods. Soil samples were dried, sieved to 1–2 mm, and 0.25 g of each soil sample was mixed with 2 ml of sodium citrate 20 mM, pH 7.0. The easily extractable glomalin (EEG) was extracted by autoclaving the mix at 121 °C for 30 min and centrifuging at 3000 rpm during 15 min. The protein in the supernatant was determined by the Bradford dye-binding assay with bovine serum albumin as

standard (Wright et al. 1996). Concentration of glomalin was extrapolated to mg g^{-1} of soil particles by correcting for the dry weight of coarse fragments >0.25 mm included in the weight of aggregates and for the volume of extractant.

At the end of the first and second irrigation periods, the mineral contents of three soil samples per treatment were determined by Inductively Coupled Plasma optical emission spectrometer (ICP-OES IRIS INTREPID II XDL). The soil was dried at room temperature for a week, ground, and sieved through a 2-mm nylon mesh before analysis. The macronutrient concentrations were determined in an extract digested with HNO3:HClO4 (2:1, *v*/*v*) by Inductively Coupled Plasma optical emission spectrometer (ICP-OES IRIS INTREPID II XDL). The concentration of Cl^- was analysed by a chloride analyzer (Chloride Analyser Model 926, Sherwood Scientific Ltd.) in the aqueous extracts obtained by mixing 100 mg of dry sample powder with 40 ml of water before shaking for 30 min and filtering.

Total nitrogen (N_T), total carbon (C_T), and organic carbon (C_{org}) concentrations of the soil samples were measured with an elemental analyser Flash EA 1112 Series- Leco Truspec. The organic matter content (OM) of the soil was determined by multiplying TOC by 1.72. Available P was also analysed colorimetrically as molybdovanadophosphoric acid (Watanabe and Olsen 1965).

Leaf mineral content and relative chlorophyll content

At the end of the first and second irrigation periods, the mineral content of leaves was determined in four plants per treatment as indicated above for the mineral content of soil. Leaves, with a medium level of development, were taken at a third of the plant height. Leaf samples were oven-dried at 80 °C and ground before analyses. Throughout the experiment, the relative chlorophyll content was determined in seven leaves per treatment using a Minolta SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan).

Measurements of plant water status and gas exchange

Seasonal changes in stem water potential (Ψ_{stem}), leaf osmotic potential at full turgor (Ψ_{100s}), stomatal conductance (g_s), and net photosynthesis (P_n) at midday were determined weekly in seven plants per treatment throughout the whole experimental period.

Stem water potential was estimated according to Scholander et al. (1965), using a pressure chamber (Model 3000; Soil Moisture Equipment Co., Santa Barbara, CA, USA) in which leaves were placed within 20 s of collection and pressurized at a rate of 0.02 MPa s^{-1} (Turner 1988). Leaves for Ψ_{stem} were taken from the north-facing side and were covered with aluminum foil for at least 2 h before measurements. The leaf osmotic potential at full turgor (Ψ_{100s}) was estimated using excised leaves with their petioles placed in distilled water overnight to reach full saturation. Then, these leaves were frozen in liquid nitrogen (−196 °C) and stored at −30 °C. After thawing, Ψ_{100s} was measured in the extracted sap using a WESCOR 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA), according to Gucci et al. (1991).

Leaf stomatal conductance (g_s) and net photosynthetic rate (P_n) were determined in attached leaves in the same plants and on the same days as the Ψ_{stem} measurements, using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, NE, USA). Leaves were taken at a third of the plant height, in the same way as for chlorophyll measurement. Gas exchange was measured around noon, fixing the conditions of CO_2 concentration at 380 ppm, the photosynthetically active radiation (PAR) at 1500 μmol m^{-2} s^{-1}, and the speed of the circulating air flow inside the system at 300 mol s^{-1}. Throughout the experiment, the average values of air temperature, relative humidity, and pressure deficit were around 22 °C, 42 %, and 1.74 KPa, respectively.

Ornamental parameters

At the end of the second irrigation period, all the plants were visually evaluated as follows: (1) PIC, percentage of plants in ideal condition; (2) PAC, percentage of plants in acceptable condition; (3) PDB, percentage of plants with dry branches; and (4) DP, percentage of dry plants. The number of flowers per plant was counted at the end of the second saline period.

Statistics

In the experiment, 20 plants were randomly attributed to each treatment. Percentage root colonization and enzymatic activities were analysed by one-way ANOVA using Statgraphics Plus for Windows 5.1 software. For the rest of the data, both variables (water quality and mycorrhizal inoculation) and their interaction were analyzed for each parameter by two-way ANOVA. Ratio and percentage data were subjected to an arcsine square-root transformation before statistical analysis to ensure homogeneity of variance. Treatment means were separated with Duncan's multiple range test ($P \leq 0.05$).

Results

Water analyses

At the beginning of the first irrigation period, the physicochemical properties of the two reclaimed wastewaters were analysed (Table 2). The sodium, chloride, and boron content of the RW1 (4 dS m^{-1}) was about 10, 9, and 3 times higher,

Table 2 Physicochemical analysis for the irrigation treatments (RW)

	Control	RW1 (4 dS m^{-1})	RW2 (6 dS m^{-1})
pH	8.25	7.78	7.80
EC (dS m^{-1})	0.92	3.87	6.06
B (mg L^{-1})	0.23	0.75	1.33
Ca (mg L^{-1})	102.70	143.00	217.32
K (mg L^{-1})	45.88	40.12	118.41
Mg (mg L^{-1})	39.73	104.60	37.56
Na (mg L^{-1})	46.92	475.50	1331.00
P (mg L^{-1})	15.74	2.29	5.63
Cl (mg L^{-1})	61.98	544.80	1006.28

Data are values from samples collected at the beginning of each saline period

respectively, than the corresponding values of the control water. The control water showed the highest *P* values while the highest Ca and Mg values were observed in the RW treatment. For the second irrigation period, the sodium and chloride content of the RW2 (6 dS m^{-1}) was about 28 and 16 times higher, respectively, than those observed for control water. RW at 6 dS m^{-1} also showed higher B, Ca, and K content and lower P than control water (Table 2).

Mycorrhizal colonization

At the end of the first and second irrigation period, the inoculated plants for control and RW treatments presented a higher percentage of root colonization than non-inoculated plants. Nevertheless, for non-inoculated plants, these percentages were relatively high, probably due to proliferation of AMF native to the field soil (Table 3). On the other hand, only AMF colonization in inoculated RW plants decreased at the end of the second irrigation period with respect to the end of the first.

At the end of the second irrigation period, nearly all the total AMF present in roots of inoculated plants remained alive (SDH staining) and active (ALP staining) (Table 3). The percentage of SDH activity was highest in the inoculated control plants, followed by the inoculated RW plants, and lowest in the non-inoculated plants. Similar behavior was observed for ALP activity. The values of both parameters were similar between them.

Glomalin and mineral content in soil

Results from soil analyses were similar for both irrigation periods; therefore, only the results for the second period are presented. The easily extractable glomalin (EEG) in the soil significantly increased following AMF inoculation but decreased under RW irrigation regardless of AMF inoculation (Table 4). In addition, there was an interaction between both factors (W×M) (Fig. 1a); the EEG content in the soil increased in the inoculated control treatment with respect to the non-inoculated control, while the lowest EEG content was observed in non-inoculated RW and values increased when plants irrigated with RW were inoculated with the AMF (Fig. 1a).

There was no effect of the irrigation water on the total nitrogen (N_T), total carbon (C_T), C_{org}, OM, and C/N ratio in soil. As regards AMF inoculation, the OM and C_{org} was higher in inoculated plants than in non-inoculated plants (Table 4). Although N_T and C_T tended to increase slight under the effect of AMF, values did not show statistical differences, nor for C/N ratios. On contrary, the available P content in soil was higher for the control than RW treatment, while AMF inoculation did not affect the available P content for either irrigation treatments (Table 4).

Leaf mineral and relative chlorophyll content

At the end of the first irrigation period, RW1 application increased Cl, Na, and Mg content in leaves while P content was decreased; AMF inoculation only had an effect on Na concentration, which decreased in leaves (Table 5). As a result of irrigating with RW2, Na, Cl, Ca, K, and B accumulated in

Table 3 Percentage of mycorrhizal root colonization at the end of the first (I) and second irrigation period (II)

Mycorrhizal inoculation	Treatments				*P*
	C	C M	RW	RW M	
% Colonization (I)	24.0±1.0 bB	83.5±4.5 a	27.1±2.0 bB	81.0±1.5 aA	***
% Colonization (II)	49.5±0.5 cA	90.4±2.7 a	33.9±1.2 dA	75.9±0.9 bB	***
% SDH (II)	44.4±4.1 b	82.5±2.6 a	31.0±3.2 b	75.3±3.1 a	*
% ALP (II)	45.3±2.6 b	73.7±0.6 a	29.9±1.6 c	67.8±4.2 a	*

Means within a row without a common lowercase letter are significantly different by $Duncan_{0.05}$ test. Means within a column without a common capital letter are significantly different by $Duncan_{0.05}$ test. P, probability level: * $P \leq 0.05$; *** $P \leq 0.001$

Percentage of fungal succinate dehydrogenase (*SDH*) and alkaline phosphatase (*ALP*) active mycorrhizal root at the end of the second irrigation period (II). Values are means±SEM ($n=3$ plants)

Table 4 Effects of the irrigation water (W) and *G. iranicum* var. *tenuihypharum* inoculation (M) on the chemical properties of soil and the easily extractable glomalin of soil (EEG) at the end of the second irrigation period. Values are means±SEM (n=3 samples)

Parameters in soil	Irrigation water (W)		Mycorrhizal inoculation (M)		Significance		
	Control	RW	+	−	W	M	W×M
Available-P (mg Kg^{-1} soil)	15.89	11.31	13.34	13.61	**	ns	ns
N_T (g 100 g^{-1})	0.15	0.13	0.15	0.13	ns	ns	ns
C_T (g 100 g^{-1})	7.15	7.71	7.62	7.24	ns	ns	ns
C_{org} (g 100 g^{-1})	1.36	1.36	1.42	1.31	ns	*	ns
OM (%)	2.34	2.34	2.43	2.25	ns	*	ns
C/N	9.49	10.28	9.81	9.96	ns	ns	ns
EEG (mg g^{-1} soil)	38.26	22.43	38.14	22.55	***	***	***

*, **, ***, and ns indicate the level of significance at $P \leq 0.05$, 0.01, 0.001 and the absence of significance, respectively, according to Duncan's multiple range test

leaves while Mg and P decreased (Table 5). The opposite occurred in AMF inoculated plants, where a decrease in Cl, Na, and K leaf content as well as an increase in Mg and P was observed (Table 5). A significant interaction between AMF inoculation and RW application was observed for Na, Ca, Mg, and B (Fig.1b); the leaf concentration of Mg and B was higher in inoculated control plants than in non-inoculated control plants, while Na, Ca, and B decreased in RW M plants with respect to non-inoculated RW plants (Fig. 1b).

From the end of the first irrigation period, the relative chlorophyll content showed significant differences between treatments (Fig. 2d), reaching the highest values in AMF inoculated control plants during both saline periods, while the lowest values occurred in non-inoculated RW plants. There were no statistical differences between mycorrhizal RW M and RW plants during the second saline period (Fig. 2d).

Measurements of plant water status and gas exchange

The Ψ_{stem} showed very similar values for all treatments during the first irrigation period, (Fig. 2a). From the beginning of the second period, non-inoculated RW plants had the lowest values and inoculated RW plants had intermediate values, week 15 coinciding with a high temperature and evaporative demand. At the end of the experiment, the inoculated RW plants reached similar values of Ψ_{stem} as control plants (Fig. 2a). As regards leaf osmotic potential at full turgor, a decrease was observed in both RW treatments (AMF inoculated and non-inoculated) with respect to both control treatments during the experiment, reaching values around −2.54 MPa for the non-inoculated RW treatment compared to −1.85 MPa for the control treatment (Fig. 2b). This parameter was hardly affected by AMF inoculation. In addition, the volumetric water content increased in soil irrigated with

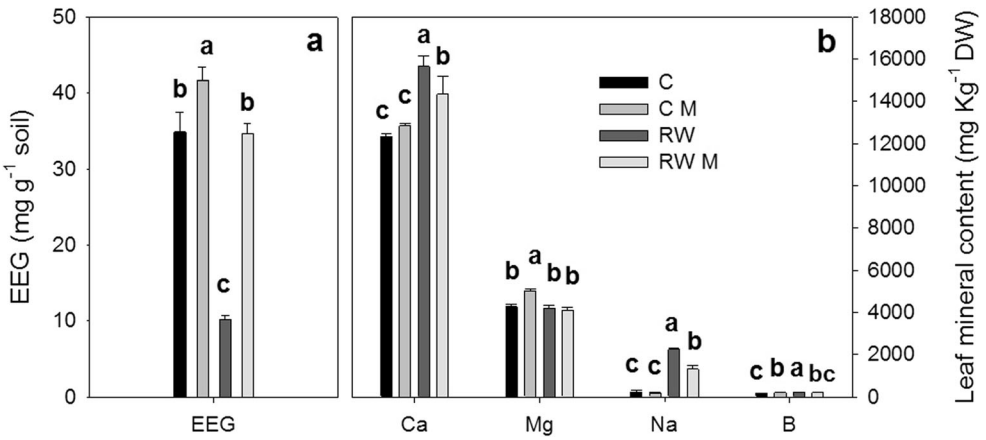

Fig. 1 Easily extractable glomalin content (EEG) in soil (**a**) and leaf mineral content (**b**) of laurustinus plants irrigated with water of different qualities (*C* control, *RW* reclaimed water), with (M) and without *G. iranicum* var. *tenuihypharum* inoculation, at the end of the second saline period. Values are mean of three soil samples for EEG and four plants for leaf mineral content. *Different lower case letters* indicate significant differences between treatments according to $Duncan_{0.05}$ test. The *vertical bars* indicate standard errors

Table 5 Effects of irrigation water (W) and *G. iranicum* var. *tenuihypharum* inoculation (M) on the mineral content of leaves at the end of the first and second irrigation (RW) period. Values are means±SEM (n=4 plants)

	Leaf analyses (g Kg^{-1} DW)	Irrigation water (W)		Mycorrhizal inoculation (M)		Significance		
		Control	RW	+	−	W	M	W×M
First RW period	Cl	2.881	5.310	3.854	3.903	**	ns	ns
	Na	0.189	0.995	0.343	0.455	**	*	ns
	Ca	4.352	4.407	4.370	4.399	ns	ns	ns
	Mg	1.260	2.151	1.716	1.620	**	ns	ns
	P	0.534	0.288	0.443	0.395	**	ns	ns
	K	4.028	4.113	4.050	4.110	ns	ns	ns
	B	0.007	0.007	0.007	0.007	ns	ns	ns
Second RW period	Cl	4.476	12.200	7.850	8.82	***	*	ns
	Na	0.202	1.771	0.743	1.230	***	***	***
	Ca	12.584	14.995	13.600	13.979	***	ns	**
	Mg	4.651	4.152	4.571	4.233	***	***	***
	P	1.746	1.459	1.702	1.502	***	***	ns
	K	12.020	15.203	13.028	14.195	***	***	ns
	B	0.197	0.202	0.202	0.205	*	ns	***

*, **, ***, and ns indicate the level of significance at $P \leq 0.05$, 0.01, 0.001 and the absence of significance, respectively, according to Duncan's multiple range test

wastewater with respect to the soil irrigated with control water throughout the experiment (Fig. 2c).

Throughout practically all of both irrigation periods, the highest g_s values were found in AMF inoculated plants (C M and RW M), this parameter being lowest in the non-inoculated RW plants (Fig. 3a). In contrast, throughout the whole experiment, the values of P_n tended to be more similar among all treatments than those observed for the g_s (Fig. 3b). A decrease in both g_s and P_n values was observed for all treatments in week 15, as a consequence of the high temperature registered

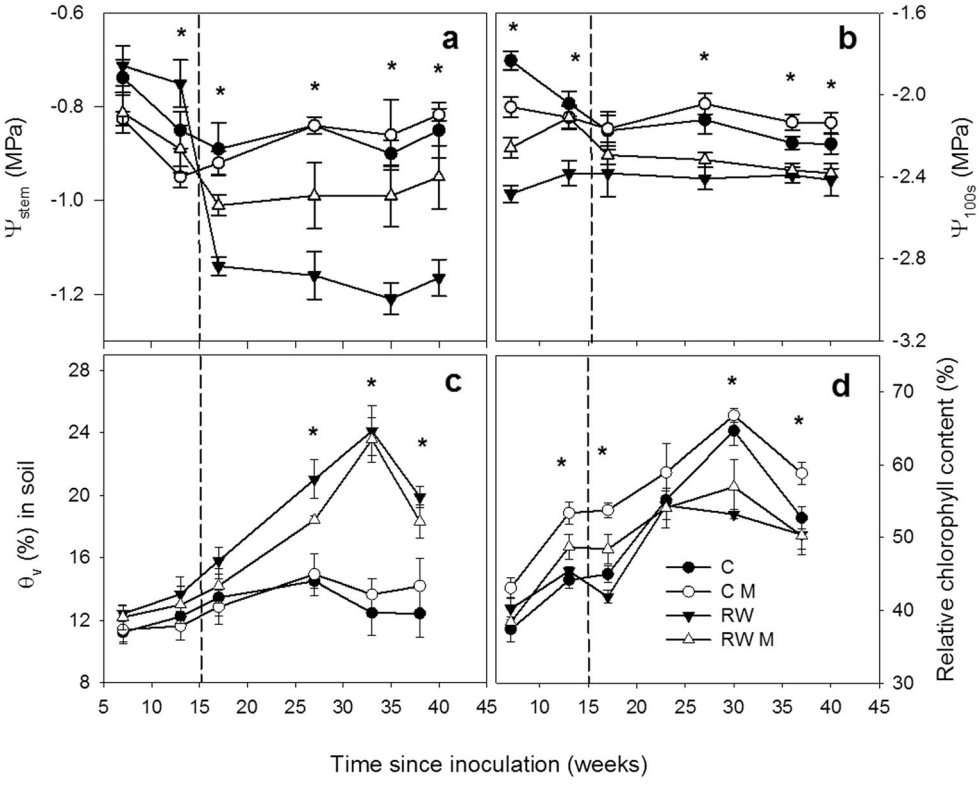

Fig. 2 Stem water potential (Ψ_{stem}) (**a**), leaf osmotic potential at full turgor (Ψ_{100s}) (**b**), volumetric water content in soil (θv) (**c**) and relative chlorophyll content in leaves (**d**) of laurustinus plants irrigated with water of different qualities (*C* control, *RW* reclaimed water), with (M) and without *G. iranicum* var. *tenuihypharum* inoculation, during the experiment. Values are means of seven plants. *Asterisks* indicate statistically significant differences between treatments by $Duncan_{0.05}$ test. The *vertical bars* indicate standard errors. *Dash lines* separate both saline periods (first at 4 dS m^{-1} and then at 6 dS m^{-1})

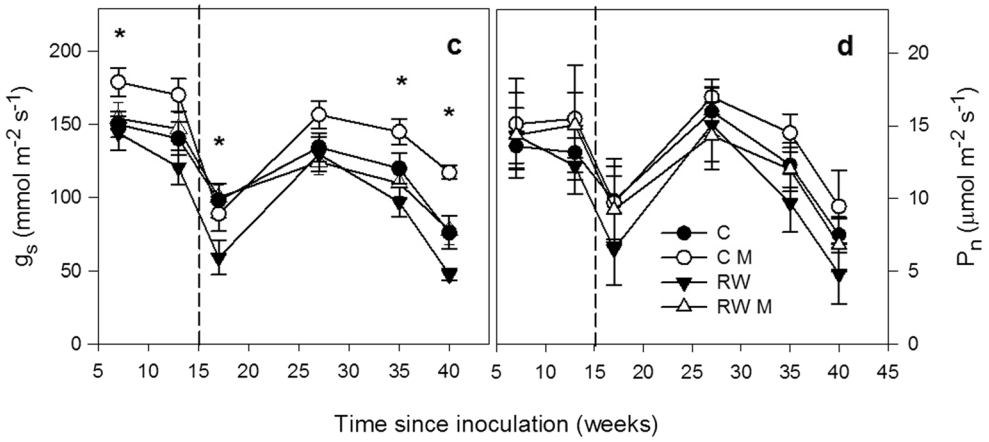

Fig. 3 Stomatal conductance, g_s (**a**) and net photosynthetic rate, P_n (**b**) at midday of laurustinus plants irrigated with water of different qualities (*C* control, *RW* reclaimed water), with (M) and without *G. iranicum* var. *tenuihypharum* inoculation, during the experiment. Values are mean of seven plants. The *vertical bars* indicate standard errors. *Asterisks* indicate statistically significant differences by Duncan$_{0.05}$ test. *Dash lines* separate both saline periods (first at 4 dS m^{-1} and then at 6 dS m^{-1})

in these days. In spite of non-significant differences between all treatments, the values of P_n in were slightly increased in C M and RW M plants as compared to non-inoculated plants.

Ornamental parameters

At the end of the experiment, inoculated control plants showed 100 % of PIC, followed by non-inoculated control plants, with 90 % of PIC (Fig. 4a). The lowest PIC and DP values were found in non-inoculated RW plants (35 and 5 %, respectively). On the other hand, AMF inoculation resulted in 65 % of PIC, 30 % of PAC and 5 % of DP in RW plants. The number of flowers was also increased by AMF inoculation, reaching the highest number of inflorescences per plant in the inoculated control plants (Fig. 4b).

Discussion

Inoculation with *G. iranicum* var. *tenuihypharum* increased mycorrhizal root colonization in laurustinus plants, regardless of the type of irrigation water used. On the other hand, non-inoculated plants presented relatively high levels of root colonization, probably due to the proliferation of native AMF present in the field soil. Similar results have been previously observed for lettuce and grapevine (Vicente-Sánchez et al. 2013; Nicolás et al. 2014). In addition, AMF colonization in *G. iranicum*-inoculated RW plants decreased slightly at the end of the second irrigation period with the saline wastewater, with respect to the end of the first saline period, which suggests that the high Na and Cl content present in the soil of the plants irrigated with RW could have negatively affected

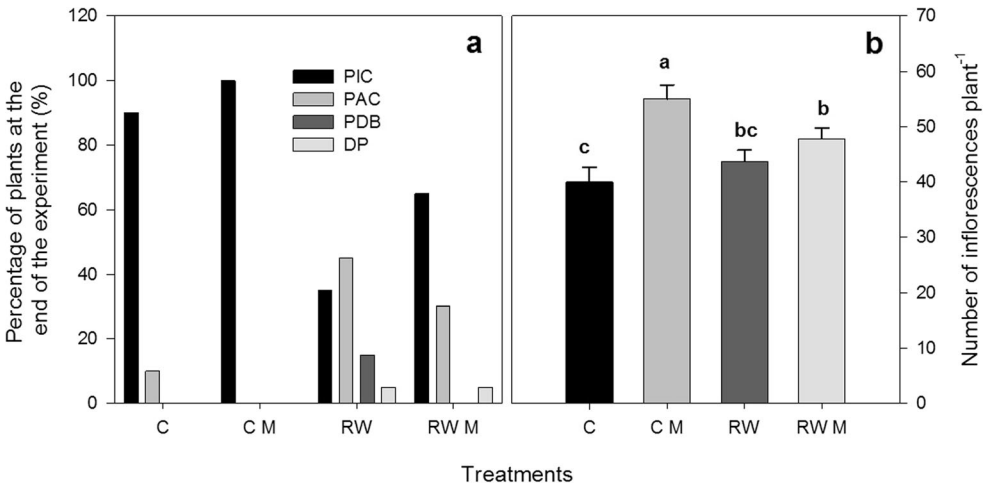

Fig. 4 Percentage of plants according to the visual characteristics (**a**) and number of inflorescences per plant (**b**) of laurustinus plants irrigated with water of different qualities (*C* control, *RW* reclaimed water), with (M) and without *G. iranicum* var. *tenuihypharum* inoculation, at the end of the second saline period. Values are mean of ten plants for number of inflorescences. *PIC* percentage of plants in ideal conditions, *PAC* percentage of plants in acceptable conditions, *PDB* percentage of plants with dry branches, and *DP* percentage of dry plants. Different *lower case letters* indicate significant differences between treatments according to Duncan$_{0.05}$ test. The *vertical bars* indicate standard errors

mycorrhizal colonization. Several authors have reported that AMF colonization of plant roots is reduced in the presence of NaCl (Tian et al. 2004; Juniper and Abbott 2006; Giri et al. 2007; Sheng et al. 2008). However, in spite of the high EC level of the wastewater (6 dS m^{-1}) applied to the laurustinus plants, AMF colonization at the end of the RW2 irrigation period remained at a fairly high level. This was probably due to the fact that *G. iranicum* var. *tenuihypharum* was resistant to the wastewater salinity as the fungus was isolated from soil under extreme saline conditions, (Fernández and Juárez 2011).

It is known that inoculating plants with AMF often improves the P nutrition of host plants growing in soils with low levels of soluble P, as the symbiotic fungi facilitate the transfer of P from the soil to roots (Evelin et al. 2009). The fungal enzyme alkaline phosphatase (ALP), identified in AMF, is considered to play a role in the processes of P acquisition by mycorrhizal plants (Gianinazzi et al. 1992; Abdel-Fattah 2001; Amaya-Carpio et al. 2009). Since P assimilation is essential to the photosynthesis, it has been suggested that higher photosynthetic activity in mycorrhizal plants is related to higher P acquisition, in part via a higher ALP activity (Abdel-Fattah 2001; Amaya-Carpio et al. 2009). In the present experiment, the available-P content of the soil was not low, according to Watanabe and Olsen (1965), and it was statistically unaffected by AMF inoculation, although it was lower after RW treatment with respect to the control treatment, possibly due to the low P content of the wastewater. The increase in leaf P content of the AMF-inoculated RW and C laurustinus plants could reflect the high fungal activity, measured by SDH and ALP, in inoculated roots.

Fungal activity not only enhanced P but also Mg content of leaves and alleviated the adverse effect of RW by suppressing accumulation of toxic ions such as Cl and Na, as previously reported in wheat (Talaat and Shawky 2014). Many authors have suggested that in mycorrhizal plants, Na may be kept inside root cell vacuoles and intraradical fungal hyphae and may not be allocated to the shoots (Cantrell and Linderman 2001; Mardukhi et al. 2011). The AMF-limited transport of Na and Cl toward laurustinus leaves would guarantee a better functionality of chloroplasts and a better photosynthetic efficiency of the inoculated plants, as shown by Rabie and Almadini (2005) and Zuccarini and Okurowska (2008) in horticultural crops. On the contrary, irrigation water x AMF inoculation interactions decreased leaf Ca content but did not increase leaf Mg content of RW plants. These results suggested that *G. iranicum* activated other mechanisms than those used to decrease toxic ions. If reclaimed water is considered as a source of nutrients, the improved nutrient uptake when plants are AMF-inoculated may be less obvious when mycorrhizal inoculation, as in the case of combining with fertilizer application (Nidchaporn 2005; Zuccarini and Okurowska 2008).

On the other hand, the high content of easily extractable glomalin (EEG) observed in both C and RW soils of *G. iranicum*-inoculated laurustinus plants could be related to the higher content of C_{org} and OM, and the tendency of N_T to increase, in the AMF-inoculated soils with respect to non-inoculated soils. Glomalin is a glycoprotein produced by AMF that protects hyphae from the losses of nutrients and water; when hyphae are no longer active, the glomalin inside of their cells is released and it accumulates in the soil (Zhu and Miller 2003; Grümberg et al. 2010). The accumulation of glomalin in the soil indicates a high percentage of edaphic carbon and nitrogen (Treseder and Turner 2007). Glomalin contribution to organic matter, carbon, and nitrogen reserves is even more significant than the microbial biomass, due to its recalcitrant behavior in soil, which permits its accumulation, creating stable aggregates (Comis 2002). Thus, this protein helps to improve soil nutrient uptake by roots and could play a key role in the structural stability of soil (Rillig 2004). The soil glomalin content was decreased by application of RW, in spite of Hammer and Rillig (2011) reporting that salinity stress induced glomalin production. However, the application of high levels of nutrients through the fertilization action of RW irrigation may have interacted with glomalin production in the present experiment.

The plants subjected to a second period of RW presented a decrease in Ψ_{stem}, as a result of the increase in EC of the soil. The results for soil θv suggested that toxic ions like Na and Cl dissolved in the wastewater could have caused extreme difficulty for roots in absorbing water from the soil (Munns 2002; Niu and Cabrera 2010). However, *G. iranicum* var. *tenuihypharum* inoculation improved the soil water status and slightly improved the g_s of inoculated RW laurustinus plants with respect to the non-inoculated plants. Similar results have been observed for the same AMF inoculated onto lettuce or grapevine (Vicente-Sánchez et al. 2013; Nicolás et al. 2014) and for mycorrhizal ornamental plants like *Arbutus unedo* (Navarro et al. 2011).

In spite of laurustinus being classified as a salt-sensitive plant (Azza et al. 2007), the values for Ψ_{100s} indicated that laurustinus plants irrigated with RW were able to develop osmotic adjustment permitting the enhancement of water status and values of P_n close to control plants throughout the experiment, in agreement with Gómez-Bellot et al. (2013). Although differences between treatments were not statistically significant, *G. iranicum* var. *tenuihypharum* inoculation slightly increased P_n values, especially for control plants, which was probably due to the AMF increasing plant P and Mg uptake, which helped to maintain the high chlorophyll content in leaves. The chlorophyll content values were similar between both RW treatments, although they tended to increase in AMF-inoculated RW plants with respect to non-inoculated RW plants. According to Evelin et al. (2009), by maintaining a high Mg/Na ratio in leaves, the chlorophyll concentration

increases and hence plant photosynthetic efficiency and growth improves. In the present study, Mg in leaves probably suppressed the effect of Na uptake in the AMF-inoculated plants so that photosynthetic capacity (estimated by chlorophyll content) improved.

At the end of the second RW irrigation period, there was a clear accumulation of toxic ions in RW plants which led to leaf chlorosis due to salts reducing the chlorophyll content in leaves. As a consequence, the lowest percentage of plants in ideal conditions was found in non-inoculated RW plants, (35 % of PIC and 5 % of DP). Nevertheless, the laurustinus plants improved their esthetic value when inoculated with *G. iranicum* var. *tenuihypharum*, and the number of flowers per plant increased. Similar increases in flowering due to AMF inoculation have been previously described for other ornamental plants (Perner et al. 2007; Navarro et al. 2012). Such an effect may be attributed to an increase in flowers of K and P concentration, both of which have been associated with bud production and flower development (Krizek and Fletcher 2005; Gaur and Adholeya 2005). Although there are no data on the flower mineral content in the present study, the stimulation of flower production could have been be related to the increased P contents of the AMF-inoculated plants, or to a possible hormonal effect induced by the presence of the AMF (Perner et al. 2007).

In conclusion, the accumulation of toxic ions in laurustinus plants after 36 weeks irrigation in a field soil with RW of high salinity, first at 4 dS m^{-1} and then at 6 dS m^{-1}, led to a high percentage of plants with chlorotic leaves. In spite of the presence of native AMF, inoculation with *G. iranicum* var. *tenuihypharum* improved the esthetic value of the laurustinus plants under both irrigation conditions, by counteracting certain negative effects of the reclaimed water with high salinity, as a consequence of a stimulation of plant physiological parameters such as stem water potential and stomatal conductance. Effective AMF associations that avoid excessive salinity could provide wastewater reuse options with economic and environmental benefits, especially when the plants are grown in soils.

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