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First Version

DETERMINATION OF ^{15}N STABLE ISOTOPE NATURAL ABUNDANCES FOR ASSESSING THE USE OF SALINE RECLAIMED WATER IN GRAPEFRUIT

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ABSTRACT

We reported the results of an isotopic study aimed at evaluating the medium to long-term effects of different water qualities and deficit irrigation strategies on the ecophysiology of grapefruit in a 7-year-old plantation in SE Spain. For a better understanding of the interaction between nitrogen and salts from reclaimed water, RW, an experiment using natural abundance (δ) of ^{15}N was conducted. This study showed that in grapefruit crop irrigated with RW leaf δ^{15}N value increased. We concluded that: (i) causal links exist between leaf δ^{15}N isotope and salt stress: positive correlation between values of this isotope and leaf salt content was showed; (ii) excess of nitrates provided by the reclaimed irrigation water were lost in the ecosystem through leaching, denitrification, etc., enriching the medium with δ^{15}N and increasing δ^{15}N values in plants. Therefore, 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. Consequently, it has been demonstrated the usefulness of isotopic discrimination measure to predict crop sustainability in the medium to long term when using water sources of different quality combined with deficit irrigation strategies.

Keywords: enrichment of δ^{15}N; gas exchange parameters; isotopic measurement; nitrogen use efficiency; saline reclaimed water.

INTRODUCTION

Increasing agricultural productivity in a sustainable way, conserving water and preventing soil pollution by nitrates, are currently the main challenges in agricultural reserch at the ecosystem level. It is well known that water is the most limiting factor for crop production, especially in areas where agriculture relies heavily on irrigation. Therefore it is necessary to evaluate alternative water sources for our irrigation systems. In this regard, reclaimed water, RW, reuse has been integrated into water resources management; it is considered as an integral part of the environmental pollution control and water management strategy. The volume of treated used for irrigation of crops in Spain RW is increasing due to the progressive implementation of the European Waste Water Directive (91/271/EEC). Moreover, frequent water-shortage periods are even forcing farmers to combine RW with deficit irrigation strategies in order to reduce water use in agriculture, such as regulated-deficit irrigation (RDI), based on the application of lower amounts of irrigation water than those needed by the crop to compensate for evapotranspiration losses (Rana et al., 2005). Murcia, 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 $dS \cdot m^{-1}$ (ESAMUR, 2013). Salinity is among the most significant environmental factors responsible for substantial losses in agricultural production worldwide, and it is one of the serious problems confronting the long-term feasibility of agriculture in production systems irrigated with RW in these semiarid regions (Ravindran et al., 2007). This is a critical problem, especially in Citrus, since they are one of the most globally important horticultural crops considered salt sensitive (Al-Yassin, 2005). Studies have shown that citrus are strongly affected by chloride and sodium (Grattan *et al*., 2013) which can be toxic to the plant. On the other hand, RW irrigation is generally considered beneficial for the crop, as a result of its macronutrients (N, K, P), and in helping to reduce the requirements for commercial fertilizer, making important savings. Therefore, RW can efficiently substitute for potable water for irrigation and simultaneously save nitrates, according to Ferreira da Fonseca et al. (2007), but requires careful management of N to obtain an optimal level of N use efficiency. Relatively little is known about the effects of saline RW irrigation 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. The stable isotope composition of bulk leaf material is mostly determined by the environmental conditions prevailing during leaf formation. Leaf nitrogen isotope enrichment, $\delta^{15}N$, is determined by the isotope ratio of the external N source and physiological mechanisms within the plant (Evans, 2001), as $^{15}N/^{14}N$ fractionations during N assimilation, N transport within plants and N loss from the plant. An improved understanding of major factors controlling leaf $\delta^{15}N$ can advance our knowledge of plant N acquisition and allocation in grapefruit crops. Some studies have evaluated the effect of water and salt stress and/or nitrogen inputs on soil/plant nitrogen isotope composition in field crops (Khelil et al., 2013ab) or pine seedlings (Marañon-Jiménez et al., 2013). However, because of the cost and time required to research on the leaf $\delta^{15}N$ value in woody crops irrigated over extened periods (i.e. multiple years) are scarce.

Our experiment is the first to evaluate the sustainability after five years of combined use of saline RW with RDI in grapefruit trees crop under field conditions by isotopic measurements in order to elucidate the relationships between salinity and ^{15}N natural abundance. The purpose here is to assess the utility of $\delta^{15}N$ as a physiological integrator and indicator of N use efficiency in grapefruit tree crops irrigated with RW combined with regulated deficit, after 5 years. Specifically, the aims are to (1) measure the variations in leaf $\delta^{15}N$ in relation to different water source and irrigation strategies (2) correlate these measurements with phytotoxic ion accumulation; and (3) assess the potential usefulness of $\delta^{15}N$ as an indicator of sustainability in the medium to long term.

MATERIALS AND METHODS

Twenty leaves per tree were sampled through the growth season in 2012, DOY from 72 to 345, 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, EEUU). Nitrogen total content ($g \cdot 100g^{-1}$) was measured too (Flash EA 112 Series, England and Leco TruSpec, Saint Joseph, USA) and this value relative to the total leaf area (N_{area}, $g_N \cdot m^{-2}$) were reported. The concentration of sodium and boron were determined by Inductively Coupled Plasma (ICP- ICAP 6500 DUO Thermo, England). Chloride ion was analyzed by ion chromatography with a Chromatograph Metrohm (Switzerland) after using a standard leaf to distilled-water ratio of 1:2.5 (w:w).

Two grapefruit leaves from ten selected trees per treatment were collected on day of year (DOY) 145, 234 and 345 for nitrogen and stable isotope determinations. Leaf $\delta^{15}N$ analysis was conducted at the University of California (Davis, EE.UU.) Stable Isotope Facility using a continuous flow, isotope ratio mass spectrometer (CF-IRMS, Europa Scientific, Crewe, UK) (http://stableisotopefacility.ucdavis.edu/). The measurements of stable nitrogen isotope ratios is expressed in thousandths (‰) following classical delta notation (δ), where $\delta^{15}N = [(R_{sample}-R_{reference})/R_{reference}] \cdot 1000$, where $R={}^{15}N/{}^{14}N$. $\delta^{15}N$ data are reported using differential notation, relative to an internationally accepted standard. The standard was atmospheric N_2 (‰). Replicate analysis of 10 plant matter samples for treatment and season showed that the precision for $\delta^{15}N$ measurements was $\leq$0.18‰.

Statistical design and analysis

Statistical analysis was performed as a weighted analysis of variance (ANOVA; statistical software IBM SPSS Statistics v. 21 for Windows. Chicago, USA). Tukey's HSD test ($P < 0.05$) was used for mean separation.

EXPERIMENTAL

Experimental conditions and plant material

The experiment was conducted at a commercial citrus orchard, located in the northeast of the Region of Murcia in Campotéjar, 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 was scheduled on the basis of daily evapotranspiration of the crop "ETc" accumulated during the previous week. ETc values were estimated as reference evapotranspiration (ETo), calculated with the Penman-Monteith methodology and a monthly local crop factor (Allen *et al.*, 1998). All trees received the same amount of fertilizers which were applied through the drip irrigation system: 263 kg N, 105 kg P_2O_5 and 155 kg K_2O $ha^{-1}.year^{-1}$. A total of 192 trees were used in this study.

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. Central trees of the middle row were used for l measurements and the rest were guard trees.

Irrigation treatments and water quality

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 water quality was different between each source of irrigation water (Table 1). Reclaimed irrigation water showed the highest values in salinity, with EC –an indicator of the salt content- values close to 3 $dS·m^{-1}$, while transfer irrigation water had an average electrical conductivity near unity (1 $dS·m^{-1}$). The 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 $mg·L^{-1}$). Moreover, in the reclaimed irrigation water there was also a higher concentration of N (NO_3^-), P and K than in the transfer irrigation water. No differences in the concentration of heavy metals were found between the different irrigation water sources (data not shown).

Two irrigation treatments were established for each water source. In the first treatment, irrigation was applied throughout the growing season according to water requirements (100% ETc), full irrigation, FI, treatment. The second treatment was regulated deficit irrigation (RDI) irrigated similarly to the FI treatment, except during the second stage of fruit growth when it received ≈50% the water amount applied to the FI treatment. The amount of water applied to full irrigation treatments was 6066 $m^3 \cdot ha^{-1}$, while the water applied in RDI treatments was 4980 $m^3 \cdot ha^{-1}$.

Table 1. Average value of chemical parameters of irrigation water in each water source: reclaimed water (RW) and Tajo-Segura transfer water (TW).

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

RESULTS AND DISCUSSION

The analysis of leaf $\delta^{15}N$ was measured on DOY 145 (24/05/2012), 234 (22/08/2012) and 345 (11/12/2012). Because of the integrative response of plant isotopic composition to multiple ecophysiological constraints through time, leaf $\delta^{15}N$ can be used to evaluate environmental conditions prevailing during leaf formation and the form (source) of N most used by plants (Querejeta et al., 2008). The growth season was divided into 3 phenological periods: Stage I (DOY 72-145), Stage II (DOY 152-234) and Stage III (DOY 247-345).

Seasonal change in $\delta^{15}N$ values

In the first analysis (DOY 145), the $\delta^{15}N$ values of the treatment TW-FI ranged from 1.81 to 1.96 ‰, with an average of 1.91±0.02 ‰. The $\delta^{15}N$ values of the treatment RW-FI were more enriched, ranging from 3.05 to 3.47 ‰ and an average of 3.23±0.05 ‰. Therefore, trees irrigated with RW resulted in a significant increase of 69.11% in the natural abundance of the isotope ^{15}N. The second analysis (DOY 234-RDI period) showed the same behavior: the treatments irrigated with TW were less enriched, with corresponding values of 1.21±0.14 and 1.16±0.13 ‰, for TW-FI and TW-RDI, respectively, and the treatment irrigated with RW reflected an increase of ^{15}N isotope, 2.68±0.06 and 2.19±0.13 ‰ for RW-FI and RW-RDI, respectively, so in this Stage II of rapid fruit growth the increase between TW-FI and RW-FI was 121.94%. As expected, the isotopic composition of leaf nitrogen of the third analysis (DOY 346) exhibited the same trend: 1.46±0.067, 1.10±0.16, 2.94±0.12 and 2.60±0.18 ‰ for TW-FI, TW-RDI, RW-FI and RW-RDI, respectively, with an increase of the abundance of ^{15}N of 101.23% in RW-FI treatment, compared to TW-FI. However, neither average leaf nitrogen total concentration nor the area-based leaf nitrogen content measured on the same leaves was statistically significant between treatments in any of the three analysis evaluated (Figure 1B and 1C). Nitrate uptake depends on internal factors related to N demand of the plant, rather than on nitrate availability in the soil volume (Cerezo et al., 2007). In this regard, greater N rates from RW did not improve total N plant uptake, in the three dates analyzed, according to recent work by Khelil et al. (2013b). Moreover, area-based leaf nitrogen content, N_{area}, had a tendency to increase with the growth season, the values measured in the first analysis (DOY 145) being significantly lower than the values of the other two analyses, mainly because all treatments reached their maximum leaf area at this stage, in accordance with the results reported by Albrigo et al. (2005).

On the one hand, the most likely explanation for the pattern in leaf ^{15}N abundance of trees irrigated with TW might be that: for plants growing under moderate nitrate-N concentration, there is negligible fractionation between ^{15}N and ^{14}N during the root uptake of nitrate and its incorporation into plant tissues (Shearer and Kohl, 1986). Under natural conditions (i.e., normal substrate nitrate concentrations) plants do not discriminate between ^{15}N and ^{14}N in the uptake and assimilation of nitrate (Mariotti et al. 1982).

On the other hand, the increase of the abundance of leaf ^{15}N in RW treatments could be argued, a *priori,* by the close relation between the availability of inorganic nitrogen in soil and leaf ^{15}N values. A general pattern is that the discrimination increases with external NO_3^- concentration (Evan, 2001). I.e., the plant becomes more ^{15}N enriched as the availability of source NO_3^- increases (Robinson, 2001). In our cause, this may be because RW has a higher concentration of nitrates, as cited in Table 1, and these treatments with a surplus of N inputs over outputs (excess N) might either be stored in soils or lost to the environment by leaching, denitrification, etc. (Bedard-Haughn et al., 2003; Craine et al., 2009; Dawson et al., 2002; Marañon-Jimenez et al., 2013; Steven et al., 2005; Watzka et al., 2006; Xu et al., 2003). All these N transformations in any ecosystem lead to N isotope fractionation and as the lighter ^{14}N isotope reacts more rapidly than the heavier ^{15}N, the residual N-source (soil) becomes enriched in ^{15}N. The close relationship between soil and plant ^{15}N observed by several authors confirms that the higher the concentration of ^{15}N in the plant, the more inefficient the system (Kriszan et al., 2009). Moreover, increased denitrification in the saline soil, possibly related to drainage (Sutherland et al., 1993), would result in enrichment with $\delta^{15}N$ in soil and plant. In summary, the increase in $\delta^{15}N$ value is a powerful indicator of long-term inefficient N usage and past N management in the terrestrial environment (Destain et al., 2010) and higher efficient N could be achieved with low N applications rates if the crop is irrigated with RW containing nitrogen.

Seasonal trends of leaf $\delta^{15}N$ for the four treatments tested are reported in Figure 1A. As shown, the natural abundance of $\delta^{15}N$ increased significantly for all trees in Stage I, regardless of the treatment, coinciding with the highest values of leaf salts content (data not shown). By contrast, the lowest ^{15}N values for the four treatments were registered in Stage II, also coinciding with the lowest average values of leaf salts (data not shown) in the fruit growth. TW-FI treatment reduced $\delta^{15}N$ levels by 36% (from 1.91‰ in Stage I to 1.21‰ in Stage II) and RW-FI treatment declined 19% (from 3.32‰ in Stage I to 2.60‰ in Stage II).

Differential translocation of nitrogen isotopes within the tree is a second alternative explanation for temporal variation in leaf ^{15}N abundance, besides the variation in leaf salts content. Studies of nutrient translocation in trees showed that nitrogen stored in woody tissues was a major source of leaf nitrogen in the spring (Luxmoore et al. 1981, Martínez-Alcántara et al., 2012). At the plant level the $\delta^{15}N$ abundance is affected, not only by the water source, but also by physiological transformation within the plant. Reallocation of N during growth should result in products with lower $\delta^{15}N$ than the original source (Evans, 2001). So during sprouting of new leaves, the plant releases a high amount of N recycled from old leaves and woody tissues to young leaves. We might expect that, compared with the older leaf tissues, the more physiologically active tissues (new leaves which are the destinations of the recycled N) have lower $\delta^{15}N$. This explains why we observed a general decrease in the abundance of leaf $\delta^{15}N$ in all treatments in Stage II: because $\delta^{15}N$ enrichment on DOY 234 was measured in mature leaves which sprouted new in spring. Accordingly, TW-FI treatment showed a higher percentage of decrease in leaf $\delta^{15}N$ value from Stage I to Stage II due to a greater mobilization of reserves, as these trees were not subjected to salt stress.

Effect of salinity on leaf $\delta^{15}N$ values

Leaf $\delta^{15}N$ was positively correlated with leaf salt level. On the one hand, the value of $\delta^{15}N$ increased with increase of Cl^- ion (Figure 2A). Considering the linear regression obtained, the increase in leaf Cl^- content of 0.3 to 0.8 $g \cdot 100g^{-1}$ would lead to an increase of 5.37 times in the natural abundance of $\delta^{15}N$. On the other hand, the presence of sodium in leaf samples also enhanced isotopic fractionation (Figure 2B). Increasing Na content of 0.02 to 0.25 $g \cdot 100g^{-1}$ in leaf tissue would cause the leaf $\delta^{15}N$ value multiply by 3.71, according to linear regression of Figure 2B. The slope that correlated sodium with $\delta^{15}N$ was greater than the slope that correlated chlorine ion with $\delta^{15}N$ (Figure 2A and 2B). Otherwise, RW-FI treatment showed a significant increase respect to RW-RDI during Stage II. This was probably caused by the significant increase of leaf sodium content in RW-FI (Stage II: 0.108 and 0.070 $g \cdot 100g^{-1}$ for RW-FI and RW-RDI, respectively). Our study showed that the salinity pattern found in leaves, and therefore in soil, was strongly present in the $\delta^{15}N$ of the leaf.

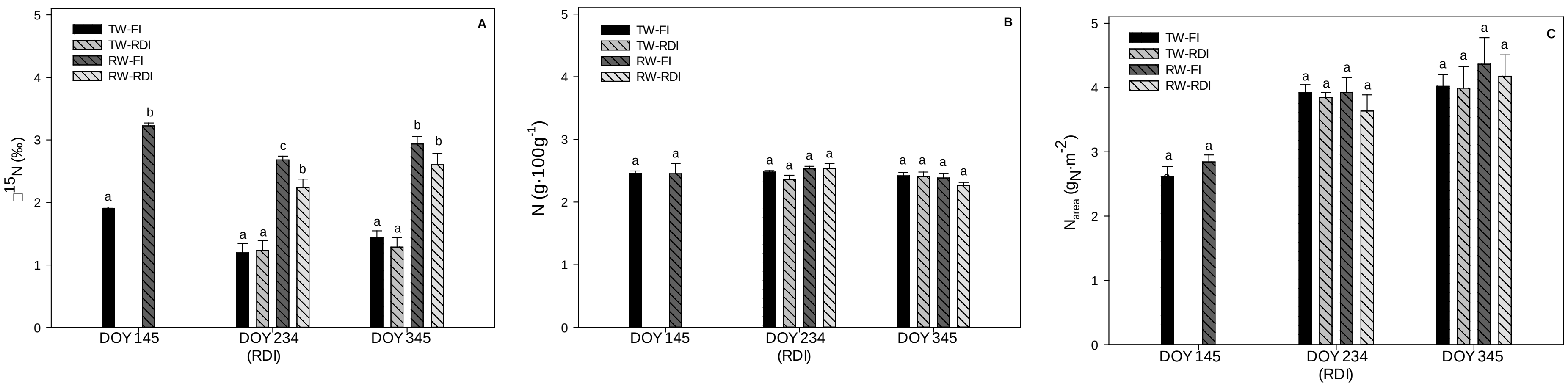

Figure 1. Seasonal change in (A) leaf δ^{15}N values, (B) leaf nitrogen total content and (C) area-based leaf nitrogen content for TW-FI (Transfer water-Full Irrigation), TW-RDI (Transfer water-Regulated Deficit Irrigation), RW-FI (Reclaimed water-Full Irrigation) and RW-RDI (Reclaimed water-Regulated Deficit Irrigation). Each value is the mean of 10 individual measurements. The values of each column followed by different letters are significantly different by Tukey´s Test ($P<0.05$). The error bars denote the standard error of the mean.

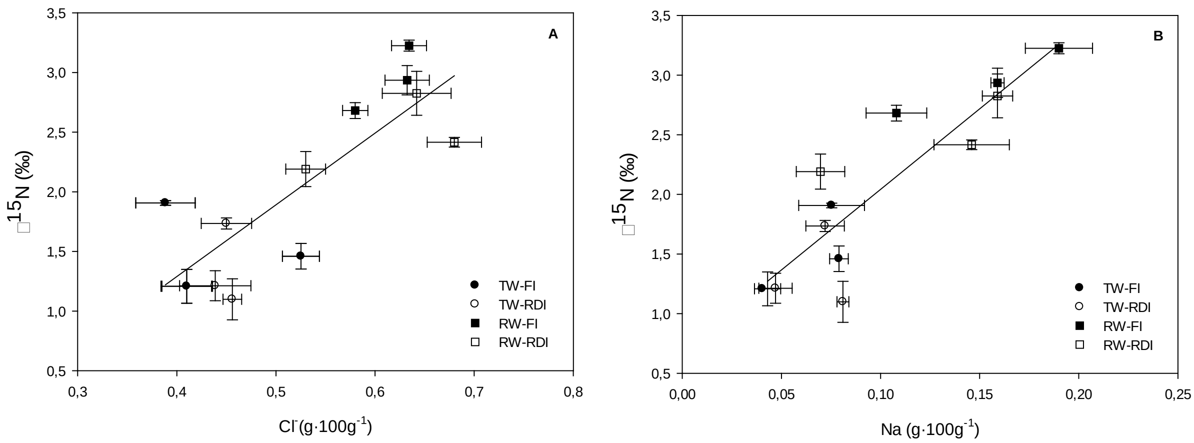

Figure 2. Relationship between leaf $\delta^{15}N$ average values (‰) with (A) average value of chlorine ion content during the previous three months of measurements of isotope for all treatments and fruit growth stages ($g\cdot100g^{-1}$) and (B) average value of sodium content during the previous three months of measurements the isotope for all treatments and stages. Each point is the average of 10 individual measurements for TW treatments and RW treatments and for the three Stages evaluated. Linear regression for (A): $\delta^{15}N=6.001\cdot Cl^{-}-1.115$ ($r^2=0.67^{**}$) ($P<0.01$) and linear regression for (B): $\delta^{15}N=13.509\cdot Na+0.691$ ($r^2=0.79^{***}$) ($P<0.001$).

CONCLUSIONS

To assess the sustainability in the medium to long term in grapefruit crops, which were irrigated with RW, combined with regulated deficit irrigation, both nutritional and structural traits measurements at leaf level and isotopic measurements were used. Our study showed that in a grapefruit crop irrigated with RW the leaf $\delta^{15}N$ value increased, most notably in RW-FI. Accordingly, we hypothesize that (i) the positive correlation between leaf $\delta^{15}N$ content and leaf salt content suggested that causal links exist between $\delta^{15}N$ and salt stress; (ii) excess of nitrates provided by the reclaimed irrigation water were lost in the ecosystem through leaching, denitrification, etc., enriching the medium with $\delta^{15}N$ and increasing $\delta^{15}N$ value in plants. Therefore, the usefulness of isotopic discrimination measure as an indicator of sustainability in the medium to long term in grapefruit irrigated with saline reclaimed water it has been demonstrated.

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