Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
http://dx.doi.org/10.5424/sjar/2014124-5495

Spanish Journal of Agricultural Research 2014 12(4): 1137-1148
ISSN: 1695-971X
eISSN: 2171-9292

RESEARCH ARTICLE

OPEN ACCESS

Assessment of the viability of using saline reclaimed water in grapefruit in medium to long term

Cristina Romero-Trigueros*, Pedro A. Nortes, Francisco Pedrero, Oussama Mounzer, Juan J. Alarcón, Jose M. Bayona and Emilio Nicolás

Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC). Departamento de Riego. P.O. Box 164. 30100 Espinardo (Murcia), Spain

Abstract

Citrus trees are strongly affected by salinity, especially in countries where irrigation is required as a semi-arid Mediterranean agronomic region. The aims of the study were i) to identify the best reliable plant-based water status indicator for field grown grapefruit trees irrigated with saline reclaimed water during five years of cultivation by measuring seasonal changes in physiological parameters (*i.e.* gas exchange and stem water potential measurements), leaf structural traits (*i.e.* leaf chlorophyll content, area-based leaf nitrogen and area-based dry mass), phytotoxic elements and yield; ii) to estimate phytotoxicity thresholds at leaf level. Our results showed that the chlorophyll content was the parameter with the highest number of measures with significant differences ($p \leq 0.05$, ANOVA) between trees irrigated with reclaimed water and control trees throughout growing stages. Moreover, Chl *a* increased linearly with area-based leaf nitrogen ($R^2 = 0.63$; $p < 0.001$) and area-based dry mass ($R^2 = 0.64$; $p < 0.001$). We also determined the salt-induced phytotoxicity thresholds at which a reduction in yields occurs; these levels were Na: 0.1 g/100 g, Cl: 0.6 g/100 g and B: 100 ppm. In conclusion, we revealed the importance of leaf chlorophyll measurements as a significance diagnostic indicator of salt stress on field grown grapefruit trees. This parameter was also related to plant-based water status indicators such as stem water potential, stomatal conductance and net photosynthesis. Additionally, a salt accumulation potential at leaf level was shown, leading to possible risk in crop sustainability in the medium to long term.

Additional key words: chlorophyll; gas exchange measurements; irrigation; phytotoxic elements; plant water status; saline reclaimed water; yield.

Introduction

Agriculture involves between 70% and 80% of the total water usage world-wide. In Europe, irrigated land has risen from 0.022 to 0.035 ha per capita (MARM, 2009). However, water is the most limiting factor for crop production, especially in areas where agriculture relies heavily on irrigation. The most recent forecast for climate change suggests a significant increment in temperature and a major reduction in the annual precipitation during the 21st century, leading to a 17% decline in the water resources available for agriculture world-wide (García-Tejero *et al*., 2010). Therefore, the current problems of shortage of water resources available for agriculture make it imperative to look for alternative water sources for our irrigation systems. In

* Corresponding author: cromero@cebas.csic.es
Received: 20-12-13. Accepted: 12-11-14.

Abbreviations used: A (net photosynthesis, μmol · m^{-2} · s^{-1}); Chl *a* (chlorophyll *a*, mg · gFM^{-1}); Chl *T* (total chlorophyll, mg · gFM^{-1}); DOY (day of year); EC (electrical conductivity, dS · m^{-1}); ETc (evaportranspiration of the crop, mm · $month^{-1}$); ETo (reference evapotranspiration, mm·$month^{-1}$); FG (fruit growth stage); g_{FM} (grams of fresh material, g); g_N (grams of nitrogen, g); g_s (stomatal conductance, mol · m^{-2}·s^{-1}); Narea (area-based leaf nitrogen, g N · m^{-2}); N_m (mass-based leaf nitrogen content); PH (postharvest period); RW (reclaimed water); SB (sprouting and bloom stage); TW (transfer water); VPD (vapour pressure deficit, kPa · $month^{-1}$); W_a (area-based dry mass, g · m^{-2}); WUE_i (water use efficiency, μmol CO_2/mol H_2O); W_w (weith-based dry mass, g · m^{-2}); WWTP (tertiary wastewater treatment plant); Ψ_{stem} (stem water potential, MPa).

this regard, the use of reclaimed water (RW) has gained considerable interest over the years (Levine & Asano, 2004). The volume of treated wastewater is in continuous increase due to environmental concerns and the progressive implementation of the European Waste Water Directive (91/271/EEC). Use of RW has considerable potential as a sustained future-supply of supplemental irrigation water, although it is not always used correctly. Among the advantages of the agricultural use of RW is the possibility to offer macronutrients (nitrogen, phosphorus and potassium) which may be positive for the crops (Levine & Asano, 2004). However, potential health risks and environmental impacts resulting from RW use for irrigation have also been documented (Angelakis *et al*., 2003). Salinity is one of the major abiotic constraints that affects agriculture, especially in countries where irrigation is required (Flowers, 2004). Murcia (SE Spain), as a semi-arid Mediterranean agronomic region, uses 100 hm^3 of RW per year, however 93% of this water has an electrical conductivity (EC) above 2 $dS \cdot m^{-1}$ and 37% has EC values above 3 $dS \cdot m^{-1}$ (Entidad Regional de Saneamiento y Depuración de Aguas Residuales, www.esamur.com). Studies have shown that citrus trees are strongly affected by salinity, especially in the Mediterranean area, and they are also one of the most sensitive crops to boron accumulation (Grattan, 2013) which can be toxic to the plant. The primary effect of high concentrations of both boron (Gimeno *et al.,* 2012) as Na and Cl (Navarro *et al.,* 2011) is stomatal closure. This causes a low transpiration rate and reduces the CO_2 availability for photosynthesis (García-Sánchez & Syvertsen, 2006), as well as increases ion accumulation (Brumos *et al*., 2009; Mouhaya *et al*., 2010) and leaf damage, usually leading to a reduction in fruit yield (Murkute *et al*., 2005). B, salinity and Cl can limit the long-term feasibility of using RW to irrigate citrus as time under exposure to salts increases. Tolerance may decline due to progressive toxic levels of Na, Cl and B that have accumulated in leaves (Reboll *et al*., 2000; Pereira *et al*., 2010). Knowledge of the role that leaf phenology, structure, photosynthetic function and plant water play in physiological function, and their interactions during the growing season, is important for assessing the productivity of fruit tree orchards (Mirás-Ávalos *et al*., 2011) and for characterizing the impact of agronomic practices.

Plant water status regulates many physiological processes including leaf physiology, which affect crop productivity in citrus (Gomes *et al*., 2004). Information on leaf physiological parameters (net photosynthesis [A], and stomatal conductance [g_s]) offers a better understanding of plant-water relationships and its effect on crop performance under saline stress. Specifying, stomatal conductance is considered a particularly suitable parameter to assess the effects of stress on the plant (Flexas *et al*., 2002) and a reduced photosynthesis in well watered but salt-stressed citrus leaves has been associated with the specific toxicity of Cl and/or Na (Levy & Syvertsen, 2004).

On the other hand, recent work has shown the importance of leaf ontogeny changes in photosynthetic process optimization (Egea *et al*., 2012), so it is important to analyze the area-based dry mass and area-based nitrogen. Moreover, the differences in the leaf chlorophyll content can be an indicator of plant vigor and photosynthetic capacity (Carter & Spiering, 2002; Wu *et al*., 2008). Healthy plants with high growth capacity are generally expected to contain high levels of chlorophyll, compared to unhealthy plants (Zarco-Tejada *et al*., 2004). Many studies have evaluated the effect of irrigation with saline water in different citrus varieties, specially developed in greenhouse pots (Lloyd *et al*., 1987; García-Sánchez & Syvertsen, 2006; Melgar, 2008; Syvertsen & Melgar, 2010) or even in vitro (Montoliu *et al*., 2009). However, to the best of our knowledge there are no reports of these parameters when saline reclaimed water is used for irrigation. Moreover, because of the cost and time required to obtain fruit yield for extended periods of time (*i.e*. multiple years), studies that evaluate tolerances of woody crops over extended periods are scarce. Here we evaluated for the first time the use of saline reclaimed water for irrigation during five years in grapefruit trees under field conditions by measuring seasonal leaf chlorophyll content, gas exchange, stem water potential and characterization of leaf structural traits and leaf phytotoxic elements.

Based on the above, the first aim of the study was to assess which sustainability indicator to plant level is the most reliable for physiologically assessing grapefruit tree crops irrigated with RW during five years of cultivation, prior to this assay. Hence, we studied changes on plant physiology and leaf ontogeny throughout the growing season and finally, the crop yield. The second aim was to estimate phytotoxicity thresholds at leaf level and assess their variation along the vegetative cycle. Furthermore, the environmental effects of salt accumulation in leaf were also evaluated.

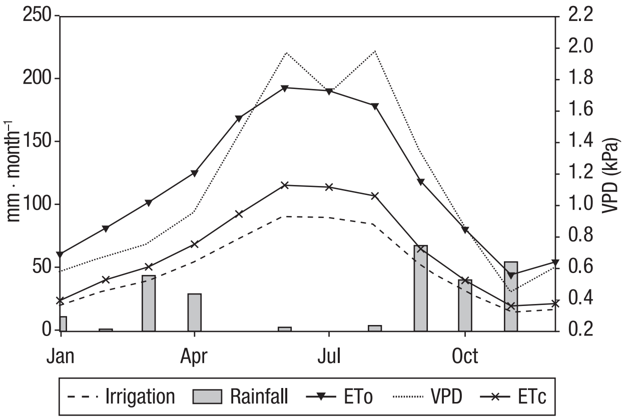

Figure 1. Seasonal evolution of rainfall (mm·month^{-1}), irrigation (mm · month^{-1}), reference evapotranspiration (ETo, mm · month^{-1}), evapotranspiration of the crop (ETc, mm · month^{-1}) and vapour pressure deficit (VPD, kPa · month^{-1}).

Table 1. Chemical parameters of irrigation water from each water source: reclaimed water (RW) and Tajo-Segura transfer water (TW). Each value is the mean of 12 individual measurements

	RW	TW
pH	7.49±0.02	8.79±0.41
EC (dS · m^{-1})	3.95±0.04	0.97±0.00
NO^{-3} (mg · L^{-1})	16.45±9.91	2.52±0.77
PO_3^{-4} (mg · L^{-1})	2.26±0.20	<1.0
K (mg · L^{-1})	42.65±4.29	4.80±1.26
Ca (mg · L^{-1})	179.00±22.22	112.36±7.25
Mg (mg · L^{-1})	134.67±24.30	53.02±5.92
B (mg · L^{-1})	0.83±0.07	0.11±0.02
Na (mg · L^{-1})	550.93±42.93	65.76±12.98
Cl (mg · L^{-1})	679.55±8.55	66.63±1.83
SO_4^{-2} (mg · L^{-1})	732.50±22.9	239.30±1.93

Material and methods

Experimental conditions and plant material

The experiment was conducted at a commercial citrus orchard, located in the northeast of the Murcia region in Campotéjar, 7 km north of Molina de Segura (38°07'18"N, 1°13'15"W) in 2012. The experimental plot of 0.5 ha was cultivated with 7 year-old 'Star Ruby' grapefruit trees (*Citrus paradisi* Macf) grafted on Macrophylla rootstock (*Citrus macrophylla*) planted at 6 × 4 m. The irrigation were scheduled on the basis of daily evapotranspiration of the crop (ETc) accumulated during the previous week. Fig. 1 shows that ETc values were estimated as reference evapotranspiration (ETo), calculated with the Penman-Monteith methodology and a monthly local crop factor (Allen *et al.*, 1998). The correction coefficient for ground cover was 1 according to Fereres & Goldhmaer (1990). All trees received the same amount of fertilizers (ha^{-1} · yr^{-1}) which were applied through the drip irrigation system: 210 kg N, 105 kg P_2O_5 and 155 kg K_2O. Pest control was that commonly used by growers, and no weeds were allowed to develop within the orchard.

A total of 96 trees were used in this study. The mean values of height and diameter of trees were: 1.94 ± 0.16 m and 2.56 ± 0.36 m, respectively. The experimental design of each irrigation treatment was four standard experimental plots distributed following a completely randomized design. Each replica was made up of 12 trees, organized in three adjacent rows. Measurements in two central trees of the middle row were used as average value for each plot while border trees were excluded from the study to eliminate potential edge effects.

Irrigation treatments and water quality

One irrigation treatment was established for each water source. Each treatment was irrigated according to water requirements (100% ETc) throughout growing season. The irrigation head was equipped and supplied with two water sources. The first was pumped from the Tajo-Segura canal (transfer water, TW) and the second water source was pumped from the North of "Molina de Segura" tertiary wastewater treatment plant (WWTP) (reclaimed water, RW). The latest is characterized by having high salt and nutrients levels (Table 1). The amount of water applied both treatments was 6066 m^3 · ha^{-1}. Reclaimed irrigation water showed the highest values in salinity, with EC values close to 4 dS · m^{-1}, while for the transfer irrigation water the EC values were lower, close to 1 dS · m^{-1}. Saline source was automatically blended at the irrigation control-head with water from TW to reduce its EC value down to ≈3 dS · m^{-1} as an intermediate value between the threshold for significant yield losses (1.5-2 dS · m^{-1}) (Maas, 1993) and the average EC of 4 dS · m^{-1} at the outlet of the WWTP. This high level of salinity observed in the RW treatment was mainly due to the high concentration of Cl (>600 mg · L^{-1}) and Na (>500 mg · L^{-1}). The boron concentration in RW was considerably higher than that in TW. Moreover, in the reclaimed irrigation water there was also a higher concentration of N, P and K than in the transfer irrigation water. The pH was more basic on TW than on RW (Table 1). No differences in the concentration of heavy metals were found between the different irrigation water sources (data not shown).

Measurements

Parameters at leaf level gas exchange (net photosynthesis [A], and stomatal conductance [g_s]) and stem water potential were determined on selected clear days with maximum evaporative demand and the measurements were made in two central trees of each replicate per treatment, on two mature leaves, fully expanded-from the mid-shoot area of each plant.

A and g_s were determined with a portable photosynthesis system (LI-6400 Li-Cor, Lincoln, NE, USA) equipped with a LI-6400/40 leaf chamber fluorometer and a LICOR 6400-01 CO_2 injector. Leaf gas exchange was measured on leaves that were placed in a 2-cm^2 leaf cuvette. The CO_2 concentration in the cuvette was maintained at 400 µmol · mol^{-1} (≈ambient CO_2 concentration). Measurements were performed at saturating light intensity of 1200 µmol · m^{-2} · s^{-1} and at ambient air temperature and relative humidity. The air flow was set to 300 mL · min^{-1}. The leaf-to-air water vapour concentration gradient ranged between 0.75 and 2.8 mmol · mol^{-1} for all measurements. Measurements were made on leaves exposed to sunlight between 08:00-10:00 GMT, depending on the season of the year, to avoid high external temperatures and low humidity and because in this period of time is when g_s reaches its highest values.

The midday stem water potential (Ψ_{stem}) was measured in leaves from the canopy close to the trunk. The leaves were wrapped within foil-covered aluminum envelopes, at least 2 h before the midday measurement. The midday stem water potential was measured at noon (12:00 h GMT), using a pressure chamber (model 3000; Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

Twenty leaves per tree were sampled in two central trees of each replicate per treatment, in the early morning, and transported in refrigerated plastic bags to determine the leaf area using an area meter (LI-3100 Leaf Area Meter, Li-cor, Lincoln, NE, USA). Leaves were washed with a detergent (0.1% alconox), rinsed with tap water, cleaned with a dilute solution of 0.005% HCl and finally rinsed with distilled water and left to drain on a filter paper before being oven dried for at least 2 days at 65°C. Then, we determined the dry weight to determine area-based dry mass (W_a, g · m^{-2}) as well as mass-based leaf area, (W_w, m^2 · g^{-1}). Nitrogen content was measured too (Flash EA 112 Series, England and Leco TruSpec, Saint Joseph, USA) and each value was either reported relative to the total leaf area (Narea, g_N · m^{-2}) or as a proportion of leaf dry mass (N_m, g_N · g^{-1}).

For the leaf chlorophyll determination, approximately 30 mg of leaves were sampled from the same avoiding major veins. Chlorophyll eluted from the leaf by submerging them in 3 mL of N, N-dymethylformamide in the dark for at least 72 h. The amount of absorbance was read at 647 nm and 664.5 nm with a Thermo Spectronic (model Helios alpha, UVA No. 092009, made in England) and used to calculate fresh mass-based chlorophyll content (mg · g_{FM}^{-1}) according to equations of Inskeep & Bloom (1985).

Dried leaves were ground and digested with a mix of acid nitric (4 mL) and hydrogen peroxide (1 mL). The concentration of sodium and boron were determined by inductively coupled plasma mass spectrometry (ICP-ICAP 6500 Duo Thermo, England). Anions, as chloride, were analyzed by ion chromatography with a Chromatograph Metrohm (Switzerland).

Statistical design and analysis

Average values of each day were analyzed by analysis of variance (ANOVA) using linear models. The analysis was conducted in SPSS software (vers. 17.0, SPSS Inc., Chicago, IL, USA). All the linear regressions were calculated using Excel. The significance of R values from linear regressions equations was indicated as Pearson correlation coefficients.

Results

Gas exchange measurements and plant water status

Linear regression analysis showed strong correlation between net photosynthesis (A), and stomatal conductance (g_s) in both treatments, which indicated that A was strongly influenced by g_s ($A = 73.301 \cdot g_s + 1.301$;

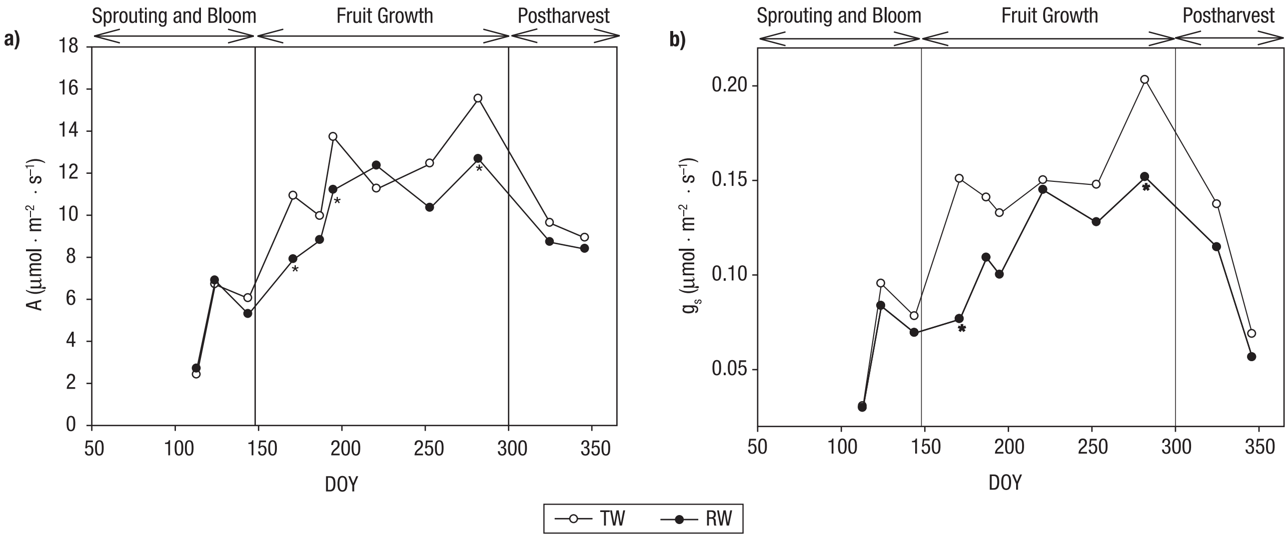

Figure 2. (a) Net CO_2 assimilation rate [A] and (b) stomatal conductance [g_s] in grapefruit trees irrigated with transfer (TW) and reclaimed water (RW). Each value is the mean of the four replicates. * indicate significant differences ($p \leq 0.05$, ANOVA) between TW and RW at each timepoint. DOY: day of year.

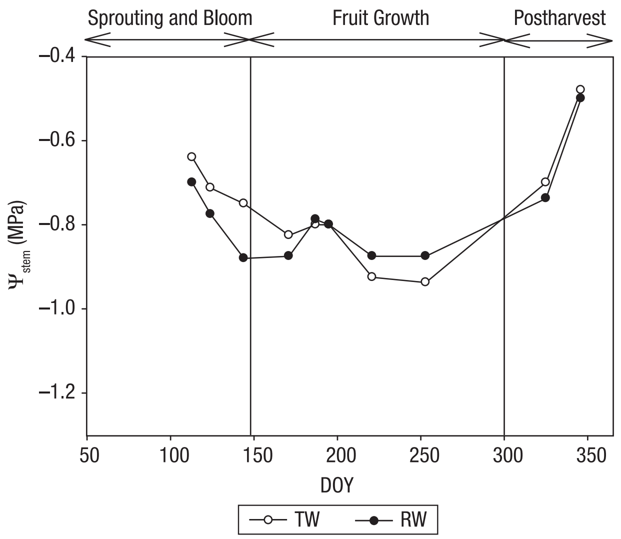

Figure 3. Stem water potential (Ψ_{stem}) in grapefruit trees irrigated with transfer (TW) and reclaimed water (RW). Each value is the mean of four replicates. DOY: day of year.

$R^2 = 0.84$, $p < 0.001$). During the growing season, gas exchange at leaf level showed a general trend to increase until end of fruit ripening period, comprised in fruit growth stage (FG stage). In particular, the highest values of both A (TW = 15.5 µmol · m^{-2} · s^{-1}; RW = 12.7 µmol · m^{-2} · s^{-1}) (Fig. 2a) as g_s (TW = 0.203 mol · m^{-2} · s^{-1}; RW = 0.152 mol · m^{-2} · s^{-1}) (Fig. 2b) were reached during the month of October. Within the same phenological stage, A and g_s showed a downward trend in July and August, being more pronounced for the RW treatment with a mean reduction of ~30-35% of A and stomatal regulation of 40-45%. Otherwise, during the sprouting and bloom stage (SB stage) there were no significant differences between treatments for any variables due to citrus flowering can be considered mostly photoperiod insensitive (Chica & Albrigo, 2013). Regardless of the water quality from the ripening phase and after harvesting, PH stage, the levels of gas exchange demonstrated a significant decrease (Figs. 2a and 2b).

The seasonal pattern of water status of the plant was indicated by $\Psi_{stem.}$ Responses of stem water potential were not significantly different between both treatments, although the grapefruits irrigated with RW had values below the TW throughout the growing season. The minimum value was obtained during the FG stage (Fig. 3).

Characterization of leaf structural traits

The pattern for greatest area-based leaf dry mass (W_a) was from November to March, reaching values of up to 188.9 g · m^{-2} and 174.2 g · m^{-2} for TW and RW, respectively. In the months from March to July, the W_a diminished progressively (TW, 108.3 g · m^{-2}; RW, 121.9 g · m^{-2}) (Fig. 4a). This downward trend was concomitant with a heavy decrease in the leaf dry mass (TW: 0.424 g · leaf^{-1} and RW: 0.353 g · leaf^{-1}) and a mild decrease in the leaf area (TW: 0.0039 m^2 · leaf^{-1} and RW: 0.0028 m^2 · leaf^{-1}).

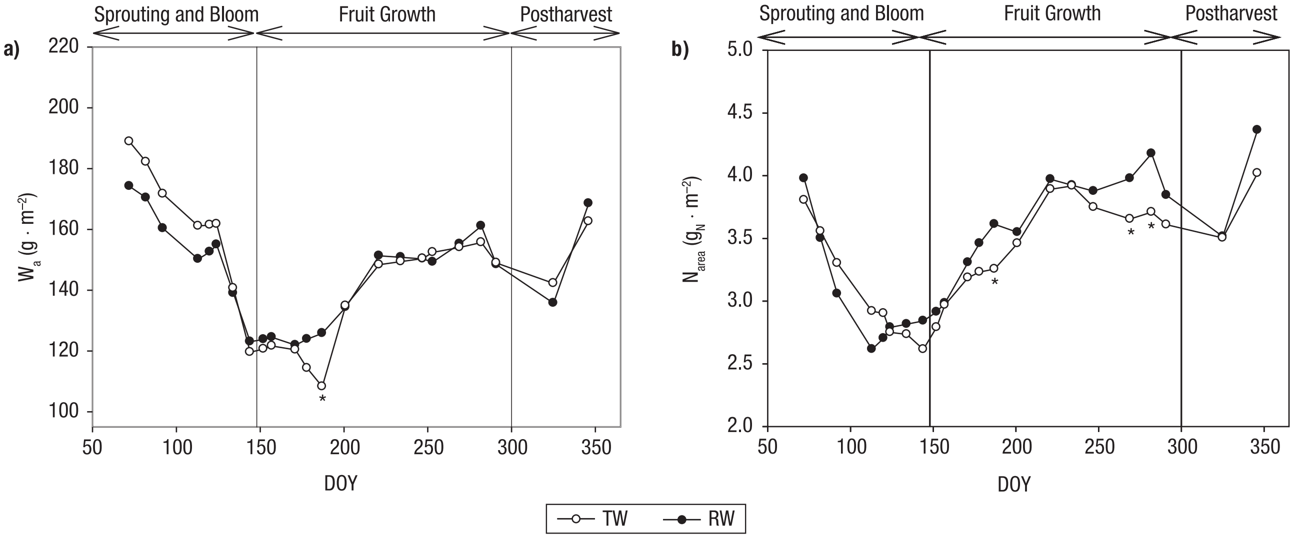

Figure 4. (a) Mass-based leaf area [W_a] and (b) area-based leaf nitrogen [N_{area}] in grapefruit trees irrigated with transfer (TW) and reclaimed water (RW). Each value is the mean of four replicates. * indicate significant differences ($p \leq 0.05$, ANOVA) between TW and RW at each timepoint. DOY: day of year.

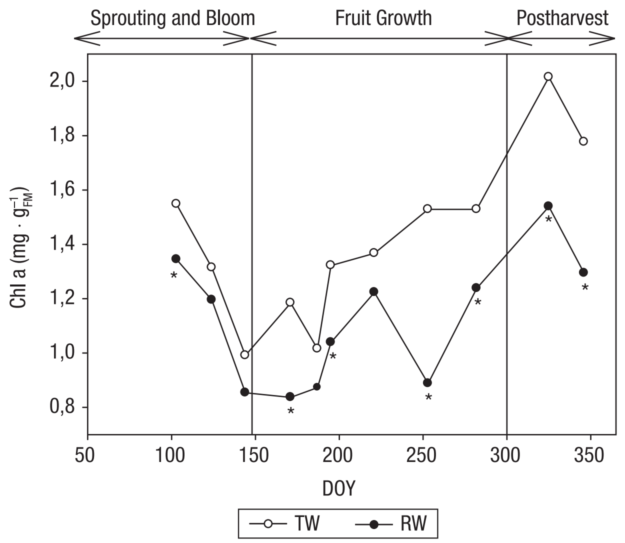

Figure 5. Seasonal concentration of chlorophyll *a* [Chl *a*] in grapefruit trees irrigated with transfer (TW) and reclaimed water (RW). Each value is the mean of four replicates. * indicate significant differences ($p \leq 0.05$, ANOVA) between TW and RW at each timepoint. DOY: day of year.

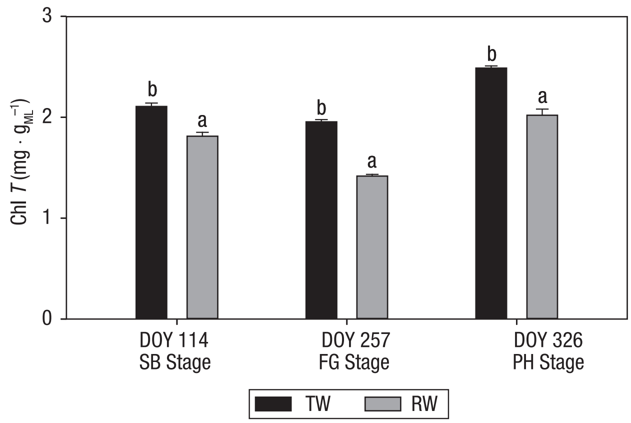

Figure 6. Total chlorophyll (Chl T) in grapefruit trees irrigated with transfer (TW) and reclaimed water (RW) at different phenological periods: SB, sprouting and bloom stage; FG, fruit growth stage; PH, postharvest period. DOY: day of year. Each histogram represents the mean of four replicates ± standard error. Different letters above the histogram bars indicate statistically significant differences among stages at $p \leq 0.05$ by ANOVA.

In contrast, the evolution of area-based leaf nitrogen content [N_{area}], showed its lowest value during the SB stage, before of June: 2.6 $g_N \cdot m^{-2}$ for both treatments. Then, it presented an upward trend throughout the growing season obtaining values of up 4.0 $g_N \cdot m^{-2}$ and 4.4 $g_N \cdot m^{-2}$ for TW and RW, respectively (Fig. 4b), being significantly greater on RW treatment than on TW treatment during FG stage.

The maximum leaf chlorophyll *a* content [Chl *a*] was presented in postharvest phase [PH stage] for both treatments and the minimum content was found at the beginning of the FG phase (Fig. 5). Moreover, significant differences in total chlorophyll content [Chl T], as well as Chl *a* were observed between trees irrigated with RW or TW throughout growing stages (Fig. 6). In particular, RW treatment exhibited a decrease in the levels studied.

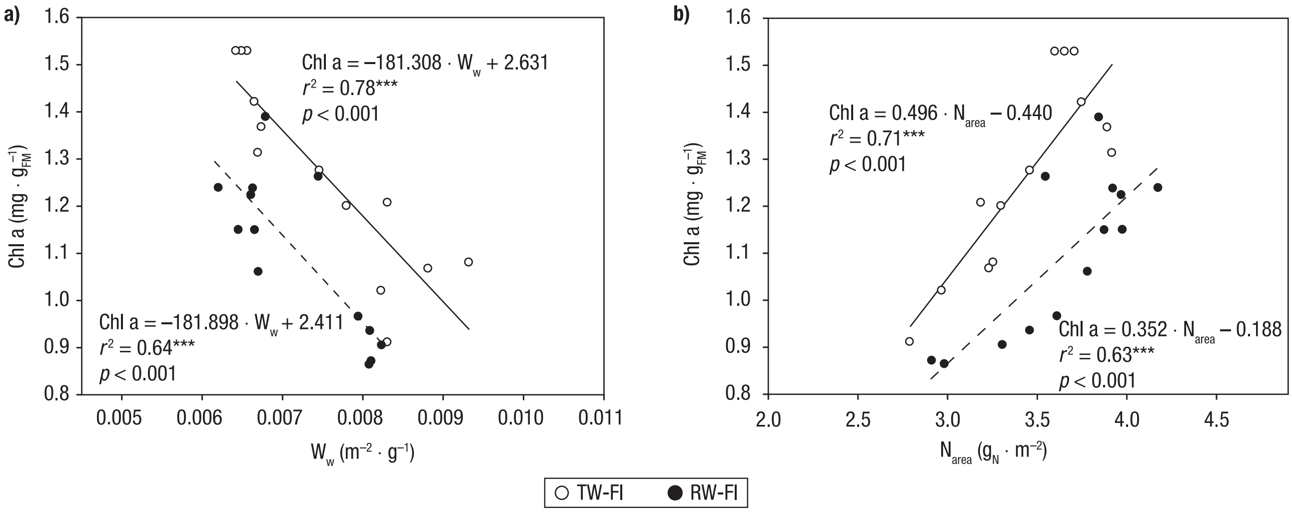

Figure 7. Relationship between chlorophyll *a* and (a) mass-based leaf area [W_w] and (b) area-based leaf nitrogen [N_{area}] in grapefruit trees irrigated with transfer (TW) and reclaimed water (RW).

Additionally, we observed that the Chl *a* decreased significantly with mass-based leaf area [W_w, $m^2 \cdot g^{-1}$] during the FG season. This was higher for the treatment irrigated with TW ($R^2 = 0.78$***, $p < 0.001$) than with RW ($R^2 = 0.64$**, $p < 0.01$) (Fig. 7a). However, during the SB stage, W_w increased progressively while the Chl *a* was practically unchanged and, therefore, their relationship was not significant (data not shown). In the same way, Chl *a* increased linearly with leaf Narea concentration throughout FG season. As before, this was higher for the treatment irrigated with TW ($R^2 = 0.71$**, $p < 0.01$) than for treatment irrigated with RW ($R^2 = 0.63$**, $p < 0.02$) (Fig. 7b). In addition, a significant correlation between A and the Chl T was found for both treatments. Specially, this relationship was more significant for the TW treatment (TW: $R^2 = 0.80$***, $p < 0.001$; RW: $R^2 = 0.47$*, $p < 0.1$). Similarly, correlation between A and leaf N content was significantly different for both TW and RW treatments (TW: $R^2 = 0.48$**, $p < 0.01$; RW: $R^2 = 0.54$**, $p < 0.02$).

Phytotoxic elements and yield

Regarding phytotoxic elements, RW treatment had foliar chloride (Cl), sodium (Na) and boron (B) concentrations significantly higher than trees irrigated with TW (Fig. 8). The highest values were found during the SB stage for trees irrigated with RW, reaching values between 0.162-0.175% and 0.635-0.720%, and 130-150 ppm for Na, Cl and B, respectively, while for trees irrigated with TW were 0.075-0.079%, 0.390-0.500% and 70-72 ppm for Na, Cl and B, respectively (Fig. 8).

Relevant differences were found between the yield observed in the TW treatment (140.8 kg · tree^{-1}) respect to the crop production measured in the RW treatment (113.8 kg · tree^{-1}) (Fig. 9). This pattern was observed in previous years too (data not shown). Salinity did not reduce the grapefruit size, due to the RW treatment decreasing the number of fruit per tree proportionally to the decrease in kg · tree^{-1}. Water productivity was 9.66 and 7.81 kg · m^{-3} for TW and RW treatments, respectively.

Discussion

Plant physiology and water relations

The present study showed that depression of A and g_s in all grapefruit trees occurred in summer (July and August) due to high temperatures and vapour pressure deficit (VPD) under atmospheric CO_2, which was consistent with previous observations on citrus (Jifon & Syvertsen, 2003) and other C_3 plant species (Díaz-Espejo *et al.*, 2007). Trees irrigated with RW presented a decrease of A and g_s throughout the FG stage. In particular, RW treatment presented a higher stomatal regulation with respect to TW due to i) phytotoxic effect of the leaf Na, Cl and B accumulate (Fig. 8) according to Lloyd *et al.* (1987), Anjum (2008), Melgar

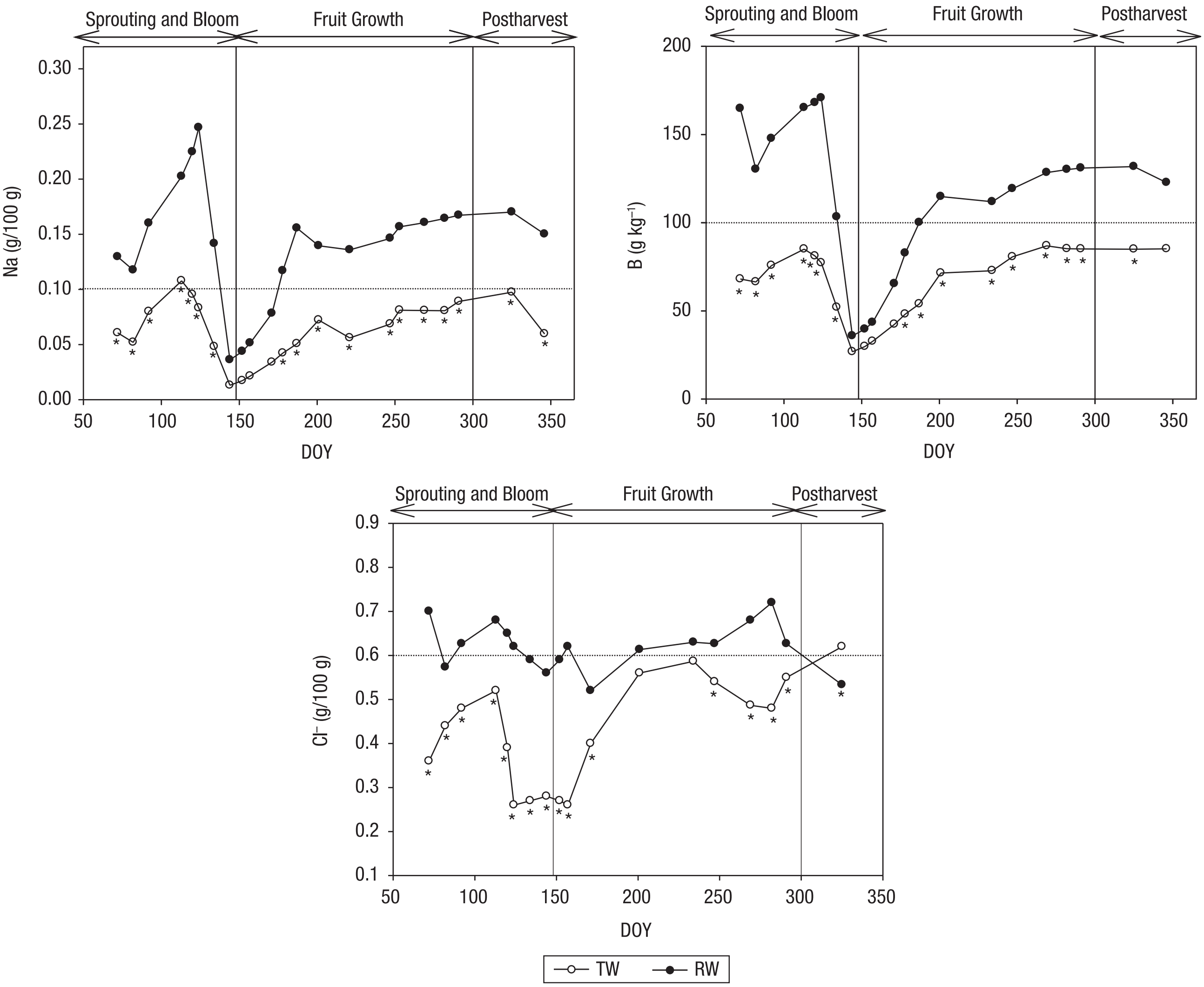

Figure 8. Seasonal concentration of sodium, chloride ion and boron in grapefruit trees irrigated with transfer (TW) and reclaimed water (RW). Horizontal dotted line indicates the phytotoxicity threshold value established by our study. Each value is the mean of four replicates. * indicate significant differences ($p \leq 0.05$, ANOVA) between TW and RW at that timepoint. DOY: day of year.

(2008), Hussain *et al*. (2012) and ii) the decrease of leaf chlorophyll content (Fig. 5 and Fig. 6) according to Zarco-Tejada *et al*. (2004). During the SB and PH stages there were no significant differences between both treatments. Under saline stress, this decline in stomatal conductance may have protective effects against stress, improving significantly intrinsic water-use efficiency (WUE_i) at leaf level by the plant as confirmed by our results (TW: 81.32 ± 3.22 µmol · mmol^{-1}; RW: 91.00 ± 4.04 µmol · mmol^{-1}) and according to Chaves *et al*. (2009). Moreover, WUE_i increased with decreasing g_s (data not shown) in the RW treatment. This indicates that grapefruit is tolerant to salinity (Syvertsen & Melgar, 2010).

All trees maintained Ψ_{stem} values above –1 MPa (threshold value of Ψ_{stem} previously reported by Pérez-Pérez *et al*. (2008)), indicating that our treatments were well irrigated. During the period 2008-2010, the Ψ_{stem} was observed in the same range in both treatments according to data published by Pedrero *et al*. (2013) for the same assay evaluated in this paper. In 2012 Ψ_{stem} declined not significantly with salinity of the RW as Paranychianakis *et al*. (2004) also observed in their own study. However, Ψ_{stem} reached its lowest value in the middle of the FG stage, when TW produced a decrease of the Ψ_{stem} greater than RW. This is because the trees irrigated with TW absorb more water from the soil profile that trees irrigated with RW (Mounzer *et al*., 2013),

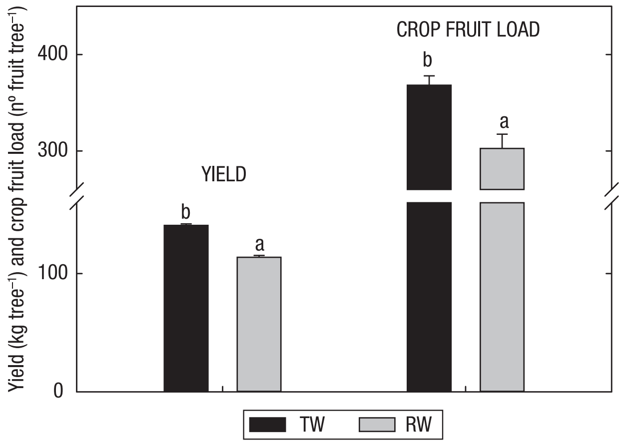

Figure 9. Yield and crop fruit load in grapefruit trees irrigated with transfer (TW) and reclaimed water (RW). Values are means of four plots with two individual trees harvest per plot. Different letters indicate significant differences at $p \leq 0.05$ by ANOVA.

reaching a greater water deficit. In RW treatment, the salinity load in the irrigation water produced a considerable reduction in the available fraction of soil water preventing absorption of the total amount of water, and subjecting the root system to additional undesired osmotic stress and ion toxicity potential risks, as chloride. This limits stomatal conductance and therefore also limits the decrease of water potential, as noted on the RW treatment (Arbona *et al*., 2009). Thus, Ψ_{stem} has demonstrated not to be a reliable indicator to assess the physiological response of grapefruit trees to salt stress.

Leaf structural traits

W_a was higher for both treatments from PH stage to SB stage. In this period the plant accumulates a larger amount of carbohydrate reserves to be utilized in floral differentiation, initial vegetative growth and fructifications. From March to July, the W_a diminished progressively due to vegetative and floral growth, besides fruit set. Narea acquired its lowest value at the end of the SB stage (Albrigo *et al*., 2005) resulting from N allocation to developing young sprouts (Egea *et al*., 2009). Neverthless, W_a continued to decline once Narea was exhausted due to fruit set (Castillo, 1996). Consequently during 144-178 DOY the relationship between W_a and Narea was not significant (data not shown). The observed fluctuations throughout the cycle in both parameters may be due to the alternation of remobilization periods of assimilation and storage periods, indicating that grapefruit leaves play an important role as a depository of assimilates. In our study, A seasonal pattern showed no significant correlation with the level of Narea (data not shown), but can be linked to a change in the internal distribution of leaf nitrogen (Yasumura *et al*., 2006).

During the winter and spring months, the maximum leaf chlorophyll content was observed, as it was shown for W_a. This helps the accumulation of carbohydrates, which will be available to sustain the following florescence, the new leaves and fructification. In addition to excess sodium, chloride and boron in RW reduced significantly the leaf Chl T and Chl *a* content throughout the growing season and, therefore, reduced photosynthesis in RW (Almansa *et al*., 2002; García-Sánchez *et al*., 2002; Levy & Syvertsen, 2004; Papadakis *et al*., 2004a,b). On the one hand, nitrogen content in the leaf is a determinant factor in the photosynthetic rate per unit of foliar area, since, if this content is high, the photosynthetic rate and chlorophyll content will be high, too (Calderón *et al*., 1997). Our data showing that Chl *a* increased linearly with leaf Narea concentration throughout the FG season (Fig. 7b), agree with the results reported by Bondada & Syvertsen (2003). TW treatment showed a stronger positive relationship between Chl *a* and Narea than RW treatment, due to the presence of phytotoxic ions in RW probably influencing the relationship. Therefore, the concentration of chlorophyll in the leaves can be an index of its nitrogen content (Daughtry *et al*., 2000). On the other hand, the differences throughout the growth season might be associated to variations in the photosynthetic rates, though they also might be due to anatomic and morphological differences in structure, like changes in leaf chlorophyll content (Reyes-Santamaría *et al*., 2000). During the FG stage, Chl *a* and W_w were significantly related too. This was probably due to the decrease of W_w being caused by the decrease foliar density and thickness, resulting in lower chloroplast number per unit area (Evans & Poorter, 2001). Specifically, the decreased of Chl *a* was more influenced by W_w in TW treatment than RW treatment. These results showed the relevance of leaf chlorophyll measurements to assess the sustainability of using RW in citrus crops. Moreover, this parameter was also related to plant water status indicators such as Ψ_{stem}, stomatal conductance and net photosynthesis, and is an important tool even for flights multispectral measurements (Zarco-Tejada *et al.,* 2004); leaf chlorophyll content is the target of studies and models linking leaf reflectance and transmittance and canopy hyperspectral reflectance imagery.

Phytotoxic elements and yield

There is disparity between phytotoxic thresholds determined at leaf level for citrus crops. Grattan (2013) established the following: for Na, 0.1-0.25 g/100 g; for Cl: 1 g/100 g and for B, 100 ppm. However, according to Embleton *et al*. (1973) and Labanauskas & Bitters (1974) the critical leaf B concentrations when B toxicity occurs falls in the 250-260 ppm. Reclaimed irrigation water showed high Na, Cl and B concentration that led to leaf Na, Cl and B levels. According to our detailed seasonal evolution, the phytotoxic thresholds, for which the yield of grapefruit crops irrigated with RW were reduced, were Na, 0.1 g/100 g; Cl, 0.6 g/100 g and B, 100 ppm. The three phytotoxic elements reached the highest values in the SB stage, despite higher rates of transpiration, and consequently higher rates of ion transport into leaves according to Walker (1986), were found in the summer period (FG). In addition, the combination of B, Cl and salinity may make the tree more susceptible to any individual stress (Yermiyahu *et al*., 2008). Nevertheless no toxicity symptoms were observed in our experiment. Similar results have been obtained by Reboll *et al*. (2000). According to Hussain *et al*. (2012), ´Star ruby´ grapefruit presented sudden symptoms of leaf fall while leaves were still green. Subsequently, new leaves emerged. This behavior could be characterized as a defense mechanism that involves an evasive action to eliminate leaves that have accumulated toxic ions (Iglesias *et al*., 2007; Arbona *et al*., 2009) and justifies the fact that there are no visible signs of toxicity.

Regarding to yield, RW decreased significantly with respect to TW treatment, approximately 20%, resulting from fewer kilograms per tree and fewer fruit. This is, salinity limits productivity of citrus (Boyer, 2001). This was closely related to the reduction in leaf chlorophyll content and photosynthetic rates and, therefore, lowers carbon availability, which affected final crop yield, as González-Altozano & Castel (2000) reported. In addition, yield losses were also due to ion accumulation and were significant before foliar injury was apparent. Similar results have been obtained by Maas & Grattan (1999). In summary, salinity, Cl and B can limit the long-term feasibility of using reclaimed water to irrigate grapefruits due to salinity and B being cumulative (Levy & Syvertsen, 2004).

After evaluating the validity of three indicators (gas exchange parameters, stem water potential and leaf chlorophyll content) to assess the sustainability of use of tertiary saline reclaimed water in grapefruit crop, our results showed the need of seasonal measurements of leaf chlorophyll content as an important diagnostic indicator of salt stress on field crops of grapefruit. This parameter is also related to plant water status indicators such as stem water potential, stomatal conductance and photosynthesis net. Also, our data accurately established phytotoxicity thresholds for Na, Cl and B that cause a decrease in citrus yields. Additionally, we observed salt accumulation in leaves leaf level that could eventually lead to possible risks in crop sustainability in the medium to long term.

Acknowledgments

This research has been supported by the projects SIRRIMED (KBBE-2009-1-2-3, Proposal No. 245159), SENECA (05665/PI/07 and 11872/PI/09), CONSOLIDER INGENIO 2010 (CSD2006-0067 MEC) and CICYT (AGL2010-17553). C. Romero-Trigueros acknowledges the financial support received from CSIC program JAE.

References

Albrigo G, Syvertsen JP, Dunlop JM, 2005. Growth conditions, crop load and fruit size affect sheepnosing in grapefruit. Proc Fla State Hort Soc 118: 28-34.

Allen RG, Pereira LS, Raes D, Smith M, 1998. Crop evapotranspiration-guidelines for computing crop water requirements. FAO Irrig Drain 56: 15-27.

Almansa MS, Hernández JA, Jiménez A, Botella MA, Sevilla F, 2002. Effect of salt stress on the superoxide dismutase activity in leaves of *Citrus limonum* in different rootstock-scion combinations. Biol Plantarum 45: 545-549.

Angelakis AN, Bontoux L, Lazarova V, 2003. Challenges and prospectives for water recycling and reuse in EU countries. Water Supply 3(4): 59-68.

Anjum MA, 2008. Effect of NaCl concentrations in irrigation water on growth and polyamine metabolism in two citrus rootstocks with different levels of salinity tolerance. Acta Physiol Plant 30: 43-52.

Arbona A, López-Climent MF, Pérez-Clemente RM, Gómez-Cadenas A, 2009. Maintenance of a high photosynthetic performance is linked to flooding tolerance in citrus. Environ Exp Bot 66: 135-142.

Bondada B, Syvertsen J, 2003. Leaf chlorophyll, net gas exchange and chloroplast ultrastructure in citrus leaves of different nitrogen status. Tree Physiol 23: 553-559.

Boyer JS, 2001. Growth-induced water potentials originate from wall yielding during growth. J Exp Bot 52: 1483-1488.

Brumos J, Colmenero-Flores A, Conesa P, Izquierdo P, Sánchez G, Iglesias MF, López-Climent A, Gómez-Cadenas A, Talón M, 2009. Membrane transporters and carbon metabolism implicated in chloride homeostasis differentiate salt stress responses in tolerant and sensitive Citrus rootstocks. Funct Integr Genomics 9: 293-309.

Calderón Z, Rodríguez A, Becerril R, Livera M, Colinas L, 1997. Fertilización foliar nitrogenada en la fotosíntesis y el desarrollo de durazno en producción forzada. Agrociencia [Mexico] 31: 291-296.

Carter GA, Spiering BA, 2002. Optical properties of intact leaves for estimating chlorophyll concentration. J Environ Quality 31: 1424-1432.

Castillo G, 1996. Fluctuación anual de carbohidratos y nutrimentos en relación al amarre de frutos de aguacate (*Persea americana* Mill) cv Colín V-33. Doctoral Thesis. Colegio de Postgraduados. Montecillo, Texcoco, Mexico.

Chaves MM, Flexas J, Pinheiro C, 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103: 551-560.

Chica EJ, Albrigo LG, 2013. Expression of flower promoting genes in sweet orange during floral inductive water deficits. J Am Soc Hortic Sci 138(2): 88-94.

Daughtry C, Walthall CL, Kim MS, Brown de Colstoun E, McMurtrey JE, 2000. Estimating corn leaf chlorophyll concentration from leaf and canopy reflectance. Remote Sens Environ 74: 229-239.

Díaz-Espejo A, Nicolás E, Fernández JE, 2007. Seasonal evolution of diffusional limitations and photosynthetic capacity in olive under drought. Plant Cell Environ 30: 922-933.

Egea G, González-Real MM, Baille A, Nortes PA, Sánchez-Bel P, Domingo R, 2009. The effects of contrasted deficit irrigation strategies on the fruit growth and kernel quality of mature almond trees. Agric Water Manage 96: 1605-1614.

Egea G. González-Real MM, Baille A, Nortes PA, Conesa M, Ruiz-Salleres I, 2012. Effects of water stress on irradiance acclimation of leaf traits in almond trees. Tree Physiol 32: 450-463.

Embleton TW, Jones WW, Labanauskas CK, Reuther W, 1973. Leaf analysis as diagnostic tool and a guide to fertilization. In: The citrus industry (Reuther W, ed), Vol 2, 2nd ed., Univ. of Calif., Berkeley (USA). pp: 184-210 and Appendix I, pp: 447-495.

Evans JR, Poorter H, 2001. Photosynthetic acclimation of plants to growth irradiance: the relative importance of specific leaf area a nitrogen partitioning in maximizing carbon again. Plant Cell Environ 24(8): 755-767.

Fereres E, Goldhamer DA, 1990. Deciduous fruit and nut trees. In: Irrigation of agricultural crops (Stewart BA & Nielsen DR, Eds.). Am Soc Agron, Madison, WI, USA. Monograph 30, pp: 987-1017.

Flexas J, Bota J, Escalona JM, Sampol B, Medrano H, 2002. Effects of drought on photosynthesis and electron transport rate regulation in grapevine. Plant Cell Environ 22: 39-48.

Flowers TJ, 2004. Improving crop salt tolerance. J Exp Bot 55: 307-319.

García-Sánchez F, Syvertsen JP, 2006. Salinity reduces growth, gas exchange, chlorophyll and nutrient concentrations in diploid sour orange and related allotetraploid somatic hybrids. J Hort Sci Biotechnol 77: 379-386.

García-Sánchez F, Syvertsen JP, Martínez V, Melgar JC, 2002. Salinity tolerance of ´Valencia´ orange tree on rootstocks with contrasting salt tolerance is not improved by moderate shade. J Exp Bot 57: 3697-3706.

García-Tejero I, Jiménez-Bocanegra JA, Martínez G, Romero R, Durán-Zuazo VH, Muriel-Fernández JL, 2010. Positive impact of regulated deficit irrigation on yield and fruit quality in a commercial citrus orchard. Agric Water Manage 97: 614-622.

Gimeno V, Simón I, Nieves M, Martínez V, Cámara-Zapata JM, García AL, García-Sánchez F, 2012. The physiological and nutritional responses to an excess of boron by Verna lemon trees that were grafted on four contrasting rootstocks. Trees 26: 1513-1526.

Gomes MMA, Lagoa AMMA, Medina CL, Machado EC, Machado MA, 2004. Interactions between leaf water potential, stomatal conductance and abscisic acid content of orange trees submitted to drought stress. Braz J Plant Physiol 13(3): 155-161.

González-Altozano P, Castel JR, 2000. Effects of regulated deficit irrigation on 'Clementina de Nules' citrus trees growth, yield and fruit quality. Acta Hortic 537(2): 749-758.

Grattan S, 2013. Evaluation of the impact of boron on citrus orchards in Riverside country. Report submitted to Riverside County Water Task Force, 78 pp.

Hussain S, Luro F, Costantino G, Ollitrault P, Morillon R, 2012. Physiological analysis of salt stress behavior of citrus species and genera: Low chloride accumulation as an indicator of salt tolerance. South Afr J Bot 81: 103-112.

Iglesias D, Cercós M, Colmenero-Flores J, Naranjo M, Ríos G, Carrera E, Ruiz-Rivero O, Lliso I, Morillon R, Tadel F, 2007. Physiology of citrus fruiting. Braz J Plant Physiol 19: 333-362.

Inskeep WP, Bloom PR, 1985. Extinction coefficients of chlorophyll a and b in N, N-dimethylformamide and 80% acetone. Plant Physiol 60: 606-608.

Jifon JL, Syvertsen JP, 2003. Moderate shade can increase net gas exchange and reduce photoinhibition in citrus leaves. Tree Physiol 23: 119-127.

Labanauskas CK, Bitters WP, 1974. The Influence of rootstocks and interstocks on nutrient concentration in Valencia oranges leaves. Amer Soc Hort Sci 99(I): 32-33.

Levine AD, Asano T, 2004. Recovering sustainable water from wastewater. Environ Sci Technol 38(11): 201A-208A.

Levy Y, Syvertsen JP, 2004. Irrigation water quality and salinity effects in citrus trees. Hortic Rev 30: 37-82.

Lloyd J, Syvertsen JP, Kriedemann PE, 1987. Salinity effects on leaf water relations and gas exchange of ´Valencia´ orange, *Citrus sinensis* (L.) Osbeck, on rootstocks with different salt exclusion characteristics. Aust J Plant Physiol 14: 605-617.

Maas EV, 1993. Salinity and citriculture. Tree Physiol 12: 195-216.

Maas EV, Grattan SR, 1999. Crop yields as affected by salinity. In: Agricultural drainage (Skaggs RW & van Schilfgaarde J, eds). Agron Monog No. 38, ASA, CSSA, SSA, Madison, WI, USA. pp: 55-108.

MARM, 2009. Estudio de la cadena de valor y formación de precios del sector cítricos. Observatorio de precios de los alimentos. Campaña 2009-2010. Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid, Spain.

Melgar JC, 2008. Leaf gas exchange, water relations, nutrient content and growth in citrus and olive seedlings under salinity. Biol Plant 52: 385-390.

Mirás-Ávalos JM, Egea G, Nicolás E, 2011. QualiTree, a virtual fruit tree to study the management of fruit quality. II. Parameterization for peach, analysis of growth-related processes and agronomic scenarios. Trees Struc Funct 25: 785-799.

Montoliu A, López-Climent MF, Arbona V, Pérez-Clemente RM, Gómez-Cadenas A, 2009. A novel in vitro tissue culture approach to study salt stress responses in citrus. Plant Growth Regul 59: 179-187.

Mouhaya W, Allario T, Brumos J, Andres F, Froelicher Y, Luro L, 2010. Sensitivity to high salinity in tetraploid citrus seedlings increases with water availability and correlates with expression of candidate genes. Funct Plant Biol 37: 674-685.

Mounzer O, Pedrero F, Nortes PA, Bayona JM, Nicolás E, Alarcón JJ, 2013. Transient soil salinity under the combined effect of reclaimed water and regulated deficit drip irrigation of Mandarin trees. Agric Water Manage 120: 23-29.

Murkute AA, Sharma S, Sing SK, 2005. Citrus in terms of soil and water salinity: a review. J Sci Ind Res 64: 393-402.

Navarro JM, García-Olmos B, Andujar S, Rodríguez-Morán M, Moreno M, Porras I, 2011. Effects of calcium on growth and nutritional state of citrus seedlings under NaCl stress. Acta Hort (ISHS) 922: 55-60.

Papadakis IE, Dimassi KN, Bosabalidis AM, Therios IN, Patakas A, Giannakoula A, 2004a. Boron toxicity in 'Clementine' mandarin plants grafted on two rootstocks. Plant Sci 166: 539-547.

Papadakis IE, Dimassi KN, Bosabalidis AM, Therios IN, Giannakoula A, 2004b. Effects of B excess on some physiological and anatomical parameters of 'Navelina' orange plants grafted on two rootstocks. Environ Exp Bot 51: 247-257.

Paranychianakis NV, Aggelides S, Angelakis AN, 2004. Influence of rootstock, irrigation level and recycled water on growth and yield of Soultamina grapevines. Agric Water Manage 69: 13-27.

Pedrero F, Alarcón JJ, Nicolás E, Mounzer O, 2013. Influence of saline irrigation reclaimed water on young grapefruits. Desalt Water Treat 51: 10-12.

Pereira F, Alves LV, Gil LF, 2010. Removal of Zn^{2+} from aqueous single metal solutions and electroplating wastewater with wood sawdust and sugarcane bagasse modified with EDTA dianhydride (EDTAD). J Hazard Water 176(1-3): 856-863.

Pérez-Pérez JG, Romero P, Navarro JM, Botia P, 2008. Response of sweet orange cv 'Lane late' to deficit irrigation in two rootstocks. I: water relations, leaf gas exchange and vegetative growth. Irrig Sci 26: 415-425.

Reboll V, Cerezo M, Riog A, Flors V, Lapena L, García-Agustin P, 2000. Influence of wastewater vs groundwater on young citrus trees. J Sci Food Agric 80: 1441-1446.

Reyes-Santamaría MI, Villegas-Monter A, Colinas-León MT, Calderón-Zavala G, 2000. Leaf specific weigth, and protein and chorophyll content in orange and tangerine leaves. Agrociencia 34(1): 49-55.

Syvertsen JP, Melgar JC, 2010. Salinity tolerance and leaf water use efficiency in citrus. J Amer Soc Hort Sci 135(1): 33-39.

Walker RR, 1986. Sodium exclusion and potassium-sodium selectivity in salt treated trifoliate orange (*Poncirus trifoliate*) and Cleopatra mandarin (*Citrus reticulate*) plants. Aust J Plant Physiol 13: 293-303.

Wu C, Niu Z, Tang Q, Huang W, 2008. Estimating chlorophyll content from hyperspectral vegetation indices: Modeling and validation. Agric For Meteorol 148: 1230-1241.

Yasumura Y, Hikosaka K, Hirose T, 2006. Seasonal changes in photosynthesis, nitrogen content and nitrogen partitioning in *Lindera umbellata* leaves grown in high or low irradiance. Tree Physiol 26: 1315-1323.

Yermiyahu U, Ben-Gal A, Keren R, Reid RJ, 2008. Combined effect of salinity and excess boron on plant growth and yield. Plant Soil 304: 73-87.

Zarco-Tejada PJ, Miller JR, Morales A, Berjón A, Agüera J, 2004. Hyperspectral indices and model simulation for chlorophyll estimation in open-canopy tree crops. Remote Sens Environ 90(4): 463-476.