



# $\delta^{15}\text{N}$ as a cultivar selection tool for differentiating alfalfa varieties under biosaline conditions

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## Abstract

**Aims** The potential of “biosaline agriculture” relies on easy-to-apply tools to select plant genotypes that are best adapted to saline conditions. We aimed to determine the effects of salinity-sodicity on the functional response of alfalfa varieties by evaluating instantaneous vs integrated plant-based measurements for the selection of alfalfa cultivars in biosaline agriculture.

**Methods** Functional responses of three alfalfa varieties were evaluated in a greenhouse study under different saline-sodic conditions. Physiological parameters included instantaneous (gas exchange and chlorophyll fluorescence) vs time-integrated (carbon isotope discrimination  $-\Delta^{13}\text{C}$ - and nitrogen isotope composition  $-\delta^{15}\text{N}$ -; specific leaf weight and chlorophyll content) measurements.

**Results** From all assessed physiological traits, only  $\delta^{15}\text{N}$  was able to effectively discriminate among genotypes WL656HQ, PGI908S and SW8421S, and showed the highest correlation with biomass production at all experimental stages. On average, the  $\delta^{15}\text{N}$  increased by a factor of 3.3 as salinity increased from the non-saline control treatment ( $\text{EC}_{\text{iw}} \sim 0.4 \text{ dS m}^{-1}$ ) to the highest salinity level ( $\text{EC}_{\text{iw}} \sim 10.0 \text{ dS m}^{-1}$ ) indicating that biological N fixation was significantly limited by salinity. Specific leaf weight was also significantly correlated with dry matter although to a much lesser extent than was  $\delta^{15}\text{N}$ .

**Conclusions**  $\delta^{15}\text{N}$  was found to be the best proxy for assessing alfalfa varieties for their adaptation potential in saline conditions. This parameter was able to discriminate alfalfa’s functional response within a narrow range of irrigation water salinity.  $\delta^{15}\text{N}$  was also capable of differentiating between alfalfa varieties classified as tolerant to salinity defined at a particular plant growth stage, making this an excellent tool for genotypic selection.

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## Introduction

While only about 1% of the water on Earth is fresh, there is an abundance of brackish water in arid and semiarid lands that could be utilized for irrigation in those regions (Rozema and Flowers 2008). Therefore, agricultural

development in arid regions requires alternative production models that rely on the use of marginal soil and water resources affected by salinity (Díaz et al. 2018; Noori et al. 2018). Biosaline agriculture, a type of farming based on plants capable of growing in saline conditions (soil and/or water), can lead to an economically viable market for salt-tolerant crops while expanding crop production in marginal lands and alleviating pressure on conventional water resources (Díaz and Grattan 2009). One of the main potential benefits of biosaline agriculture is the production of forage for livestock which is usually a scarce commodity in many arid and semi-arid regions (Masters et al. 2007).

Alfalfa (*Medicago sativa* L.), one of the most important high-quality forages in arid and semi-arid regions across the world (Djilianov et al. 2003; Noori et al. 2018), with a global production close to 454 million tons per year (Baha and Bekki 2015), has been proposed as a forage species of choice in biosaline agriculture (Grattan et al. 2004; Ayars et al. 2011; Cornacchione and Suarez 2015). Alfalfa has been traditionally classified as “moderately sensitive” to salinity – reported to tolerate up to  $2 \text{ dS m}^{-1}$  (electrical conductivity in saturated soil-paste extract;  $\text{EC}_e$ ) where productivity suffers a 7.3% yield decline for each unit increase in  $\text{EC}_e$  above this threshold value (Maas and Grattan 1999). However, recent studies have shown that some alfalfa varieties can thrive in saline environments (e.g.  $\text{EC}_e \sim 10 \text{ dS m}^{-1}$ ;  $\text{EC}_{iw} \sim 5 \text{ dS m}^{-1}$ ) without suffering significant reductions in biomass and quality compared to non-saline conditions (Putnam et al. 2017; Díaz et al. 2018). Moreover, alfalfa’s nutritional quality can be increased under saline conditions compared to those grown in non-saline environments (Ferreira et al. 2015). Since not all alfalfa varieties will perform equally under biosaline agriculture, it is essential to understand the physiological and biochemical mechanisms linked to salt stress in order to identify potential alfalfa varieties that could be particularly suited for saline-sodic conditions.

Plants under salt stress develop processes to preserve normal cellular metabolism and prevent damage (Munns and Tester 2008; Acosta-Motos et al. 2017). These adaptations to saline environments can be assessed by instantaneous measurements that provide a snapshot in time of plant stress such as photosynthetic activity (Álvarez et al. 2018), stomatal conductance (Anand et al. 2000), chlorophyll fluorescence (Dąbrowski et al. 2016), or water potential (Gómez-Bellot et al. 2018); and by those that provide an

integrated index of the plant’s stress history such as carbon and nitrogen isotope discrimination (Yousfi et al. 2012; Ariz et al. 2015), chlorophyll accumulation (Yousfi et al. 2009; Badran et al. 2015), or specific leaf weight (Zhang et al. 2012). Although all of them have been widely used as selection criteria in breeding programs for different species, in the case of alfalfa under saline conditions, application of a combination of these methods, particularly isotope discrimination measurements, have been studied to a much lesser extent.

A greenhouse study was designed to assess the effects of salinity-sodicity on the performance of three varieties of alfalfa marketed as moderately tolerant to salinity. Several physiological parameters (instantaneous and integrated measurements) were used to evaluate their potential as ecophysiological tools for the selection of alfalfa cultivars under different salinity conditions. Saline-sodic soils and sodium-chloride dominated saline groundwater are common in arid and semi-arid parts of the Canary Islands as well as in other arid regions of volcanic nature around the world (Tejedor et al. 2007).

Information gained from this study will have direct application to brackish water management strategies in arid regions, including the easternmost Canary Islands. Not only does this arid region lack quality water, but it has an abundance of salt-affected soils and groundwater, as well as a high demand for livestock feed, which is one of the main economic activities in these areas. Specific aims were: *i*) to determine the time-course effects of salinity-sodicity on the functional response of alfalfa varieties; and *ii*) to compare instantaneous vs integrated plant-based measurements for the selection of alfalfa cultivars in biosaline agriculture.

## Material and methods

### Experimental design

From November 2014 to April 2016, a completely random two-way factorial greenhouse experiment (1 soil type \* 3 alfalfa varieties \* 5 irrigation water qualities \* 4 replications;  $n = 60$ ) was developed at the Canarian Institute for Agricultural Research facilities (Tenerife Island, Spain). Soil was packed in containers 40 cm in diameter and 50 cm in height (mesocosm level) with a soil bulk density of approximately  $1.2 \text{ g cm}^{-3}$ . The bottom of each pot contained a 3-cm gravel layer to

facilitate drainage. Air temperature and relative humidity ranged from 9 to 47 °C and from 17 to 94%, respectively, during the study period.

Water quality treatments consisted in five salinity levels (electrical conductivity of the irrigation water,  $EC_{iw} \sim 0.4, 2.5, 5.0, 7.5$  and  $10.0 \text{ dS m}^{-1}$ ). The lowest salinity treatment,  $0.4 \text{ dS m}^{-1}$ , was used as the control, and corresponded to reverse osmosis desalinated seawater. The other treatments simulated the quality of chloride-dominated saline groundwater ( $\sim 2.5$  to  $10 \text{ dS m}^{-1}$ ) frequently found in coastal groundwaters in the Canary Islands (Spain). The boron concentration was set at  $2.5 \text{ mg L}^{-1}$  for all treatments, the maximum concentration found in the groundwaters of these areas. This was done by adding boric acid ( $B(OH)_3$ ) to each treatment water. The treatment saline waters were produced by adding various salts (i.e.  $NaCl, MgSO_4, CaSO_4, Na_2SO_4, KNO_3, NaHCO_3$ ) to desalinated seawater. The different irrigation waters were analysed fortnightly, according to official methods (APHA 1998), to ensure salt concentration targets were met. Table 1 shows the average chemical composition of the waters used for irrigation during the experiment. The salinity obtained with the different treatments ( $EC_{iw}$ ) differed slightly from the target concentrations ( $EC_{iw}$  target). The pH of the desalinated seawater averaged 7.1, while in synthetic treatment waters, it was slightly alkaline and varied between 7.8 and 8.7. This slightly basic pH is common in the chloride-dominated ground waters of coastal parts of the Canaries. The sodium adsorption ratio (SAR) increased with water salinity by a factor of six between the control and the maximum salinity treatment. Levels of  $NO_3^-$  were always under  $1 \text{ mmol}_c \text{ L}^{-1}$ , and the average B concentration was  $2.2 \text{ mg L}^{-1}$  for all treatments (Table 1). Water was applied using an automatic drip irrigation system, with four pressure-compensating drips per container, each with a flow of  $2 \text{ L h}^{-1}$ . The Christiansen Coefficient of Uniformity was determined monthly with values consistently  $>98\%$ . Monitoring of volumetric water content with EC-5 sensors (Decagon Devices) provided us with the required irrigation dosages to maintain the soils close to field capacity throughout the whole study period. All pots received approximately 448 L of water treatment over the study.

A clay loam soil ( $380 \pm 10 \text{ g kg}^{-1}$  clay,  $388 \pm 8 \text{ g kg}^{-1}$  silt,  $232 \pm 10 \text{ g kg}^{-1}$  sand) classified as Calcic Haplosalids from the island of Lanzarote was used in this study (Soil Survey Staff 2014). This soil was extremely saline ( $EC_e \sim 54 \pm 5 \text{ dS}$

$\text{m}^{-1}$ ), sodic ( $ESP \sim 40 \pm 1\%$ ), calcareous ( $\sim 170 \text{ g CaCO}_3 \text{ kg}^{-1}$ ), and with a high B content ( $\sim 7.3 \text{ mg kg}^{-1}$ ). The collected soil also had a very low organic carbon and nitrogen content ( $\sim 6.8$  and  $0.8 \text{ g kg}^{-1}$  respectively) which is common for soils in arid regions. The low nutrient content made necessary the use of basal fertilisation (300 g of fertiliser NPK 21:10:10, 30 g of gypsum and 500 g of goat manure per pot) to prevent nutrient deficiencies which could potentially mask the salinity effects. No pots were inoculated with rhizobia. Table 2 shows the chemical composition of soil ( $\sim 0$ –45 cm pot depth) after 430 days of application of the different treatments. Soil salinity ( $EC_e$ ) decreased significantly compared to the initial soil, regardless of the treatment applied, while the exchangeable sodium percentage (ESP) increased slightly compared to the initial soil in the saline treatments above  $2.5 \text{ dS m}^{-1}$ , largely as a result of the sodium chloride-dominated nature of the irrigation water. Accumulation of B in the soils was greater under the least saline treatments ( $0.4$  and  $2.5 \text{ dS m}^{-1}$ ), possibly as a result of the greater use of water by the plants under these treatments, which translated to lower leaching fractions (Díaz and Grattan 2009). Slight increases were observed in organic C and TN, which may have been due to the addition of manure and nitrogen fertiliser at the beginning of the experiment. Soil physicochemical characterisation was performed in accordance with standard methods (Soil Survey Staff 1996).

Three varieties of alfalfa (*Medicago sativa*; varieties WL656HQ, SW8421S, PGI908S) were selected based on their reported salt tolerance (NAFA 2020). PGI908S has been classified as salt tolerant during germination and forage production, SW8421S as salt-tolerant only during forage production, and WL656HQ as tolerant only during germination (NAFA 2020). In regard to the fall dormancy (FD) rating (scaled from FD 1 - lowest fall growth-, to FD 11 -greatest fall growth-), SW8421S is rated as FD 8 non-dormant variety, while PGI908S and WL656HQ are rated as FD 9 non-dormant varieties (NAFA 2020). All of these varieties are classified as highly resistant to several alfalfa diseases (NAFA 2020), and adapted to areas of warm and hot temperatures.

A total of 60 seeds were planted per pot. To foster germination and crop establishment, irrigation with desalinated seawater was applied in the first stage of the

**Table 1** Chemical characteristics of the desalinated seawater and simulated groundwater treatments throughout the experiment; mean  $\pm$  standard deviation;  $n = 35$ ;  $EC_{iw}$ , electrical conductivity of the irrigation water

Targeted $EC_{iw}$ treatment; $dS\ m^{-1}$	pH	Actual $EC_{iw}$ $dS\ m^{-1}$	SAR ( $mmol\ L^{-1}$ ) <sup>0.5</sup>	$NO_3^-$ $mmol_c\ L^{-1}$	B $mg\ L^{-1}$
0.4	7.1 $\pm$ 0.6	0.4 $\pm$ 0.1	5.3 $\pm$ 0.8	0.0 $\pm$ 0.0	2.2 $\pm$ 0.2
2.5	8.2 $\pm$ 0.2	2.9 $\pm$ 0.2	16.9 $\pm$ 1.7	0.2 $\pm$ 0.0	2.2 $\pm$ 0.2
5.0	8.4 $\pm$ 0.3	5.3 $\pm$ 0.3	23.8 $\pm$ 1.7	0.5 $\pm$ 0.2	2.2 $\pm$ 0.1
7.5	8.4 $\pm$ 0.1	7.8 $\pm$ 0.2	29.2 $\pm$ 1.0	0.7 $\pm$ 0.2	2.2 $\pm$ 0.1
10.0	8.4 $\pm$ 0.1	9.9 $\pm$ 0.3	33.6 $\pm$ 0.7	0.9 $\pm$ 0.2	2.2 $\pm$ 0.1

experiment to reduce the salinity in the upper soil profile. Following this initial period (60 days post seeding), the number of plants were thinned to approximately 28 per pot and the application of the treatments, which lasted for 430 days, was commenced. A total of 15 cuts were performed on the forages during the experimental period to determine the total production of dry matter (DM). Harvest times were established when the control treatment plants reached a flowering status of approximately 10%. The cut height was established at 5–6 cm above the soil surface. The collected plant material was weighed, oven dried at 60 °C for 72 h and weighed again to determine dry matter production expressed in grams per square metre.

#### Stable carbon and nitrogen isotope signatures

Dry plant material collected from each cut was finely crushed to analyse C and N isotope composition. From this dry ground material, three combined samples were obtained by combining the plant material from every 5 consecutive cuts. Thus, the first sample comprised plant material from cuts 1 to 5, corresponding to the first 124 days from the commencement of the treatment

application; the second sample comprised of combined cuts 6 to 10, corresponding to days 124 to 269 from commencement; and the third consisted of combined cuts 11 to 15, corresponding to days 269 to 430.

The stable carbon ( $^{13}C/^{12}C$ ) and nitrogen ( $^{15}N/^{14}N$ ) isotope ratios in alfalfa leaf tissues were measured by mass spectrometry (ANCA-SL Stable Isotope Analysis System, Europa Scientific, Crewe, UK) with a sample precision of  $\pm 0.03 \times 10^{-3}$ . Analyses were conducted at the UC Davis Isotope Lab. Each treatment tissue sample was processed in duplicate. The  $^{13}C/^{12}C$  isotope ratios were expressed in  $\delta$  notation determined as:  $\delta^{13}C = (^{13}C/^{12}C)_{\text{sample}} / (^{13}C/^{12}C)_{\text{standard}} - 1$ , where ‘sample’ refers to treated plant material and ‘standard’ to the Pee Dee Belemnite calcium carbonate international standard. The same  $\delta$  notation was used for the  $^{15}N/^{14}N$  ratio expression ( $\delta^{15}N$ ), using in this case the standard referred to  $N_2$  in air (Yousfi et al. 2012). Carbon isotope discrimination ( $\Delta^{13}C$ ) was calculated as:  $\Delta^{13}C (\text{‰}) = (\delta a - \delta p) / (1 + \delta p)$ , where  $\delta a$  and  $\delta p$  are the isotopic compositions of air and plant material, respectively (Farquhar et al. 1989). The isotopic composition of air was assumed to be  $-8.0 \times 10^{-3}$ .

**Table 2** Soil chemical properties at the end of the experiment (~0–45 cm depth); mean  $\pm$  standard deviation;  $n = 12$ ; different letters in the same column denote significant differences ( $p < 0.05$ ) between treatments according to a one-way ANOVA and post hoc

Targeted $EC_{iw}$ $dS\ m^{-1}$	$EC_e$ $dS\ m^{-1}$	$pH_e$	ESP %	Organic C $g\ kg^{-1}$	TN $g\ kg^{-1}$	$B_{HWSB}$ $mg\ L^{-1}$
0.4	2.1 $\pm$ 1.1 a	8.4 $\pm$ 0.2 b	11.2 $\pm$ 2.6 a	8.7 $\pm$ 0.9 a	1.0 $\pm$ 0.1 b	14.3 $\pm$ 4.0 c
2.5	12.6 $\pm$ 2.1 b	8.0 $\pm$ 0.1 a	38.5 $\pm$ 2.3 b	8.8 $\pm$ 0.7 a	1.0 $\pm$ 0.1 b	12.8 $\pm$ 5.1 bc
5.0	16.8 $\pm$ 1.8 c	8.0 $\pm$ 0.1 a	46.2 $\pm$ 3.3 c	7.9 $\pm$ 0.8 a	1.0 $\pm$ 0.1 b	11.3 $\pm$ 3.5 bc
7.5	19.1 $\pm$ 1.5 d	8.2 $\pm$ 0.2 a	48.5 $\pm$ 0.9 c	7.9 $\pm$ 1.4 a	0.9 $\pm$ 0.1 ab	8.6 $\pm$ 4.0 ab
10.0	19.3 $\pm$ 2.0 d	8.1 $\pm$ 0.1 a	48.6 $\pm$ 2.0 c	7.8 $\pm$ 1.0 a	0.9 $\pm$ 0.1 a	6.4 $\pm$ 0.9 a

Tukey test.  $EC_e$ , electrical conductivity in saturated soil-paste extract;  $pH_e$ , pH in saturated soil-paste extract; ESP, exchangeable sodium percentage;  $B_{HWSB}$ , hot water soluble boron

### Specific leaf weight

The day before each cutting the fully expanded 3rd trifoliate from the top (one leaf per pot) were taken in the early morning for determination of specific leaf weight (SLW). The projected leaf area was determined by mean of a portable Area Meter (AM100, ADC, UK). Samples were immediately transported to the laboratory in a plastic container and placed in an oven at 60 °C for 72 h for subsequent weighing in order to calculate the SLW ( $\text{g cm}^{-2}$ ).

### Chlorophyll content

Chlorophyll content was measured along the study with a chlorophyll meter (CCM 200, ADC, UK), using the same leaflets (three readings from each leaflet) used to measure SLW. Calibration of SPAD readings with leaf chlorophyll concentrations were performed previously. Chlorophyll concentrations were carried out following the method proposed by Lichtenthaler (1987) in pure acetone as a solvent. Relationships between SPAD readings and chlorophyll content per leaf area ( $\mu\text{mol m}^{-2}$ ) were fitted to the formula  $\text{Chl (a + b)} = 2.437 \text{ SPAD value} + 305.13$ .

### Gas exchange measurements

Gas exchange measurements were made using a Portable Photosynthesis System Li-6400 (Li-Cor, Lincoln, NE, USA) under saturated PPFD conditions ( $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) to 25 °C and  $\text{CO}_2$  chamber concentration of  $400 \mu\text{mol mol}^{-1}$ . For each pot, measurements were made on the same leaves used for SLW determination. The parameters measured were: photosynthetic rate ( $A_{\text{sat}}$ ), stomatal conductance ( $g_s$ ), and transpiration rate ( $T_r$ ). The intrinsic water use efficiency ( $\text{WUE}_{\text{int}}$ ) was calculated as the ratio  $A_{\text{sat}} / g_s$ .

### Chlorophyll a fluorescence

Maximum quantum efficiency of PSII (Fv/Fm) was determined using a portable fluorimeter (Handy PEA, Hansatech, UK) after 30 min of dark adaptation. Basal fluorescence (Fo) and the maximum fluorescence (Fm) were determined after saturating red light pulse (650 nm,  $3000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) flashed by an array of six light-emitting diodes on a homogeneous irradiation area. From

these parameters, the maximum photochemical efficiency (Fv/Fm) was calculated as the ratio  $(F_m - F_o)/F_m$  according to Genty et al. (1989). Measurements were carried out at the same time and in the same leaves type used to measure the gas exchange.

### Statistical analysis

Statistical methods were implemented using Statistical Package for the Social Sciences (SPSS; version 25.0). The level of significance for all tests was set to  $p < 0.05$ . Assumptions of normality (Kolmogorov Smirnov test) and homogeneity of variance (Levene test) were met for each analysis. A general linear model (GLM) univariate analysis was used to determine the effect of time, water quality and alfalfa variety on biomass, instantaneous and integrated measurements. Pearson's correlation coefficients were calculated to check for significant relationships between physiological traits and biomass production.

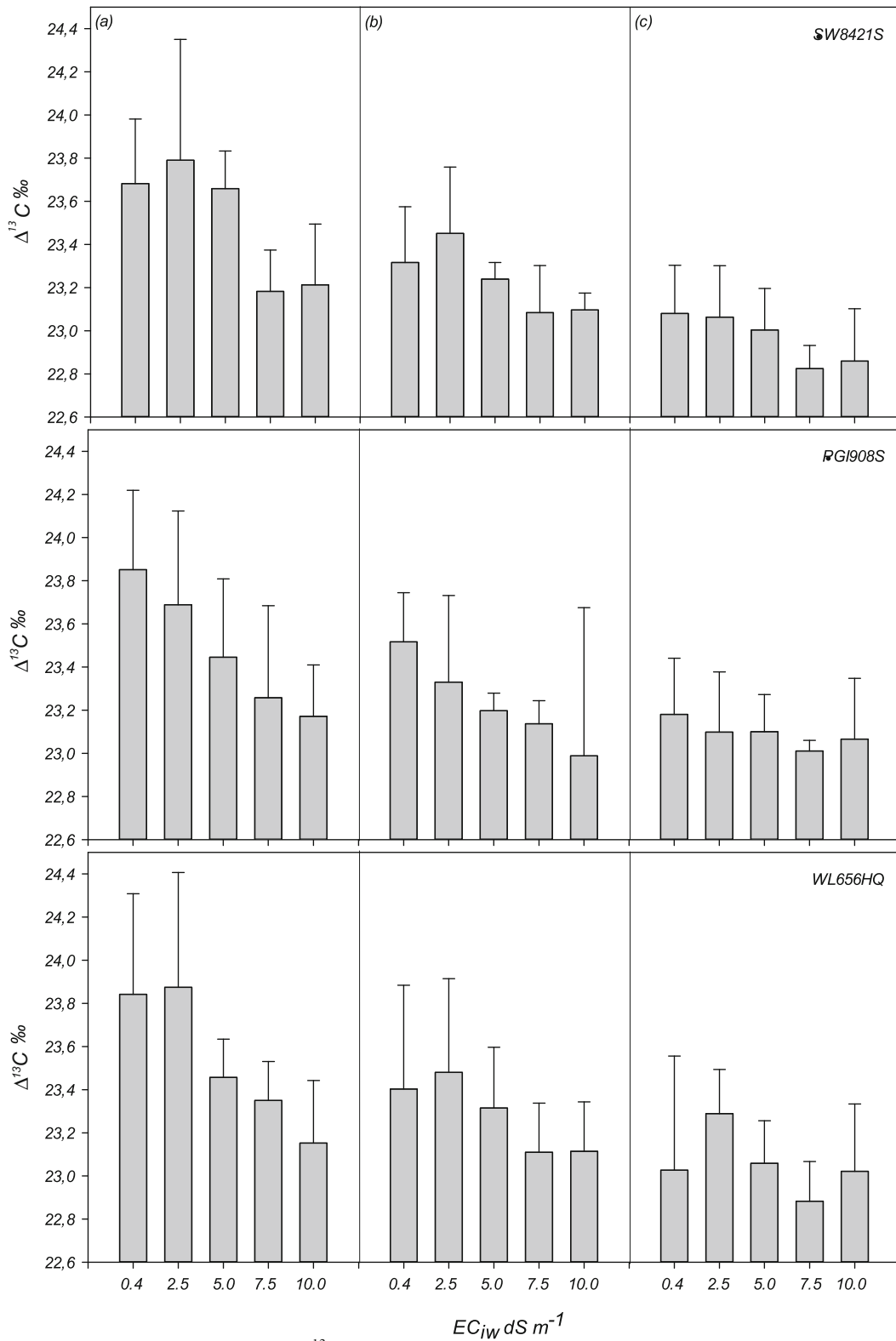
## Results

### Biomass

Cumulative biomass at the end of the experimental period for the three alfalfa cultivars under different salinity levels in the irrigation water are presented in Table 3. Statistical analysis reveals that biomass production was affected by the salinity treatment and variety. The variety with the lowest production was WL656HQ, compared to SW8421S and PGI908S. Increased irrigation water salinity reduced biomass production for all varieties particularly when the salinity treatment exceeded  $5.0 \text{ dS m}^{-1}$ . More detail on biomass effects within this study can be found in companion paper (Díaz et al. 2018).

### Integrated measurements

Tissue  $\Delta^{13}\text{C}$  values for alfalfa varieties at the three growth stages of the experiment are illustrated in Fig. 1. General linear model results showed that  $\Delta^{13}\text{C}$  was primarily influenced by salinity and plant age. No significant differences among alfalfa varieties were observed (Table 4), neither interactions between fixed factors were statistically significant. Mean values were significantly different under treatments 0.4 and 2.5 dS



**Fig. 1** Stable carbon isotope discrimination ( $\Delta^{13}C$ ) in alfalfa varieties under irrigation with contrasting water qualities at different growth stages of the experiment: **a** ~1–124 days after

salinization, **b** ~125–269 days after salinization, **c** ~270–430 days after salinization; bars represent means and standard deviation;  $n = 4$



**Table 3** Cumulative biomass production of three alfalfa cultivars under irrigation with different simulated groundwater quality at the end of the experiment; mean ± standard deviation; n = 4; different

letters in the same row denote significant differences (p < 0.05) between treatments according to a one-way ANOVA and post hoc Tukey test

Alfalfa variety/ EC <sub>iw</sub> (dS m <sup>-1</sup> )	0.4	2.5	5.0	7.5	10.0
	g dry matter m <sup>-2</sup>				
SW8421S	5690 ± 727 a	5503 ± 493 a	4889 ± 248 ab	4018 ± 144 bc	3159 ± 462 c
PGI908S	5912 ± 447 a	5592 ± 469 a	4797 ± 175 b	4087 ± 141 c	3247 ± 564 d
WL656HQ	5845 ± 479 a	5113 ± 503 ab	4338 ± 318 bc	3846 ± 83 cd	3028 ± 625 d

m<sup>-1</sup> with regard to 7.5 and 10.0 dS m<sup>-1</sup> (Table 4). Overall, Δ<sup>13</sup>C decreased as the season progressed, regardless of treatment (Fig. 1). Significant differences were found between the three experiment stages, where Δ<sup>13</sup>C values averaged 23.51, 23.25 and 23.04 ‰ at first, second and third growth periods, respectively. Δ<sup>13</sup>C values were poorly correlated with biomass production and were not significant (r values ranged from 0.047 to 0.395; p > 0.05).

Nitrogen isotope composition (δ<sup>15</sup>N) values in relation to biomass production are shown in Fig. 2. Statistical analysis demonstrated that δ<sup>15</sup>N was significantly affected by salinity level, plant age and alfalfa variety (Table 4), and there was also a significant salinity\*time interaction. In general, δ<sup>15</sup>N increased linearly with increased irrigation water salinity (Fig. 2). All treatments were significantly different except 0.4 and 2.5 dS m<sup>-1</sup>, which displayed similar results (Table 4). δ<sup>15</sup>N values decreased under all salinity treatments through the experimental season, showing mean values of 4.56, 3.19 and 1.26 ‰ for the first, second and third time period, respectively. Results among alfalfa varieties were important, with WL656HQ being significantly

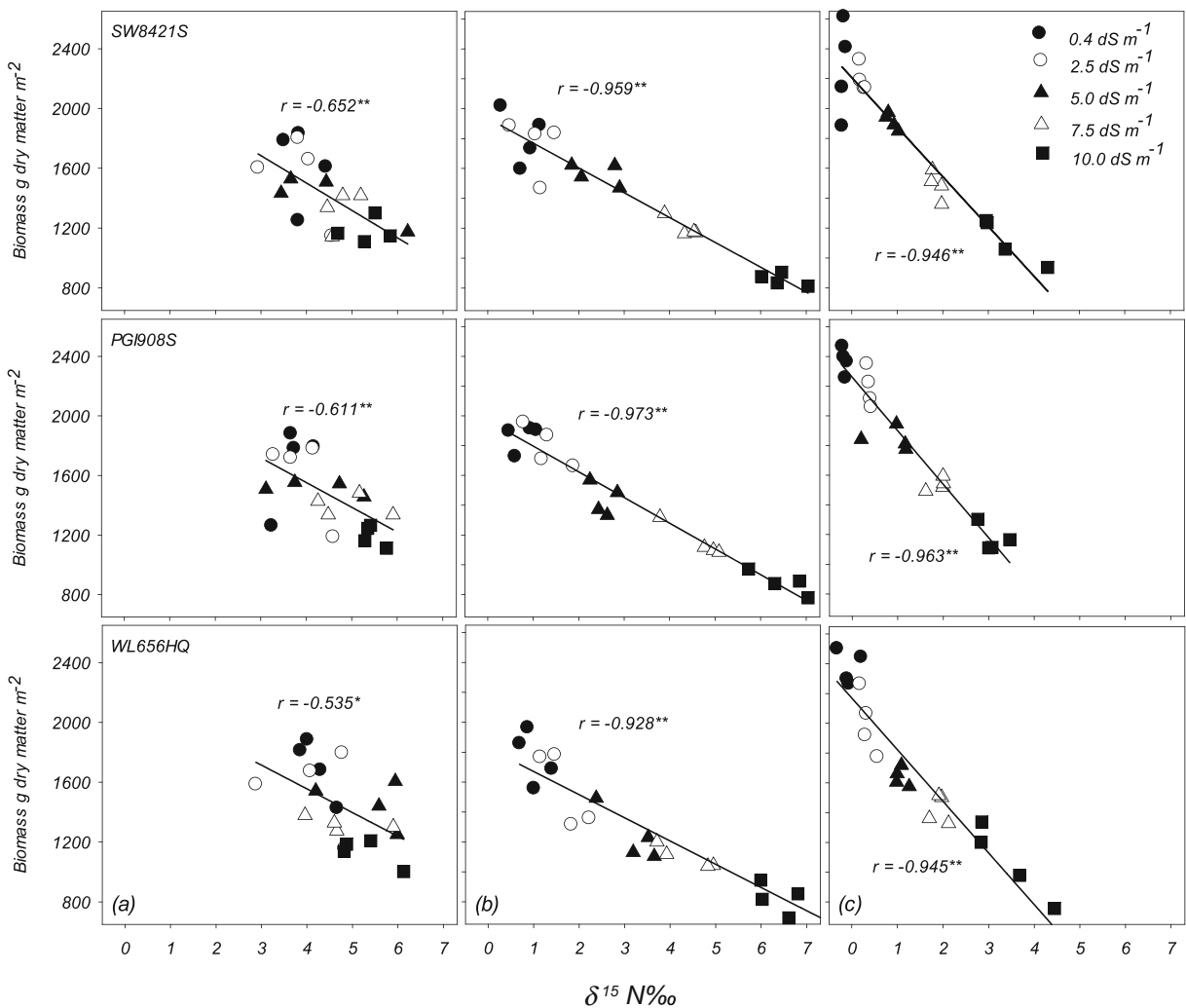
different from PGI908S and SW8421S varieties (mean δ<sup>15</sup>N ~ 3.18, 2.94 and 2.90 ‰, respectively). Notably, δ<sup>15</sup>N was highly negatively correlated with biomass production at all experimental growth stages (r values ranged from -0.535 to -0.973; p < 0.05 and < 0.01 respectively). In addition, the relative effect of salinity on biomass production became more pronounced at later cuttings as reflected by the degree of separation between the control and high saline treatments along the ‘y’ axis (Fig. 2).

Specific leaf weight (SLW) from the three alfalfa varieties at different experiment stages are presented in Fig. 3. Salinity treatment and time factors had a significant effect on SLW, but no differences were found among varieties (Table 4), or interactions between fixed factors. With regard to salinity levels, plants treated with 0.4 dS m<sup>-1</sup> were significantly different from those treated with 5.0, 7.5 and 10.0 dS m<sup>-1</sup>. However, those plants treated with 2.5 dS m<sup>-1</sup> only differed from those treated with the highest salinity, 10.0 dS m<sup>-1</sup> (Table 4). An increase in SLW was observed over time with average values of 0.0026, 0.0032 and 0.0034 g cm<sup>-2</sup>, at the first,

**Table 4** Results of the GLM univariate analysis comparing integrated measurements from alfalfa varieties under several irrigation water salinity levels throughout the experiment; WL variety WL656HQ, PG variety PGI908S, SW variety SW8421S; 1st

period ~1–124 days after salinization, 2nd period ~125–269 days after salinization, 3rd period ~270–430 days after salinization; n = 36–60

Parameter	Variety	Time (experiment stage)	Treatment (EC <sub>iw</sub> ; dS m <sup>-1</sup> )
Δ <sup>13</sup> C ‰	ns	1st > 2nd > 3rd (F = 34.042; p = 0.000)	2.5 = 0.4 = 5.0 ≥ 5.0 = 7.5 = 10.0 (F = 11.853; p = 0.000)
δ <sup>15</sup> N ‰	WL > PG = SW (F = 4.647; p = 0.011)	1st > 2nd > 3rd (F = 561.795; p = 0.000)	10.0 > 7.5 > 5.0 > 2.5 = 0.4 (F = 256.654; p = 0.000)
SLW g cm <sup>-2</sup>	ns	3rd > 2nd > 1st (F = 56.354; p = 0.000)	10.0 = 7.5 = 5.0 ≥ 7.5 = 5.0 = 2.5 ≥ 2.5 = 0.4 (F = 9.494; p = 0.000)
Chlorophyll μmol m <sup>-2</sup>	ns	3rd > 2nd > 1st (F = 470.516; p = 0.000)	7.5 = 5.0 > 10.0 > 2.5 > 0.4 (F = 207.180; p = 0.000)



**Fig. 2** Cumulative shoot biomass from alfalfa varieties under irrigation with contrasting water qualities at different growth stages of the experiment in relation to  $\delta^{15}\text{N}$ ; **a** ~1–124 days after

salinization, **b** ~125–269 days after salinization, **c** ~270–430 days after salinization; \* significant at  $p < 0.05$ ; \*\* significant at  $p < 0.01$ ;  $n = 20$

second and third cutting periods, respectively. Significant, negative correlations were exhibited between SWL and biomass production for all three experiment stages with the exception of variety WL656HQ during the first period ( $r = 0.077$ ;  $p > 0.05$ ).

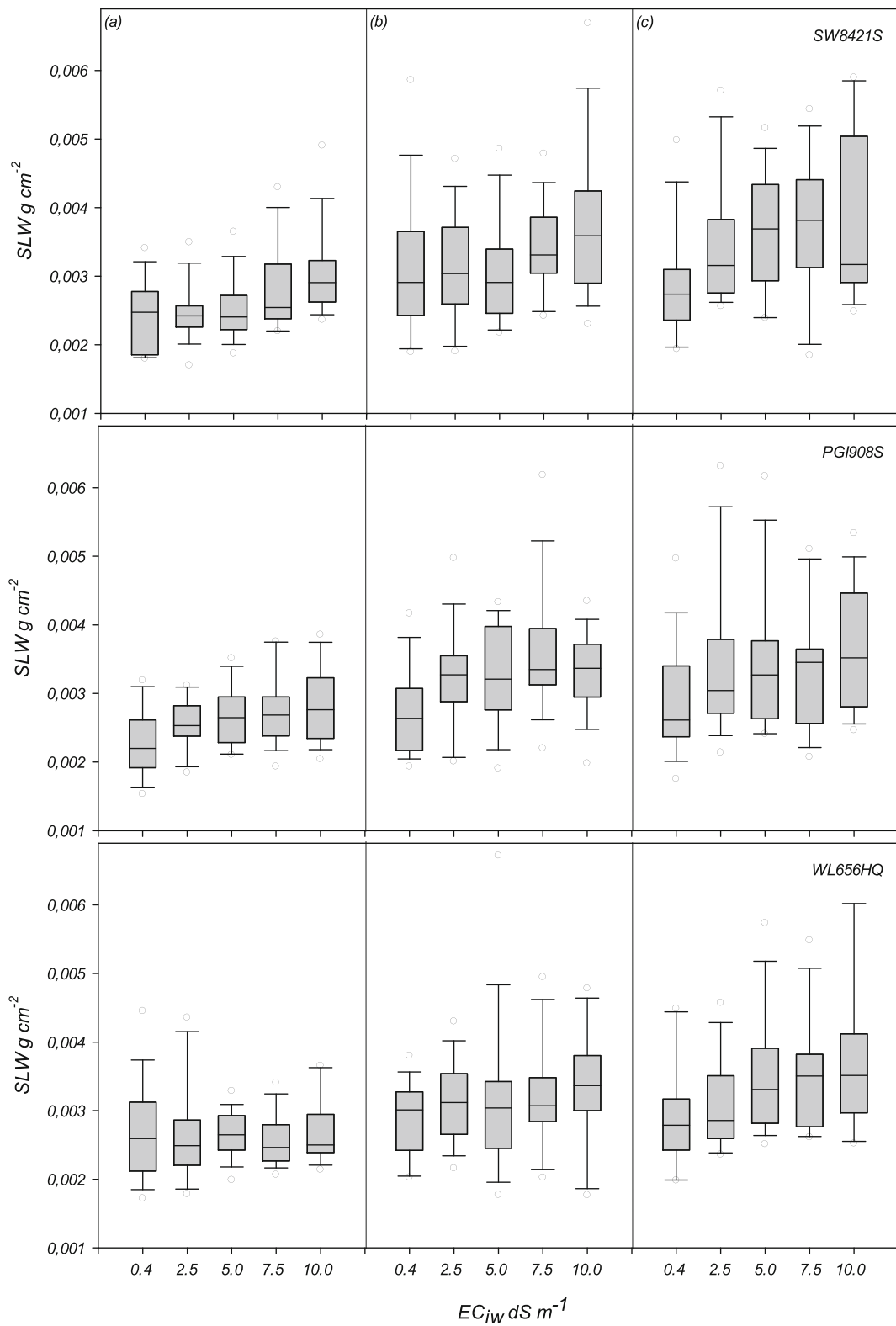
Chlorophyll content showed no differences between alfalfa varieties, but a significant effect of irrigation water salinity and time was observed (Fig. 4; Table 4). Plants treated at all salinity levels exhibited significant differences between them, except those treated with 5.0 and 7.5  $\text{dS m}^{-1}$  which were similar and also presented the highest content ( $\sim 493 \mu\text{mol m}^{-2}$ ). Chlorophyll

increased significantly with time, showing an average of 430, 468 and 505  $\mu\text{mol m}^{-2}$  for the 1st, 2nd and 3rd growth period respectively. Biomass production negatively correlated with chlorophyll content at stage 2nd only for PGI908S variety ( $r = -0.522$ ;  $p = 0.046$ ), and at stage 3rd for PGI908S and WL656HQ varieties ( $r = -0.523$  and  $-0.799$ ;  $p = 0.045$  and  $0.001$ , respectively).

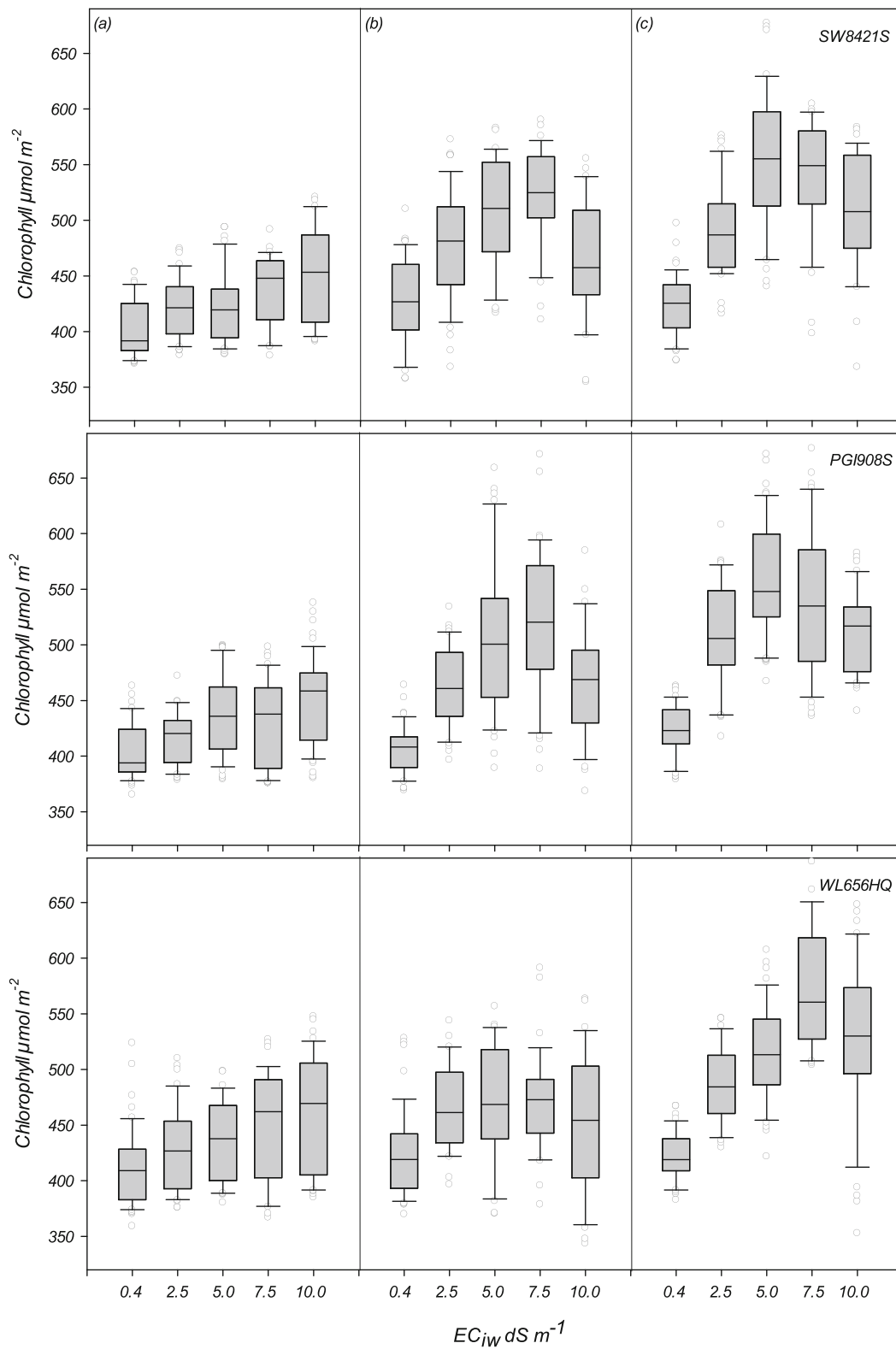
#### Instantaneous measurements

Five different parameters related with photosynthetic activity are reported in Table 5. None of these parameters were significantly different among alfalfa





**Fig. 3** Specific leaf weight (SLW) from alfalfa varieties under irrigation with contrasting water qualities at different growth stages of the experiment: **a** ~ 1–124 days after salinization, **b** ~ 125–269 days after salinization, **c** ~ 270–430 days after salinization;  $n = 20$



**Fig. 4** Chlorophyll content in alfalfa varieties under irrigation with contrasting water qualities at different growth stages of the experiment: **a** ~ 1–124 days after salinization, **b** ~ 125–269 days after salinization, **c** ~ 270–430 days after salinization;  $n = 20$

**Table 5** Effect of alfalfa variety, irrigation water salinity level, and time exposed to salinity on leaf net CO<sub>2</sub> assimilation ( $A_{\text{sat}}$ ), stomatal conductance ( $g_s$ ), transpiration rate ( $T_r$ ), water use efficiency (WUE), and fluorescence (Fv/Fm); mean  $\pm$  standarddeviation;  $n = 180\text{--}300$ ; values followed by different letters were significantly different ( $p < 0.05$ ) by GLM univariate analysis (post hoc Tukey's test)

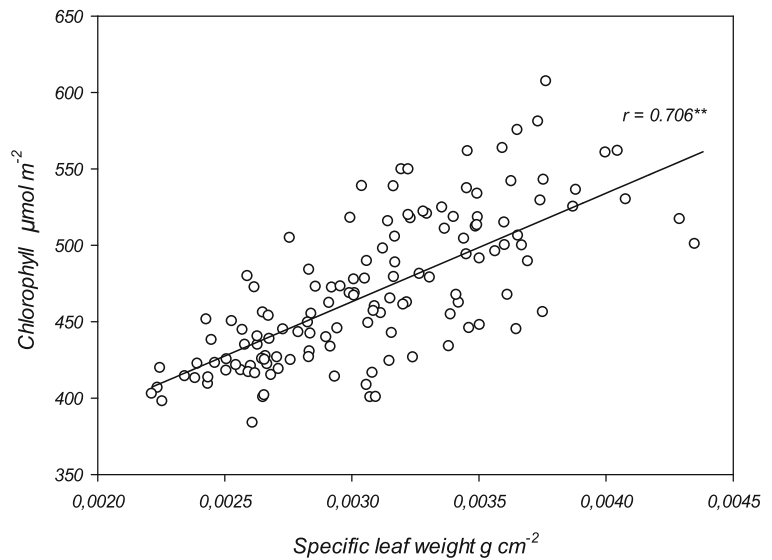
	$A_{\text{sat}}$ $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$	$g_s$ $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$	$T_r$ $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$	WUE <sub>i</sub> $\mu\text{mol CO}_2 \text{ mmol H}_2\text{O}$	Fv/Fm
Variety					
PGI908S	28.4 $\pm$ 8.9 a	0.44 $\pm$ 0.21 a	8.89 $\pm$ 3.44 a	73.4 $\pm$ 28.7 a	0.852 $\pm$ 0.013 a
SW8421S	27.4 $\pm$ 8.8 a	0.47 $\pm$ 0.21 a	9.09 $\pm$ 3.39 a	67.6 $\pm$ 29.8 a	0.847 $\pm$ 0.027 a
WL656HQ	29.1 $\pm$ 9.2 a	0.45 $\pm$ 0.19 a	9.20 $\pm$ 3.52 a	70.9 $\pm$ 25.2 a	0.851 $\pm$ 0.022 a
EC <sub>iw</sub> target					
0.4 dS m <sup>-1</sup>	25.3 $\pm$ 8.8 a	0.37 $\pm$ 0.18 a	7.67 $\pm$ 3.09 a	79.3 $\pm$ 31.7 b	0.849 $\pm$ 0.027 a
2.5 dS m <sup>-1</sup>	30.8 $\pm$ 9.5 b	0.47 $\pm$ 0.21 bc	9.54 $\pm$ 3.71 cd	73.1 $\pm$ 26.1 b	0.850 $\pm$ 0.027 a
5.0 dS m <sup>-1</sup>	30.5 $\pm$ 8.9 b	0.54 $\pm$ 0.19 c	10.52 $\pm$ 3.30 d	61.4 $\pm$ 22.8 a	0.852 $\pm$ 0.011 a
7.5 dS m <sup>-1</sup>	29.0 $\pm$ 8.2 b	0.46 $\pm$ 0.19 b	9.07 $\pm$ 3.27 bc	70.7 $\pm$ 27.3 ab	0.848 $\pm$ 0.024 a
10.0 dS m <sup>-1</sup>	24.8 $\pm$ 8.0 a	0.40 $\pm$ 0.19 ab	7.94 $\pm$ 3.07 ab	72.5 $\pm$ 30.5 b	0.852 $\pm$ 0.011 a
Experiment stage					
1–124 days	23.5 $\pm$ 5.6 a	0.39 $\pm$ 0.13 a	7.89 $\pm$ 2.16 a	66.3 $\pm$ 23.4 a	0.856 $\pm$ 0.013 b
125–269 days	27.6 $\pm$ 8.5 b	0.45 $\pm$ 0.24 b	10.0 $\pm$ 4.05 b	75.3 $\pm$ 32.7 b	0.846 $\pm$ 0.022 a
270–430 days	33.5 $\pm$ 9.3 c	0.53 $\pm$ 0.20 c	9.46 $\pm$ 3.65 b	71.3 $\pm$ 27.6 ab	0.849 $\pm$ 0.026 a

varieties ( $p > 0.05$ ). Similarly, none of the interactions among the three fixed factors (variety \* EC<sub>iw</sub> \* experiment stage) were significant ( $p > 0.05$ ).  $A_{\text{sat}}$ ,  $g_s$ ,  $T_r$  and WUE<sub>int</sub> in plants were primarily affected by irrigation water salinity ( $p < 0.01$ ). Overall the lowest  $A_{\text{sat}}$ ,  $g_s$  and  $T_r$  values were observed in plants treated with the lowest and highest salinity level (i.e. 0.4 and 10.0 dS m<sup>-1</sup>), with the highest values observed at intermediate salinity levels (Table 4). Conversely, plants from the low-salinity control treatment (0.4 dS m<sup>-1</sup>) showed the highest WUE<sub>int</sub>, while those in treatment 5.0 dS m<sup>-1</sup> showed the lowest (Table 5). Maximum quantum yield of PSII (Fv/Fm) was not affected by irrigation water salinity level ( $p > 0.05$ ). All gas exchange parameters were also influenced by experiment stage (plant age) (Table 4). With the exception of Fv/Fm, the rest of assessed parameters showed lower values during the 1st study period, but increased later throughout the experimental period (Table 5). Significantly higher levels of Fv/Fm were observed in the 1st study period. The photosynthetic parameter that exhibited a better correlation with biomass production was WUE, although only significant at 2nd periods for PGI908S variety ( $r = 0.694$ ;  $p = 0.004$ ).

## Discussion

Different functional parameters were analysed in order to understand the physiological basis of salt tolerance in alfalfa using integrated and instantaneous measurements. With regard to **integrated measurements**, stable isotopes have been used as time-integrated indicators of response under stress conditions (Dawson et al. 2002) and provide a valuable quantitative index of the cumulative stress experience of the plant (Poss et al. 2000). Environmental stress-causing factors such as salinity could affect the fractionation of carbon isotope composition in the plant tissue due to its effect on CO<sub>2</sub> fixation and transpiration (Farquhar et al. 1989; Ehleringer et al. 1993), and therefore it has been used as a parameter to see the plant response to different salinity conditions (Yousfi et al. 2009). Theoretically, it can be assumed that the lowest level of salinity (0.4 dS m<sup>-1</sup>) in irrigation water constitutes optimal conditions for alfalfa growth, and an increase of salinity could potentially produce stressful conditions to the plant, thereby reducing stomatal conductance and affecting CO<sub>2</sub> ratios inside/outside the leaf, and leading to a decrease in  $\Delta^{13}\text{C}$  (Farquhar et al. 1989). In our study, a decrease in  $\Delta^{13}\text{C}$  was observed between control and highest salinity

**Fig. 5** Relationship between leaf chlorophyll content and specific leaf weight from the three alfalfa varieties through the experiment;  $n = 140$ ; \*\* significant at  $p < 0.01$



treatments, with a significant decrease from 7.5 dS m<sup>-1</sup> (Fig. 1; Table 4), which is in accordance with results obtained in alfalfa by Isla and Aragüés (2009). However, salinity treatments moved in a narrow range within moderate salinity (2.5 to 10 dS m<sup>-1</sup>) and  $\Delta^{13}\text{C}$  it was not a measurement sensitive enough to distinguish between adjoining salinity levels (e.g. 0.4, 2.5 and 5.0 dS m<sup>-1</sup> or 5.0, 7.5 and 10.0 dS m<sup>-1</sup>).

The poor correlation observed between  $\Delta^{13}\text{C}$  and dry matter has also been reported by others in crops such as wheat (Yousfi et al. 2010, 2012) suggesting biomass production could be mediated through factors affecting N metabolism (Yousfi et al. 2012). Regardless of irrigation water salinity,  $\Delta^{13}\text{C}$  decreased as the experimental season progressed indicating that the overall stress experience was greater when the plants were older. Even the biomass differences between low and high salinity treatments became larger at later times. The  $\Delta^{13}\text{C}$  decrease in saline treatments over time is likely an effect of cumulative soil salinization and specific ion toxicity (i.e., Na; Díaz et al. 2018). But the reduction of  $\Delta^{13}\text{C}$  in plants from the control treatment over time could be related to other factors that affect the water relations of the plant-system. For example, at lowest salinity treatment, sodicity may have impacted soil physical conditions. Poor physical conditions are related to soil waters that are low in salinity and high in SAR (Suarez et al. 2006). At low salinity, transpiration and net CO<sub>2</sub> assimilation were adversely affected. This stress could very well be related to a deterioration of soil structure leading to anoxia and a low flux of soil water to the

roots. Boron toxicity could be another factor to consider. Díaz et al. (2018) reported forage mineral composition data from alfalfa tissue collected from this same experiment and found a significantly higher shoot B concentration in control plants than those from saline treatments, an effect observed by various authors examining different crops (Yermiyahu et al. 2008; Díaz and Grattan 2009). This increased tissue B could affect the  $\Delta^{13}\text{C}$ .

Unlike the effect with  $\Delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  gave a better indication of genotypic differences in the response of alfalfa to salinity, it was able to discriminate between all salinity levels (except between 0.4 and 2.5 dS m<sup>-1</sup>), and was highly correlated with biomass production (Table 4; Fig. 2). Other authors have also found shoot  $\delta^{15}\text{N}$  a better indicator than  $\Delta^{13}\text{C}$  for genotypic differences in the response of wheat to salinity, reporting a strong correlation with biomass (Yousfi et al. 2009). Additionally, Ariz et al. (2015) reported that leaf  $\delta^{15}\text{N}$  was a sensitive integrator of combined environmental stresses such as elevated [CO<sub>2</sub>] and low water availability on N<sub>2</sub>-fixing alfalfa plants. Where nodulated legumes grow in a medium free of mineral N and/or organic N, relying only upon symbiotic N<sub>2</sub> fixation for growth, the isotopic composition of that plant would be expected to be similar to that of atmospheric N<sub>2</sub> (i.e.  $\delta^{15}\text{N} \sim 0\text{‰}$ ; Unkovich et al. 2008). For non N<sub>2</sub>-fixing plants growing in soil containing mineral and/or organic N, its  $\delta^{15}\text{N}$  value should resemble that of the soil N taken up by the plant (usually  $\delta^{15}\text{N} > 0\text{‰}$ ; Unkovich et al. 2008). In this study, our nodulated alfalfa plants, growing in a soil where the source of available N is both from applied

mineral and fixated atmospheric  $N_2$ , the N in the tissue could be a combination of both sources, and therefore its shoot  $\delta^{15}N$  should lie between the values of those two individual N sources. Our results show that low salinity treatments led to a high  $N_2$  fixation in alfalfa plants, overall at the later stages in the experiment. As irrigation water salinity increased the use of soil N and probably a decrease in  $N_2$  fixation took place. Over time, regardless salinity treatment,  $\delta^{15}N$  values decreased likely as a consequence of progressive decline in soil N (addition of N fertilizer and organic matter was made only at the beginning of the experiment). Apart from plant N uptake, other N processes such as volatilization, denitrification, and leaching could contribute to soil N depletion (Garg and Geetanjali 2007). Numerous authors have reported that biological nitrogen fixation in alfalfa crops is affected by salinization (Noori et al. 2018). The symbiotic relationship between *Rhizobiaceae* and legumes causes the development of root nodules where bacteria fix atmospheric nitrogen that the host plant incorporates as organic molecules (Plá and Cobos-Porras 2015). Although rhizobia are usually more resistant to saline conditions than their plant host, nodule formation is highly sensitive to salt stress (Plá and Cobos-Porras 2015). The initial phases of bacterial colonization and infection processes are particularly inhibited, and nodule number, weight and functioning decrease significantly (Bruning and Rozema 2013; Plá and Cobos-Porras 2015). The negative effect of salinity on symbiosis is related to the inhibition of a specific nitrogenase activity mainly by oxidative stress (Delgado et al. 1993). Liu et al. (2011) and Bruning and Rozema (2013) reported that productivity and capacity of nodule formation and nitrogen-fixation of alfalfa could be affected by the saline stress as low as of 50 mM NaCl. According to the high correlation between  $\delta^{15}N$  and dry matter production found in our study, it can be inferred that the effects of salinity on nitrogen metabolism play an important role in alfalfa performance. Although a full understanding of biochemical processes involved in nitrogen isotopic fractionation has not yet been reached, the natural variation in  $\delta^{15}N$  linked to nitrogen metabolism could be potentially useful for identifying genotypic differences under salinity conditions (Yousfi et al. 2012). To our knowledge there are no reports on genotypic relationships between  $\delta^{15}N$  and alfalfa biomass under salinity stress.

SLW levels indicate morphological changes towards enhancement of leaf thickness in response to salt stress.

Increasing in SLW with salinity may reflect thicker cell walls or greater volume into which salts could be sequestered (Negrao et al. 2017). This SLW increase indicates a better performance in terms of the plant's ability to accumulate more dry matter per unit leaf area under salt stress (Veneklaas et al. 2002; Sarabi et al. 2019). Similar results have been reported in *Beta vulgaris* (Taghizadegan et al. 2019), cowpea (Wilson et al. 2006) and soybean (Bai et al. 2019).

Conflicting results have been found with regard to chlorophyll content in alfalfa, with a decrease (Petcu et al. 2007; Farissi et al. 2013; An et al. 2016), an increase (Ashrafi et al. 2014; Sandhu et al. 2017), or unchanged (Ashrafi et al. 2014) content responses to salinity. In the current study, the chlorophyll content was significantly higher in saline-treated plants as compared to those from the control treatment. These results could be in accordance with the morphological changes, particularly leaf thickening, in response to the salt tolerance of these varieties (Pandey et al. 2009; Qiu et al. 2017). Therefore, the expected decrease in plant growth in response to salinity could be offset by concentrating chlorophyll in mesophyll cells (Yousfi et al. 2012). This suggestion is supported by the high correlation observed between chlorophyll content and SLW ( $r = 0.706$ ;  $p = 0.000$ ; Fig. 5). Only a decrease in chlorophyll was observed in plants at the highest salinity treatment in the last periods of the experiment indicating that a pigment photooxidation (Gomes et al. 2011), loss chloroplast membranes (Ceccarelli et al. 2010), slower synthesis and/or faster breakdown or dissociation (Bonales-Alatorre et al. 2013), or damage by reactive oxygen species (Gill and Tuteja 2010), could be induced at this salinity level.

With regard to **instantaneous measurements**, under saline conditions most plant species show lower  $CO_2$  uptake and this decrease in photosynthetic capacity is mainly the result of stomatal and/or non-stomatal limitations (Chaves et al. 2012; Sarabi et al. 2019). Our results indicate that with regard to photosynthetic activity the three alfalfa varieties responded to salt stress in a similar way. An increase in  $A_{sat}$ ,  $g_s$  and  $T_r$  was observed with regard to control treatment at intermediate levels of salinity (2.5 and 5.0  $dS\ m^{-1}$ ) followed by a decline at higher saline conditions (7 and 10  $dS\ m^{-1}$ ). Similar results, at low salinity treatment, were reported by Anand et al. (2000) in alfalfa, attributing it to the increased photosynthate demand by the plant to meet the additional energy expenditure imposed by adjustment to

increased moderate salinity (von Caemmerer and Farquhar 1984). Additionally, in our case, as it was mentioned above, soil structural stability could potentially be affected under low saline - high ESP conditions (i.e. control treatment) which reduces infiltration, O<sub>2</sub> transport to the roots and a subsequent reduction in water uptake. As of 5 dS m<sup>-1</sup> a decrease in A<sub>sat</sub>, g<sub>s</sub> and T<sub>r</sub> was observed, although to a different extent depending on the parameter, while WUE<sub>int</sub> was increased. For example, at 10 dS m<sup>-1</sup>, A<sub>sat</sub>, g<sub>s</sub> and T<sub>r</sub> were reduced on average by 19, 24 and 25% respectively, with regard to 5 dS m<sup>-1</sup> treatment. This greater decrease in g<sub>s</sub> and T<sub>r</sub> compared to A<sub>sat</sub> led to a 18% increase in the WUE<sub>int</sub>, indicating stomatal closure reduced water loss to a higher degree than it did affecting CO<sub>2</sub> exchange. The enhancement in WUE may have contributed to the adaptation of these varieties to imposed stress throughout a better photosynthesis performance. This is in accordance with fluorescence results where a maintained activity of PSII was observed. The poor correlation founded between photosynthesis parameters and biomass production suggests that the effect of salinity on growth was not mainly mediated through effects on photosynthesis activity (Anand et al. 2000).

No significant changes in maximum quantum use efficiency of photosystem II (Fv/Fm) were observed in this study. Some authors have shown the salt stress inhibits PSII activity in *Medicago* species (Elfanssi et al. 2018; Farissi et al. 2018; Najjar et al. 2019), whereas other have reported no changes in Fv/Fm under salinity stress (Panta et al. 2016). Chaves et al. (2012) described that the photochemical efficiency is only occasionally affected under high salt stress. These inconsistent results may be a consequence of different sensitivities of plant species and/or experimental conditions. In our study the high Fv/Fm maintained under unfavourable conditions claims the salt tolerance of these varieties at mid-term.

## Conclusions

Among the physiological traits examined, δ<sup>15</sup>N represented the best proxy as a cultivar selection tool for differentiating alfalfa varietal response under biosaline conditions, as it was able to discriminate alfalfa's functional response within a narrow range of irrigation water salinity. These results highlight the relevance of N metabolism in alfalfa adaptation to salinity. Specific leaf

weight, although not found here to be useful for genotypic selection, can be used as a good proxy for biomass production under saline conditions. Particular characteristics of this study, e.g. soil sodicity, led to several factors interacting with one another affecting the water relations of the plant-system and those interactions could change over time. These interacting factors likely explain the complexity of results obtain for Δ<sup>13</sup>C and instantaneous measurements. Our results reveal the significance of long-term (e.g. more than one year) functional studies under salt stress conditions for perennial forages.

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