Evaluación de la eficiencia del uso del nitrógeno en pomelos regados con agua regenerada salina mediante la medida de la abundancia natural del isótopo estable ^{15}N

^{15}N Stable isotope natural abundance for assessing nitrogen use efficiency using saline reclaimed water in grapefruit

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Resumen

El objetivo de este trabajo fue evaluar los efectos a medio-largo plazo del riego con agua regenerada salina (AR) en la ecofisiología de pomelos mediante la medida de la abundancia natural del isótopo estable ^{15}N. Los resultados revelaron que el riego con AR disminuyó la conductancia estomática y la eficiencia del uso del nitrógeno (EUN) e incrementó los valores de $\delta^{15}N$ y de pH del suelo. Debido a ello, se concluye que i) el exceso de nitratos procedente del AR fue perdido en el ecosistema por lixiviación, desnitrificación, etc., dando lugar al enriquecimiento del medio en $\delta^{15}N$ y, por consiguiente, de la planta; ii) el aumento del pH del suelo provocó una disminución de "NH_3 agotado en ^{15}N" debido a la volatilizacion de NH_4^+; (iii) el $\delta^{15}N$ y la EUN de los árboles regados con AR se correlacionaron significativamente con su conductancia estomática. El estudio muestra el papel clave que la salinidad procedente del AR juega en la asimilación de N por parte de la planta y, consecuentemente, en la discriminación isotópica del nitrógeno.

Abstract

We reported the results of an experiment using natural abundance (δ) of ^{15}N aimed at evaluating the medium to long-term effects of irrigation with saline reclaimed water (RW) on the ecophysiology of grapefruit trees. This study showed that the use of RW decreased stomatal conductance and photosynthetic nitrogen use efficiency (NUE) and increased leaf $\delta^{15}N$ value and soil pH. Therefore, we concluded that (i) excess of nitrates provided by RW were lost in the ecosystem through leaching, denitrification, etc., enriching the medium with $\delta^{15}N$ and increasing $\delta^{15}N$ values in plants; (ii) enrichment in $\delta^{15}N$ of soil mineral N because of loss of "^{15}N depleted NH_3" through volatilization from aqueous NH_4^+, because of a high pH; (iii) $\delta^{15}N$ and PNUE for RW treatment was strongly influenced by stomatal conductance. The results of this study highlight the key role that salt content from RW can play in N uptake by plants and, hence, isotopic discrimination of leaf N.

Keywords: enrichment of $\delta^{15}N$; gas exchange; nitrogen use efficiency; saline reclaimed water, stable isotope.

Palabras claves: agua depurada salina; eficiencia del uso del nitrógeno; enriquecimiento de $\delta^{15}N$; intercambio gaseoso; isótopo estable.

Introduction

Increasing agricultural productivity of *Citrus* in a sustainable way, conserving water and preventing soil pollution by nitrates, are currently one of the main challenges in agricultural research at the ecosystem level. In semi-arid agronomic regions, reclaimed water (RW) is beginning to be integrated into water resources management because it is generally considered beneficial for the crop, as a result of its macronutrients (NKP), and in making important savings in fertilizers. However, RW requires careful management of

N to obtain an optimal level of photosynthetic N use efficiency, NUE. It is known that the assimilation of NO_3^-, the predominant form of N available in an aerobic environment, is critical if plants are to grow in saline conditions (Aslam et al., 1984). In this sense, Murcia uses 100 hm^3 of RW per year and 93% of this water has an electrical conductivity (EC) above 2 $dS \cdot m^{-1}$ and 37% has EC values above 3 $dS \cdot m^{-1}$ (ESAMUR, 2013). Studies have shown that gas exchange of *Citrus* is strongly affected by chloride and sodium (Grattan et al., 2013). In addition, it has been documented that salinization inhibits net NO_3^- uptake in *Citrus* seedlings (Camañes et al., 2009) but in woody crops has not been studied. Therefore, relatively little is known about the effects of RW irrigation on NUE and, hence, of salinity stress on N cycling in agroecosystems. In this regard, stable isotope methods have emerged as one of the more powerful tools for advancing understanding of relationships between plants and their environment. Leaf nitrogen isotope enrichment, $\delta^{15}N$, is determined by the isotope ratio of the external N source and physiological mechanisms within the plant, as $^{15}N/^{14}N$ fractionations during N assimilation, N transport within plants and N loss from the plant (Evans, 2001). Our experiment is the first to evaluate the sustainability after five years of use of saline RW in grapefruit trees crop under field conditions by measuring leaf gas exchange, characterization of leaf structural traits and isotopic measurements in order to elucidate the relationships between NUE, salinity and ^{15}N natural abundance.

Material and Methods

The experiment was conducted at a commercial *Citrus* orchard, located in the northeast of the Region of Murcia, 7 km north of Molina de Segura (38º07'18”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 x 4 metres. The irrigation head was equiped and supplied with two water sources. The first was pumped from the Tagus-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), characterized by generating a highly saline effluent and higher nutrient levels. The experimental design of each irrigation treatment was 4 standard experimental plots and distributed following a completely randomized design. Each replica was made up of 12 trees, organized in 3 adjacent rows. The middle row were used for measurements and the rest were guard trees. Parameters at leaf level gas exchange (net photosynthesis, A, and stomatal conductance, g_s) were determined with a portable photosynthesis system (LI-6400/40 Li-Cor, Lincoln, NE, USA) immediately before the leaves were cut in order to analyze their isotopic content. Nitrogen total content ($g \cdot 100g^{-1}$) was measured too (Flash EA 112 Series, England and Leco TruSpec, Saint Joseph, USA) and value relative to the total leaf area (N_{area}, $g_N \cdot m^{-2}$) was reported. NUE were calculated as net photosynthesis per area-based leaf nitrogen content (A/N_{area}). Leaf $\delta^{15}N$ analysis was conducted at the Stable Isotope Facility of University of California (Davis, EE.UU) using a continuous flow isotope ratio mass spectrometer (CF-IRMS, Europa Scientific, Crewe, UK). Soil pH was measured from soil saturation extraction. Grapefruit leaves from ten selected trees per treatment were collected for nitrogen and stable isotope determinations. Previously, instantaneous gas exchange parameters in these same leaves was measured.

Statistical analysis was performed as a weighted analysis of variance (ANOVA; statistical software IBM SPSS Statistics v. 21 for Windows. Chicago, USA). The significance of R values from linear regressions equations was indicated as Pearson correlation coefficients.

Results and Discussion

The data presented here were collected on day of year (DOY) 234 in 2012.

On the one hand, irrigation with RW caused a significantly (i) decrease in stomatal conductance and in NUE; (ii) increase in the presence of the heavy isotope ^{15}N and in the soil pH (Table 1). This trend was also observed in DOY 145 and DOY 345 (data not shown). Therefore, taking into account the results shown in Romero-Trigueros et al. (2014a) about this same experiment, a standard model was established: enrichment of $\delta^{15}N$ isotope led to decreases in NUE on RW treatment.

Two possible complementary explanations there were: i) Excess of nitrates provided by RW were lost in the ecosystem through leaching, denitrification, etc., enriching the medium with $\delta^{15}N$ and increasing $\delta^{15}N$ value in plants, as it was explained more widely in Romero-Trigueros et al. (2014a). Therefore, a higher NUE could be achieved with low N applications rates if the crop is irrigated with RW; (ii) Increasing the soil pH resulted in a enrichment in $\delta^{15}N$ of soil mineral N because of loss of "^{15}N depleted NH_3" through volatilization from aqueous NH_4^+, according to Tisdale et al., 1993. Moreover, this can be combined with decrease in NO_3^- assimilation because of salinity (Khelil et al., 2005).

On the other hand, gas exchange provides a snapshot measure of plant physiological activity, as it is strongly affected by the prevailing environmental conditions at the time of measurement (Querejeta et al., 2008). By contrast, the isotopic approach provides a time-integrated measure over the entire period of formation of plant tissues (Dawson et al., 2002) although also could be influenced by environmental and physiological conditions at the moment when leaf was collected. To delve into this aspect, we observed the distribution of each measure used to calculate the average value shown in Table 1. Two different patterns were observed for each water quality: 1) TW treatment showed a negative correlation (P<0.001) between leaf $\delta^{15}N$ and NUE, as expected, and a positive relationship between leaf nitrogen total content in the dry matter and leaf $\delta^{15}N$ content (Figure 1A,B). Furthermore, there were no differences between treatments in leaf area (data not shown), hence, as leaf nitrogen total content decreased (and also ^{15}N), the NUE (A/N_{area}) increased. Therefore, plants was more efficient: not reduce its photosynthetic rate when the leaf total nitrogen was less. 2) On RW treatment $\delta^{15}N$ became positively correlated with NUE (Figure 1A). This did not mean that the data shown here contradict the standard model (in general terms: the greater isotopic fractionation in the system, the lower NUE). A possible hypothesis for this distribution of the data is discussed below.

There was no correlation between the stable isotope ^{15}N and total N in leaf (Figure 1B), unlike TW treatment. That is to say, it could not be affirmed that increases in the isotope $\delta^{15}N$ were due to leaf N. Hence, the only way to explain this behavior is by influencing gas exchange at leaf level. Firstly, trees of both treatments have strong correlation between A and g_s ($A=73.301 \cdot g_s+1.301$; $r^2=0.84$***, P<0.001) according to Romero-Trigueros et al. (2014b) which indicated than A was strongly influenced by g_s.

Improving efficiency (A/N_{area}) with increasing ^{15}N abundance (Figure 1A) was mainly due to increased levels of gas exchange, since increased leaf ^{15}N isotope did not lead to the increase in leaf N total content (Figure 1B). This was verified by finding a significant positive correlation between stomatal conductance with isotopic discrimination (Figure 2A) and with NUE (Figure 2B) for RW treatment. The trees irrigated with RW were subjected to salt stress which resulted in lower gas exchange, stomatal conductance and photosynthesis rates, compared to TW treatment, despite having more N available in the medium. Furthermore, the distribution of data within the RW treatment gave us information about the origin of small fluctuations in NUE values: they were not due to the increase/decrease in leaf N total content, but were mainly caused by the increase/decrease of g_s, and therefore the A. It was observed that the higher the stomatal opening, the more enriched the leaf tissue in the heavy isotope ^{15}N. This was probably because of a higher light isotope ^{14}N volatilization (Groenigen et al., 2002). Because of this, NUE and ^{15}N were positively correlated.

Summarizing, the improvement in NUE for RW treatment was determined by the stomatal conductance; hence, the $\delta^{15}N$ was associated with partial closing of stomata due to salinity from RW. However, for treatment irrigated with TW the highest NUE rate was due to lower leaf N total content.

Conclusions

It has been demonstrated the usefulness of isotopic discrimination measure, in accordance with gas exchange measurement for assessing levels of leaf NUE in grapefruit crops and predicting crop sustainability in the medium to long term when using water sources of different quality. The results showed that (i) excess of nitrates provided by the RW were lost in the ecosystem through leaching, denitrification, etc., enriching the medium with $\delta^{15}N$ and increasing $\delta^{15}N$ value in plants (ii) enrichment of "^{15}N depleted NH_3" in soil, because of a high pH, combined with a relative increase of NH_4^+ uptake because nitrate uptake by the roots of plants was probably decreased by salinity; (iii) $\delta^{15}N$ and the NUE for RW treatment was strongly influenced by stomatal conductance. Future research must also be directed at demonstrating that the processes of NO3- assimilation field trials in *Citrus* are affected by salinity. Thus, the plant opts to assimilate N in other forms, such as ammonia, urea, etc. This will influence the type of fertilization in *Citrus* irrigated with saline water.

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Table 1. *Physiological traits (net CO_2 assimilation rate, (A), stomatal conductance (g_s), photosynthetic nitrogen use efficiency (NUE), ^{15}N isotope abundance and soil pH. *Indicate significant differences at $P \leq 0.05$ by ANOVA. Average values and standard error of 10 individual measurements.*

Treatment	A ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	g_s ($mol \cdot m^{-2} \cdot s^{-1}$)	NUE ($\mu mol \cdot g_N^{-1} \cdot s^{-1}$)	$\delta^{15}N$ (‰)	Soil pH
TW	11.491±0.676	0.124±0.005	3.150±0.225	1.184±0.133	7.7±0.100
RW	10.272±0.669	0.103±0.006*	2.616±0.176*	2.465±0.106*	8.4±0.050*

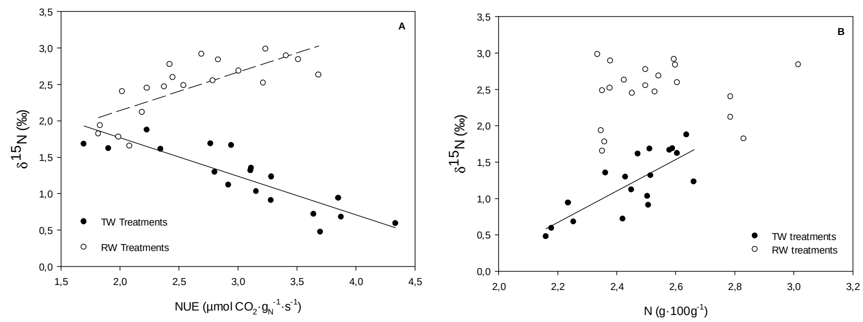

Figure 1. *Relationship obtained between leaf $\delta^{15}N$ value (‰) with (A) NUE, (B) N for both water qualities. Linear regressions for (A): $\delta^{15}N=-0.528 \cdot NUE+2.822$ ($r^2=0.75$***) ($P<0.001$) for TW treatment and $\delta^{15}N=1.086 \cdot NUE+0.527$ ($r^2=0.57$**) ($P<0.01$) for RW treatment. Linear regressions for (B): $\delta^{15}N =2.163 \cdot N-4.086$ ($r^2 = 0.61$**) ($P<0.01$) for TW treatment.*

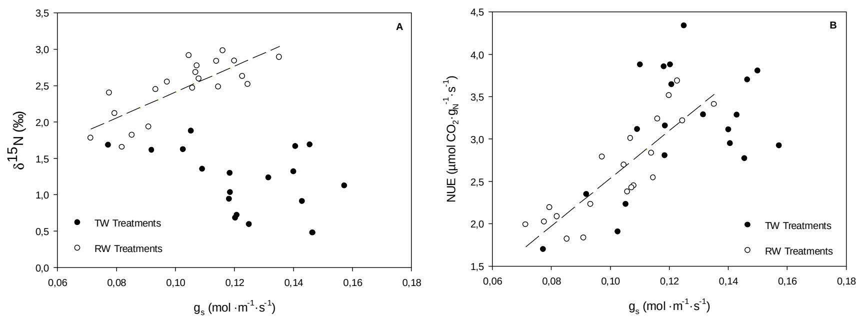

Figure 2. *Relationship obtained between g_s with (A) leaf $\delta^{15}N$ value and (B) NUE for both water qualities. Linear regressions for (A): $\delta^{15}N=13.452 \cdot g_s+1.081$ ($r^2=0.50$**) ($P<0.02$) for RW treatment. Linear regressions for (B): NUENUE=$28.169 \cdot g_s-0.281$ ($r^2=0.74$***) ($P<0.001$) for RW treatment.*