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Comparative effect of salinity on growth, grain yield, water use efficiency, $\delta^{13}C$ and $\delta^{15}N$ of landraces and improved durum wheat varieties☆

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ABSTRACT

Supplemental irrigation with low-quality water will be paramount in Mediterranean agriculture in the future, where durum wheat is a major crop. Breeding for salinity tolerance may contribute towards improving resilience to irrigation with brackish water. However, identification of appropriate phenotyping traits remains a bottleneck in breeding. A set of 25 genotypes, including 19 landraces and 6 improved varieties most cultivated in Tunisia, were grown in the field and irrigated with brackish water (6, 13 and 18 dSm^{-1}). Improved genotypes exhibited higher grain yield (GY) and water use efficiency at the crop level (WUE_{yield} or 'water productivity'), shorter days to flowering (DTF), lower N concentration (N) and carbon isotope composition ($\delta^{13}C$) in mature kernels and lower nitrogen isotope composition ($\delta^{15}N$) in the flag leaf compared with landraces. GY was negatively correlated with DTF and the $\delta^{13}C$ and N of mature kernels and was positively correlated with the $\delta^{15}N$ of the flag leaf. Moreover, $\delta^{13}C$ of mature kernels was negatively correlated with WUE_{yield}. The results highlight the importance of shorter phenology together with photosynthetic resilience to salt-induced water stress (lower $\delta^{13}C$) and nitrogen metabolism (higher N and $\delta^{15}N$) for assessing genotypic performance to salinity.

1. Introduction

Durum wheat is one of the most cultivated herbaceous crops in the southern and eastern Mediterranean basin (www.fao.org/statistics/yearbook). These environments are characterized by 'terminal stress' in the sense that drought develops during the last part of the crop cycle. One of the ways of increasing productivity in these semiarid environments is irrigation; however, this may expose soils to progressive salinization as a consequence of inappropriate irrigation practices [1,2]. At the same time, competition for water resources among different social and economic sectors is growing, with agriculture being progressively forced to use lower quality water [3], and this may compromise yield and critically expose soils to progressive salinization [4]. In arid and semi-arid regions, water and soil salinity are among the main factors limiting plant productivity. Tunisia is a Mediterranean country burdened by this salinity problem. It is estimated that saline soils cover over 1800,000 ha [5], representing 11.6% of the total surface of the country. In Tunisia, most durum wheat is commonly grown on marginal soils under rainfed conditions [6]. While supplemental irrigation may be a method to increase yield in durum wheat it might also expose the crop to additional salinity. In that context, selecting more salt tolerant genotypes is a way of improving durum wheat performance in the Mediterranean and other dry areas [2].

The use of stable isotope variation in plant research has grown steadily during the past two decades. This trend will continue as researchers realize that stable isotopes can serve as time-integrated indicators of how plants interact with and respond to their abiotic and biotic environments [7]. In that context, analysis of the natural abundances of the stable isotopes of carbon (^{12}C, ^{13}C) and nitrogen (^{14}N, ^{15}N) in plants is of potential interest for studies on salinity resilience [8–10].

The stable carbon isotope fractionation ($\delta^{13}C$) by plant matter (frequently expressed as discrimination from the surrounding air, $\Delta^{13}C$) integrates over time the ratio of intercellular to atmospheric CO_2 concentration and thus the balance between the net photosynthetic assimilation and the stomatal conductance (i.e. the intrinsic water use efficiency) in C_3 species such as wheat [11–13].

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Conditions inducing stomatal closure, such as water stress and salinity, restrict the CO_2 supply to carboxylation sites, which then increases $\delta^{13}C$ (or decreases $\Delta^{13}C$) and the intrinsic water use efficiency of the plant [9,12,14,15]. Moreover, genotypic variability for $\delta^{13}C$ in wheat under drought and salinity has also been reported, with resilient genotypes exhibiting lower $\delta^{13}C$ in kernels (or other organs developed during the last part of the crop cycle) and thus lower intrinsic water use efficiency [8,16–19]. However, the concept of water use efficiency is diverse [20] which implies that time integrated intrinsic water use efficiency not necessarily parallels the water use efficiency at the crop level (or 'water productivity') formulated as the ratio of total crop biomass or grain yield per unit of water used (evapotranspired).

Natural variation in the plant N isotope signature (commonly expressed as a composition, $\delta^{15}N$) as response to water stress and salinity has been reported. Moreover, $\delta^{15}N$ has been proposed for genotypic screening under drought [21,22] or salinity [8,23] because it is linked to N metabolism [10]. The fractionation of nitrogen (N) occurs during N uptake, assimilation, recycling and redistribution within the plant [24,25]. A change in the environmental conditions that impact on metabolism can cause a substantial change in the isotopic content of metabolites [26,27]. However, reported environmental and genotypic effects are diverse, including increases and decreases in plant $\delta^{15}N$ as response to growing conditions or related with genotypic resilience [10,27].

A previous study on durum wheat under field conditions in Tunisia has shown the value of carbon and nitrogen isotope compositions in assessing the genotypic performance of durum wheat under different water regimes [28]. However, to the best of our knowledge there are few studies evaluating the genotypic tolerance and the related physiological traits of field-grown durum wheat to salinity. In this study we compared the response of a set of Tunisian landraces and several of the modern (i.e. improved) durum wheat genotypes most cultivated in this country to different levels of salinity imposed by irrigating with brackish water under field conditions. Main objective was to determine which physiological traits are potentially useful as a phenotypic indicator to assess genotypic tolerance to irrigation with different levels of salinity. Second objective was to determine what differences exist between landraces and modern varieties in adaptation to salinity. To that end crop yield and water use efficiency at the crop level (WUE_{yield} or 'water productivity', formulated as the ratio between grain yield and water evapotranspired) were assessed together with some agronomical yield components, the $\delta^{13}C$ and the $\delta^{15}N$ and nitrogen content of flag leaves and grains, the chlorophyll content of the flag leaves and the number of days from sowing to anthesis. This study analysed samples collected from a recent field work, where genotypic variability in grain yield and it agronomical components have been evaluated [29].

2. Materials and methods

2.1. Plant material and growth conditions

The durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] genotypes used in this study consisted of 19 landraces and 6 improved cultivars (Supplemental Table S1). These genotypes were chosen on the basis of the available information about genetic diversity and tolerance of landraces to salinity [30] and the current commercial varieties most cultivated in Tunisia [29]. Main study was undertaken during the 2011 crop season at three irrigated sites in central Tunisia characterized by water supplies with different levels of salinity: Echbika (35° 37′ N, 9° 56′ E) exposed to medium saline conditions (irrigation water of 6 dS m^{-1}), Barrouta (35° 34′ N, 10° 02′ E) exposed to medium/severe saline conditions (irrigation water of 13 dS m^{-1}) and Sidi Bouzid (35° 02′ N, 9° 33′ E) exposed to very severe salinity (irrigation water of 18 dS m^{-1}). Precipitation and temperatures during the growing season were collected from the weather station of the National Institute of Meteorology of Tunisia closest to the site. Given their proximity, for Echbika and Barrouta sites the same weather station was used (Supplemental Fig. S1). Seeding was carried out in the 19 and 21 November 2011 in the Echbika and Barrouta and at 23 November in the Sidi Bouzid at a seeding rate of 300 viable seeds m^2. A completely randomized design was used to accommodate the two-way factorial experiment, with genotype and salinity as the main factors. Twenty-five genotypes and three replicates per genotype were used in each site totalling 75 plots per site and 225 plots for the total study. Each plot was 2 m^2, with 20 cm between rows, and 50 cm between plots. Irrigation was supplied via a drip system. In order to ensure a homogeneous water supply, line-source emitters were installed in each sowing row. The emitter discharge was 4 l h^{-1} at 1.0 bar operating pressure and there was 30 cm spacing between emitters of the same line. Irrigation water was provided from sowing to the grain filling stage. Physiological maturity was achieved during the second half of May and harvest was performed about 1 month later. The three experimental sites exhibited very similar climate conditions in terms temperature, photothermal quotient, radiation and vapor pressure deficit (Supplemental Fig. S1). For the analysis of soil salinity, five soil cores of 100 cm depth were collected per trial. Each core was divided into five different sections (0–20, 20–40, 40–60, 60–80, and 80–100 cm). For each soil depth, the corresponding sections of the five cores were mixed, dried and sieved at 2 mm. Then, 5 g of soil was suspended with 25 ml of distilled water and agitated during 1 h. Subsequently, soil salinity was measured using an electrical conductivity meter (dS m^{-1}). The analyses of stable isotopes and other physiological traits used in this study were performed in samples from 2011. In addition, the grain yield of the 2010 and 2012 crop seasons, from the same set of genotypes and experimental conditions, and reported in a previous publication [29], were also used in this study.

2.2. Crop phenology, leaf chlorophyll and ion content

The number of days from sowing to flowering (DTF) was recorded when approximately 50% of the spikes at each plot exhibited extruded anthers. The chlorophyll content of the flag leaf was measured in 10 plants per plot at anthesis. Measurements were performed using a portable meter (Minolta SPAD 502 meter, Plainfield, IL, USA). Plant height was measured around one week after anthesis as the distance from ground to the spike tip. The blade area was then measured from five flag leaves per plot using a portable laser leaf area meter (CID Bio-Science, CI-202, USA). For the analysis of ions content, ten flag leaves per plot were sampled just after flowering in each experimental site. The three replicates of each genotype in each experimental site were pooled together, oven-dried at 70 °C for 48 h and finely ground. Samples of 0.5 g were incubated for 12 h with 10 ml concentrated nitric acid (HNO_3) and 3 ml of chlorate acid ($HClO_3$) and then digested at 300 °C for 6 h. The amount of Na^+ and K^+ was then determined with an Inductively Coupled Plasma Emission Spectrometer (ICPES, Flame Photometer 410, Sherwood, UK).

2.3. Grain yield, agronomical components and water use efficiency

Harvest was performed during the second half of June, with 1 m^2 of each plot being hand harvested and the number of spikes per unit area counted and the total shoot biomass (including kernels) weighed. Grains were collected using a shredder (Wentersteiger,

LD-180, Germany) and the grain number per unit area and thousand kernel weight were estimated. Harvest index was then calculated as the ratio of grain yield to total shoot biomass. Additional information about plant sampling along with determination of grain yield and agronomical components are detailed in [29]. Crop water use in each trail was calculated as the initial (i.e. at planting) soil water content (mm) plus the accumulated precipitation (mm) and accumulated irrigation (mm) from planting to maturity minus the soil water content at maturity (mm). Water use efficiency (WUE_{yield}) was then estimated as the ratio between grain yield and water use. Gravimetric water content was measured before sowing and again at maturity in order to calculate water use and WUE_{yield}. The gravimetric water content was measured in 6 plots per block, on the same six genotypes across the three repetitions of each trial and values were averaged. For this purpose, soil samples were obtained from the upper 40 cm of the soil profile, samples were weighted fresh and later placed in a forced-air oven at 70 °C during a minimum of 48 h and weighted again to assess the humidity content. The volumetric water content was obtained using the gravimetric soil water content and soil bulk density.

2.4. Total nitrogen and stable carbon and nitrogen isotope analyses

For each plot, the five flag leaves used for blade area determination as well as mature grains were dried at 70 °C for 24 h and finely ground for further stable carbon and nitrogen isotope analysis. The total nitrogen concentration and the stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios were analysed in flag leaves and mature grain samples of the 6 dS m^{-1} and 13 dS m^{-1} experimental sites using an elemental analyser (Flash 1112 EA; Thermo-Finnigan, Bremen, Germany) coupled with an isotope ratio mass spectrometer (EA-IRMS, Delta C IRMS, Thermo-Finnigan) operating in continuous flow mode. Samples of ~1 mg and reference materials were weighed into tin capsules, sealed, and then loaded into an automatic sampler (Thermo-Finnigan) before EA-IRMS analysis. Measurements were conducted at the Scientific Facilities of the University of Barcelona. Nitrogen was expressed as a concentration (g N per g dry weight). The $^{13}C/^{12}C$ ratios were expressed in δ notation [31]: $\delta^{13}C = (^{13}C/C^{12})_{sample}/(^{13}C/^{12}C)_{standard} - 1$ [13], where 'sample' refers to plant material and 'standard' to Pee Dee Belemnite (PDB) calcium carbonate. The same δ notation was used for the $^{15}N/^{14}N$ ratio ($\delta^{15}N$), but in this case the standard referred to N_2 in air. Atropine was used as a system check in the elemental analyses of nitrogen. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose, and USGS 40 L-glutamic acid) were used for calibration to a precision of 0.1‰. For nitrogen, isotope secondary standards of known $^{15}N/^{14}N$ ratios (IAEA N_1 and IAEA N_2 ammonium sulphate and IAEA NO_3 potassium nitrate) were used. Samples of mature kernels from 18 dS m^{-1} were grounded, weighted in tin capsules and the $\delta^{13}C$ further analysed with a −IRMS (Delta V advantage, Thermo Scientific, USA) coupled with an elemental analyzer (Flash EA1112 HT) in the facilities of the Centre National des Sciences et Technologies of Tunisia. Secondary standards used were again IAEA CH6 and CH7. In the case of 18 dS m^{-1}, for each genotype and growing condition samples from the three replications were pooled, grounded and two analytical replications done.

2.5. Statistical analyses

Analysis of variance (ANOVA) was performed using the GLM procedure to calculate the effects of salinity level and genotype. A bivariate correlation procedure was used to calculate the Pearson correlation coefficients. Multiple linear regression analysis (stepwise) was used to analyse the relationship between the variables studied. Data were analysed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using a Sigma-Plot 11.0 program for Windows (Systat Software Inc., Point Richmond, CA, USA).

3. Results

Soil salinity (measured as electrical conductivity, EC) increased at harvest, with the most stressed site (18 dS m^{-1}) showing the highest EC (Supplemental Fig. S2) and Na^+ concentration values and the lowest K^+ concentrations [29]. The concentrations of Na^+ and K^+ and the K^+/Na^+ ratio in the dry matter of shoots sampled at flowering exhibited significant differences across the three trials. Thus Na^+ concentration increased from 90.9 μmol g^{-1} at 6 dS m^{-1}, to 131.3 μmol g^{-1} at 13 dS m^{-1} and reaching 144.3 μmol g^{-1} at 18 dS m^{-1} (pooled values across all the genotypes within a trail), whereas K^+ concentration decreased from 24.1 μmol g^{-1} at 6 dS m^{-1} to 16.7 μmol g^{-1} at 13 dS m^{-1} and 8.2 μmol g^{-1} at 18 dS m^{-1}. Consequently, the K^+/Na^+ ratio decreased from 0.265 to 0.057 as the level of salinity in the irrigation water increased from 6 dS m^{-1} to 18 dS m^{-1}. The shoot ion concentrations under distinct salinity irrigations were in the range reported in previous studies of durum and bread wheat [2,9,32,] exposed to salinity stress.

3.1. Effect of salinity on grain yield, growth parameters and water use efficiency

The increase in salinity significantly affected all the growth traits studied (Table 1). The effect of salinity in decreasing plant growth through a water stress effect has been extensively reported [2,33]. An increase in the salinity of the irrigation water from 6 dS m^{-1} to 18 dS m^{-1} significantly decreased grain yield (GY), total biomass at harvest, the number of kernels per m^2 and the thousand kernel weight (TKW), whereas the pattern for the harvest index was less clear. Decreases in GY and agronomical components, such as spike length, number of spikelets per spike, number of grains per spikelet and TKW have been reported in the past in response to increases in salinity [34–36]. Plant height, flag leaf blade area and the leaf chlorophyll content were also affected significantly by salinity, with blade area being the trait most affected. While such a decrease in chlorophyll content may be the consequence of salt [2,33] it was small in relative terms, which agrees with previous studies in durum wheat [8] and suggests the existence of compensatory mechanisms such as an increase in leaf thickness and compaction as a response to salinity. Moreover, the number of days from sowing to anthesis (DTF) also decreased as salinity increased. Crop duration was strongly reduced in response to salinity, which agrees with previous reports [37]. Crop water use efficiency (WUE_{yield}), in terms of GY per unit of water evapotranspired also decreased as the level of salinity in the irrigation water increased (Fig. 1), with the values achieved being in general comparable [38–40] or somewhat lower [41,42] than those reported for durum and bread wheat irrigated with non-saline water.

3.2. Effects of salinity on $\delta^{13}C$, $\delta^{15}N$ and nitrogen concentration

The salinity levels significantly affected the stable nitrogen isotope composition ($\delta^{15}N$) as well as the N concentration on both the flag leaf and the mature grains (Table 2). Salinity and water stress usually increase $\delta^{13}C$, as reported elsewhere [8,9,43–45]. Contrarily, in the current work, the flag leaves at the site irrigated with 13 dS m^{-1} exhibited a slightly lower (i.e. more negative) $\delta^{13}C$ than the site with the less brackish water (with 6 dS m^{-1}) (Table 2) and a decrease was also observed for the $\delta^{13}C$ of grains from the less (6 dS m^{-1}) to the most saline condition (18 dS m^{-1})

Table 1
Genotypic groups and environment effects on the number of days to flowering (DTF), plant height, flag leaf area (FLA), leaf chlorophyll content (LC), number of kernels per square meter (Kernel m^{-2}), thousand kernels weight (TKW), total aerial biomass (TB) at harvest, grain yield (GY) and the harvest index (HI) of the set of landraces and improved cultivars of durum wheat genotypes grown under different levels of salinity (6 dS m^{-1}; 13 dS m^{-1} and 18 dS m^{-1}). Genotypic groups values are the means of 171 measurements for the landraces (19 genotypes, 3 environments and 3 replicates per regime) and 54 measurements in the case of improved cultivars (6 cultivars, 3 environments and 3 replicates per regime), while environment values are the means of the 75 measurements (25 genotypes and 3 replicates per genotype). *F* values are presented. Levels of significance are as follows: **P < 0.01 and ***P < 0.001. ns, not significant.

	DTF	Plant height (cm)	FLA (cm^2)	LC	Kernels m^{-2}	TKW (g)	TB at harvest ($t\,ha^{-1}$)	GY ($t\,ha^{-1}$)	HI
Genotypic groups									
Landraces	147.67	124.28	11.45	51.65	4971.46	46.74	10.24	2.51	0.26
Improved cultivars	136.61	90.6	8.77	53.23	7175.81	45.57	10.01	3.59	0.37
Environments									
Echbika 6 dS m^{-1}	153.28	122.7	12.90	54.31	5885.51	55.87	12.47	3.32	0.27
Barrouta 13 dS m^{-1}	144.00	122.9	11.71	53.02	5382.14	43.72	11.56	2.87	0.26
Sidi Bouzid 18 dS m^{-1}	137.78	103.0	7.81	48.77	5233.87	39.76	6.52	2.23	0.32
Level of significance									
G, Genotypic groups (df = 1)	5022.78^{***}	636.01^{***}	68.60^{***}	12.02^{***}	87.39^{***}	2.01^{ns}	0.52^{ns}	176.78^{***}	158.12^{***}
E, Environment (df = 2)	505.60^{***}	110.18^{***}	49.50^{***}	38.86^{***}	8.50^{***}	118.97^{***}	131.26^{***}	89.96^{***}	21.60^{***}
G × E interaction (df = 2)	0.28^{ns}	4.62^{*}	23.18^{***}	4.72^{**}	5.95^{**}	3.94^{*}	2.90^{***}	2.95^{*}	3.00^{*}

Table 2
Genotypic groups and environment effects on carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope composition and on nitrogen concentration of flag leaves and grains of the set of landraces and improved cultivars of durum wheat grown under different levels of salinity (6 dSm^{-1}; 13 dSm^{-1}). Genotypic groups values are the means of 114 measurements for the landraces (19 genotypes, 2 environments and 3 replicates per salinity regime) and 36 measurements for the improved cultivars (6 genotypes, 2 environments and 3 replicates per environment), while environment regime values are the means of the 75 measurements (25 genotypes and 3 replicates per genotype). *F* values are presented. Levels of significance are as follows: **P < 0.01 and ***P < 0.001. ns, not significant.

	$\delta^{13}C_{flagleaf}$ (‰)	$\delta^{13}C_{grain}$ (‰)	$N_{flagleaf}$ (%)	N_{grain} (%)	$\delta^{15}N_{flagleaf}$ (‰)	$^{15}N_{grain}$ (‰)
Genotypic groups						
Landraces	−28.41	−25.96	3.66	2.73	3.24	4.27
Improved cultivars	−28.51	−26.82	3.81	2.11	2.11	3.76
Environments						
Echbika 6 dS m^{-1}	−28.18	−26.26	3.80	2.75	3.54	4.87
Barrouta 13 dS m^{-1}	−28.69	−26.05	3.59	2.41	2.39	3.42
Level of significance						
G, Genotypic groups (df = 1)	2.06^{ns}	46.14^{***}	4.76^{***}	84.36^{***}	16.32^{**}	7.97^{***}
E, Environment (df = 2)	18.38^{***}	2.10^{ns}	15.61^{*}	23.01^{***}	7.87^{***}	54.48^{**}
G × E interaction (df = 2)	0.31^{ns}	0.15 ns	2.38^{ns}	0.06^{ns}	6.40^{***}	1.67^{ns}

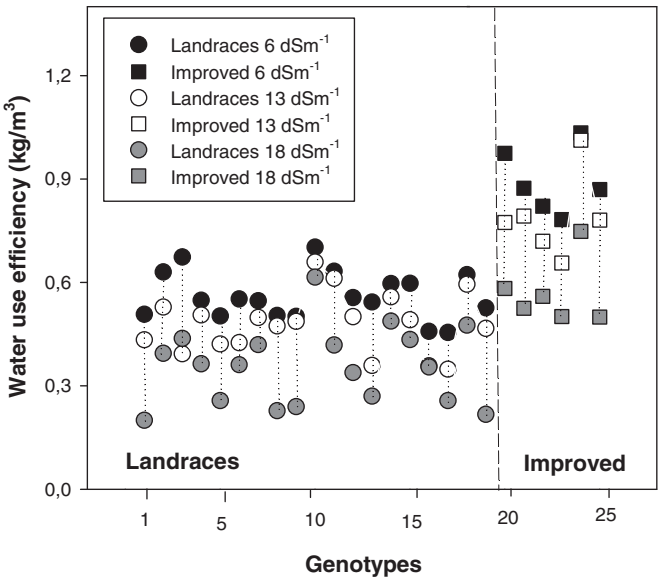

Fig. 1. Crop water use efficiency (WUE_{yield}) of the set of 19 landraces and 6 improved genotypes under the three different growing conditions assayed. Values were calculated as the ratio between the grain yield achieved for each genotype divided by the total amount of water received from November to May of the next year for the landraces (6561, 6036 and 5081 m^3ha^{-1} in Echbika [6 dSm^{-1}], Barrouta [13 dSm^{-1}] and Sidi Bouzid [18 dS m^{-1}], respectively) and one week before for the improved cultivars (5676, 5151 and 4879 m^3ha^{-1} in Echbika, Barrouta and Sidi Bouzid, respectively).

(Supplemental Fig. S3). In addition, we found a decrease in the $\delta^{15}N$ and the N concentration in both the flag leaves and mature grains under 13 dS m^{-1} compared to 6 dS m^{-1}. Decreases in shoot N concentration and $\delta^{15}N$ have been reported in cereals as a consequence of salinity [8,9] and deficit irrigation [21,46] and suggest that these stress conditions influence N uptake and/or assimilation [8,9,22,23].

3.3. *Genotypic effect on plant growth, grain yield, WUE, $\delta^{13}C$, $\delta^{15}N$ and nitrogen concentration*

The genotypic effect was highly significant for all the growth traits studied, except thousand kernel weight (TKW) and total biomass at harvest (Table 1). Improved genotypes exhibited lower plant height but higher grain yield and number of kernels per m^2 regardless of the level of salinity considered (Table 3). Except for the most severe salinity (18 dSm^{-1}), landraces exhibited slightly higher TKW values than improved genotypes (Table 3). In addition, the landraces showed a greater number of days from sowing to anthesis regardless of the level of salinity (Table 3). Leaf chlorophyll content exhibited significant genotypic effects only under the severe salinity (18 dSm^{-1}), with landraces showing lower values compared to the improved cultivars (Table 3). The genotypic groups by environment (G × E) interaction was not significant only for DTF (Table 1). Moreover, modern genotypes exhibited higher WUE compared with landraces regardless of the salinity condition (Fig. 1).

Table 3
Effect of growing environment on the growth parameters, the grain yield and the different stable isotope compositions and nitrogen concentration analysed in the two durum wheat genotypic groups (df = 1). Data shown are means of 19 landraces and 6 modern varieties. Means are significantly different (P < 0.05) according to the factorial analysis of variance (ANOVA). *F* values are presented. Levels of significance are as follows: **P < 0.01; ***P < 0.001. ns, not significant.

	Echbika 6 dS m^{-1}			Barrouta 13 dS m^{-1}			Sidi Bouzid 18 dS m^{-1}		
	Landraces	Improved cultivars	*F*	Landraces	Improved cultivars	*F*	Landraces	Improved cultivars	*F*
DTF	155.84	145.18	231.39***	146.73	135.33	199.46***	140.45	129.33	378.82***
Plant height (cm)	129.37	101.44	157.89***	131.70	95.22	212.66***	111.78	75.19	276.58***
FLA (cm^2)	13.96	9.57	52.84***	12.70	8.61	40.90***	7.71	8.14	1.09^{ns}
LC	54.22	54.62	0.31^{ns}	52.82	53.61	0.44^{ns}	47.92	51.46	6.72**
TB at harvest (t ha^{-1})	12.33	12.90	1.07^{ns}	11.87	10.59	3.50^{ns}	6.52	6.54	0.002^{ns}
Kernels m^{-2}	5080.76	8433.86	64.88***	5002.09	6585.62	23.86***	4831.52	6507.94	12.65***
TKW (g)	56.79	53.00	6.52**	44.08	42.57	1.01***	39.33	41.14	2.09^{ns}
GY (t ha^{-1})	3.02	4.28	137.52***	2.58	3.78	77.42***	1.92	2.73	21.64***
$\delta^{13}C_{flagleaf}$ (‰)	−28.13	−28.35	1.40^{ns}	−28.67	−28.77	0.66^{ns}	/	/	/
$\delta^{13}C_{grain}$ (‰)	−26.07	−26.89	21.49***	−25.84	−26.75	24.65***	/	/	/
$\delta^{15}N_{flagleaf}$ (‰)	3.99	2.15	26.32***	2.50	2.07	0.96^{ns}	/	/	/
$\delta^{15}N_{grain}$ (‰)	5.05	4.31	8.02**	3.49	3.22	1.24^{ns}	/	/	/
$N_{flagleaf}$ (%)	3.74	3.99	9.96**	3.57	3.62	0.15^{ns}	/	/	/
N_{grain}(%)	2.90	2.27	44.20***	2.56	1.96	40.19***	/	/	/

DTF, the number of days to flowering; FLA, flag leaf area; LC, leaf chlorophyll content; TB, total aerial biomass at harvest; Kernel m^{-2} number of kernels per square meter; TKW, thousand kernels weight; GY, grain yield; $\delta^{13}C$, stable carbon isotope composition of flag leaves ($\delta^{13}C_{flagleaf}$) and grains ($\delta^{13}C_{grain}$); $\delta^{15}N$, stable nitrogen isotope composition of flag leaves ($\delta^{15}N_{flagleaf}$) and grains ($\delta^{15}N_{grain}$); N, concentration of flag leaves ($N_{flagleaf}$) and grains (N_{grain}).

The genotypic groups effect was significant for the $\delta^{15}N$ as well as the N concentration on both flag leaves and mature grains (Table 2), and for the $\delta^{13}C$ only in mature grains (Table 2), with values for all these traits being higher in landraces than in improved genotypes. The same pattern of a lower $\delta^{13}C$ of grains in improved genotypes compared with landraces was also found at 18 d Sm^{-1} (Supplemental Fig. S3). Moreover, the genotypic groups × environment interaction (G × E) was only significant for $\delta^{15}N$ of the flag leaves (Table 2). Genotypic differences between landraces and improved cultivars for the set of stable isotope signatures and N concentrations were also examined within each growing condition (Table 3). Except for the $\delta^{13}C$ of flag leaves, there were significant differences for all traits studied ($\delta^{13}C$ of grains and $\delta^{15}N$ and N of the flag leaves and grains) under 6 dS m^{-1}, but only for $\delta^{13}C_{grain}$ and N_{grain} under 13 dS m^{-1}.

3.4. Relationships of grain yield to growth, $\delta^{13}C$, $\delta^{15}N$ and N concentration

Grain yield was strongly negatively correlated across genotypes and within each growing condition with DTF as well as with flag leaf area and plant height (Fig. 2A–C), whereas TKW did not correlate with GY (data not shown). In addition, GY was negatively correlated with the $\delta^{13}C$ and N concentration of the kernels at both 6 dS m^{-1} and 13 dS m^{-1} (Fig. 3A, C). In addition, the three salinity levels showed a similar pattern of negative correlation between $\delta^{13}C_{grain}$ and the average GY across the three years and across the 25 genotypes (Supplemental Fig. S3). All the three salinity levels (6 dS m^{-1}, 13 dS m^{-1} and 18 dS m^{-1}) showed similar patterns in their negative relationship between GY and the $\delta^{13}C$ of grains, which suggests that $\delta^{13}C$ is a powerful trait in terms of monitoring the genotypic response to the osmotic stress associated with salinity [8,45]. GY did not correlate with N concentration or the $\delta^{13}C$ of the flag leaves at any of the two salinity levels tested (data not shown). Finally, GY was significantly correlated with $\delta^{15}N$ only at 6 dSm^{-1} and with flag leaves (Fig. 3B). Moreover, GY was negatively correlated with the $\delta^{13}C$ of kernels within landraces and improved cultivars separately when both saline conditions (6 dS m^{-1} and 13 dS m^{-1}) were combined, but the correlation across improved cultivars was much stronger than across landraces (Fig. 4A). However, GY was correlated positively with $\delta^{15}N$ of grains only within landraces (Fig. 4B). A positive relationship across genotypes between $\delta^{15}N$ and biomass has already been reported in durum wheat growing under different salinity conditions in a hydroponic system [8]. In addition, $\delta^{13}C$ was negatively correlated with WUE_{yield} across genotypic groups within each of the three saline conditions (Fig. 5).

Table 4
Pearson correlation coefficients of the relationships between crop duration, measured as number of days from sowing to flowering (DTF) and the different stable isotope compositions and the total N concentrations. Data are the means of all genotypic groups and repetitions in each environment. Levels of significance are as follows: ns, not significant; **P < 0.01 and ***P < 0.001. Abbreviations for variables as defined in Table 2.

	Echbika 6 dS m^{-1}	Barrouta 13 dS m^{-1}
DTF versus $\delta^{13}C_{flagleaf}$	0.47^{ns}	0.23^{ns}
DTF versus $\delta^{13}C_{grain}$	0.71***	0.77***
DTF versus $\delta^{15}N_{flagleaf}$	0.74***	0.26^{ns}
DTF versus $\delta^{15}N_{grain}$	0.51**	0.32^{ns}
DTF versus $N_{flagleaf}$	−0.35^{ns}	−0.06^{ns}
DTF versus N_{grain}	0.73***	0.71***

3.5. Relationships of N concentration with $\delta^{13}C$ and $\delta^{15}N$

The nitrogen concentration of mature grains was positively correlated with the $\delta^{13}C$ of kernels across genotypes at both 6 dSm^{-1} and 13 dSm^{-1} (Supplemental Fig. S4A), while the $\delta^{13}C$ and $\delta^{15}N$ of the flag leaves only correlated with N concentration in mature grains at 6 dSm^{-1} (Supplemental Fig. S4B, C), and the $\delta^{15}N$ of kernels did not correlate (data not shown). In addition, the nitrogen concentration of mature grains was correlated positively with the $\delta^{15}N$ of kernels within both landraces and improved cultivars when both saline conditions were combined (Supplemental Fig. S5C), while the $\delta^{13}C$ of the flag leaves only correlated with the N concentration of mature grains for landraces (Supplemental Fig. S5B).

3.6. Relationships of days from sowing to flowering with $\delta^{13}C$, $\delta^{15}N$ and N concentration

DTF was positively correlated with the $\delta^{13}C$ and N of kernels at both the lower and medium salinity levels (Table 4). However, for the $\delta^{15}N$ of the flag leaves and the grains had a significant positive correlation with DTF only at 6 dSm^{-1}. In addition, the nitrogen concentration of mature grains was correlated positively with DTF

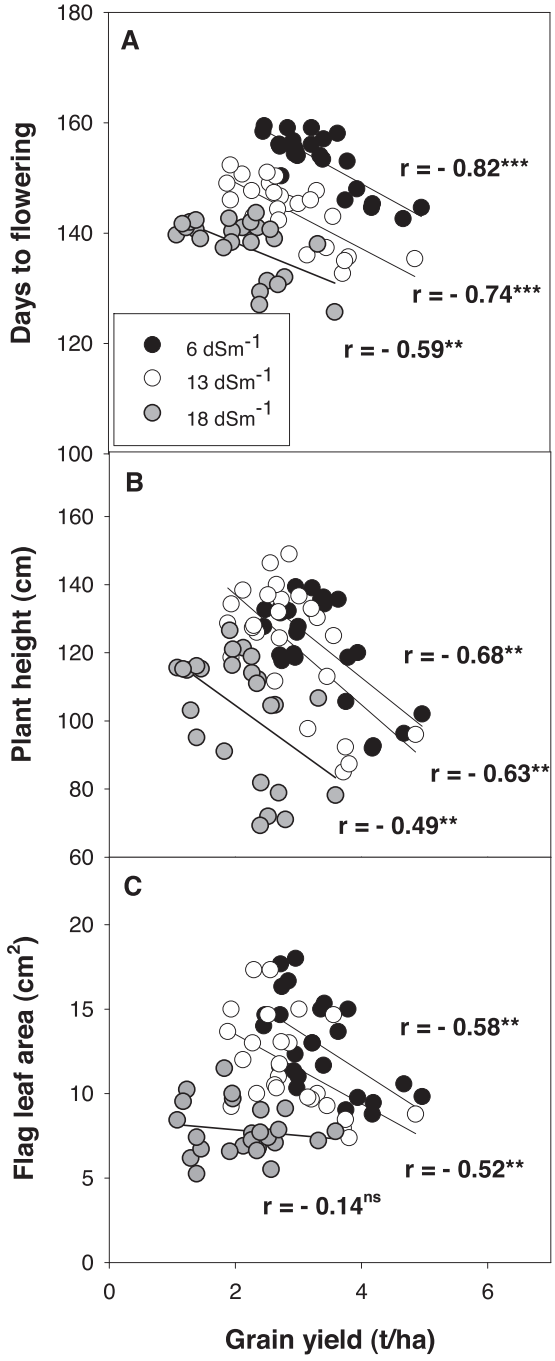

Fig. 2. Relationships of grain yield (GY) to (A) days from sowing to flowering, (B) plant height and (C) flag leaf area within 6 dSm^{-1}, 13 dSm^{-1} and 18 dSm^{-1}. Each point represents the averaged value of one genotype. Levels of significance: ns, not significant; **P < 0.01; ***P < 0.001.

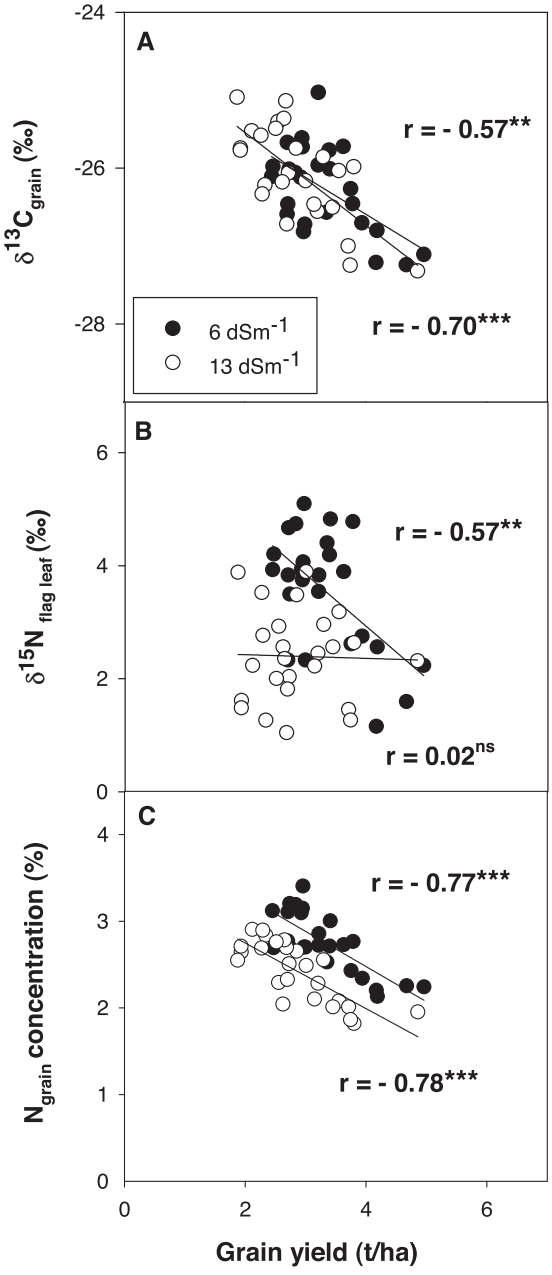

Fig. 3. Relationships of grain yield (GY) to (A) carbon isotope composition of mature grains ($\delta^{13}C_{grain}$), (B) nitrogen isotope composition of the flag leaf ($\delta^{15}N_{flagleaf}$) and (C) nitrogen concentration of mature grains (N_{grain}), within 6 dSm^{-1} and 13 dSm^{-1}. Each point represents the averaged value of one genotype. Levels of significance: ns, not significant; **P < 0.01; ***P < 0.001.

within both landraces and improved cultivars when both saline conditions were combined (Supplemental Fig. S5A).

3.7. Stepwise analysis of grain yield across genotypes

A multiple linear regression (stepwise) was performed that explained GY variations as the dependent variable across the set of 25 genotypes at the lower (6 dSm^{-1}) and medium (13 dSm^{-1}) saline conditions assayed. Phenology together with the growth parameters (other than total biomass and the agronomical yield components), $\delta^{13}C$, $\delta^{15}N$ and N concentration of both the flag leaves and the grains were used as independent variables (Table 5). The DTF was the first variable selected by the model in both growing conditions. It alone accounted for around 78% and 72% of the genotypic variation in grain yield at 6 dSm^{-1} and 13 dSm^{-1}, respectively. The N concentration of grains was the second variable chosen by the model to explain GY in both salinity conditions. Leaf chlorophyll content and the $\delta^{15}N$ of kernels were also selected by the model but with a minor role in accounting for the differences in GY (Table 5).

Stepwise analyses were also performed for the two subsets of genotypes (Supplemental Table S2). For landraces, only the N of the flag leaves was chosen at 6 dSm^{-1} to explain 30% of the variability of GY, whereas at 13 dSm^{-1}, the N concentration in mature grains was the first variable chosen by the model (33% of the variability), followed by leaf chlorophyll, the $\delta^{13}C$ of kernels and plant height. Regarding the improved cultivars, only the $\delta^{13}C$ of kernels was cho-

Table 5
Multiple linear regressions (stepwise) explaining grain yield (GY) variation across genotypic groups in each environment as a dependent variable, and all the growth traits (excluding total biomass and agronomical yield components), number of days to flowering, leaf chlorophyll content, nitrogen concentration and stable isotope signatures in the same particular growing condition as independent. Levels of significance: ***P < 0.001. Abbreviations for variables as defined in Tables 1 and 2.

Dependent Variable	Environment	Variable chosen	R^2	Final stepwise model
GY	Echbika 6 dS m^{-1}	DTF	0.78***	GY = −0.08 DTF −0.38 N_{grain} + 18.46
		DTF; N_{grain}	0.80***	
GY	Barrouta 13 dS m^{-1}	DTF	0.72***	GY = −0.05 DTF − 0.68 N_{grain} − 0.07 LC −0.16 $\delta^{15}N_{grain}$ + 9.23
		DTF; N_{grain}	0.76***	
		DTF; N_{grain}; LC	0.79***	
		DTF; N_{grain}; LC; $\delta^{15}N_{grain}$	0.81***	

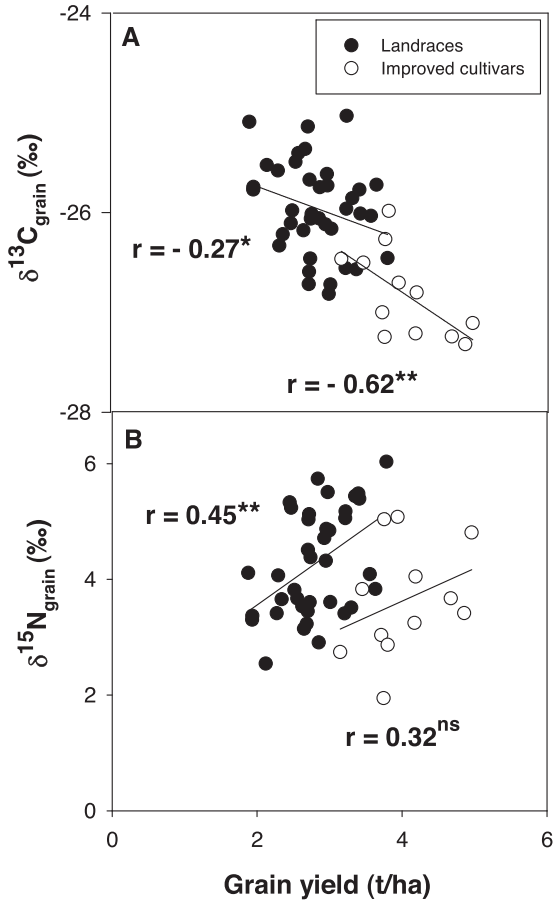

Fig. 4. Relationships of grain yield (GY) to (A) carbon isotope composition ($\delta^{13}C$) and (B) nitrogen isotope composition ($\delta^{15}N$) of mature grains within landraces (filled symbols) and improved genotypes (open symbols) across 6 dS m^{-1} and 13 dS m^{-1} together. Each point represents the averaged value of one genotype and growing condition. Levels of significance: ns, not significant; *P < 0.05; **P < 0.01.

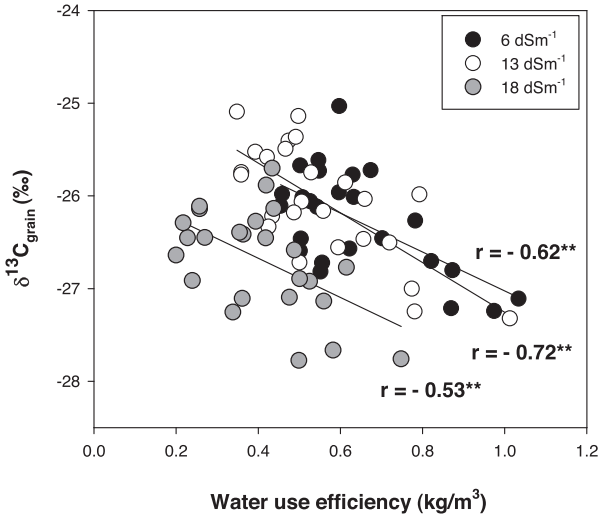

Fig. 5. Relationship between carbon isotope composition ($\delta^{13}C$) of mature kernels and crop water use efficiency (WUE_{yield}) under the three different growing conditions assayed: (6 dS m^{-1}, 13 dS m^{-1} and 18 dS m^{-1}). Levels of significance: **P < 0.01.

sen by the model at 13 dSm^{-1} to explain 60% of the variability of GY, whereas at 6 dSm^{-1} no variable was chosen.

4. Discussion

4.1. Growth, crop duration and grain yield as genotypic indicators of tolerance to salinity

Salinity of irrigation water and genotypes both significantly affected plant height and leaf area, traits that can be considered useful for screening durum wheat germplasm under salinity and water stress [33,47,48]. Within a given salinity level genotypes with smaller flag leaf and plant height were the more yielding. These morphological traits are characteristics of improved (i.e. post Green Revolution) cultivars that possess a higher yield potential [49], which somehow may still translate to a higher yield under stress conditions. Moreover, plants with smaller leaves may have reduced transpirative surfaces and thus a lower accumulation of toxic ions in the shoot, which is one of the mechanisms plants use to tolerate the reduced ion uptake effects under soil salinity [50]. Leaf chlorophyll content, has been proposed as a screening criterion for wheat tolerance to salinity [33]. However, in our study it only correlated (positively) with GY at the most severe salinity level, corresponding to the treatment with the lowest chlorophyll content in the flag leaves. Previous studies in durum wheat under controlled conditions have failed to find any correlation between leaf chlorophyll content and plant growth [8].

In our study salinity decreased GY, mainly through a decrease in TKW and to a lesser extent in the number of kernels per m^{-2}. In this context, it has been reported that the effect of salinity was most pronounced on the yield components, which were undergoing development at the time of the salt stress [51]. Conversely the yield components, which were stressed by salinity during their development, made less of a contribution to grain yield [52]. Our results also showed that within each growing condition GY correlated highly and negatively with days to flowering (DTF) with shorter duration genotypes having a greater yield. In this context, it has been reported that under salt stress, phenological escape is one of the strategies employed by plants to survive such environmental conditions [37].

4.2. Genotypic tolerance to salinity, stable isotope composition and N content

The study reported genetic variability in GY, $\delta^{13}C$, $\delta^{15}N$ and N concentration for durum wheat under different salinity conditions. Genetic variability to salinity for these traits has been reported before for durum wheat under controlled pot conditions [8]. Studies on other cereals like barley and wheat under field conditions have also reported genetic variability in GY and $\delta^{13}C$ under salinity [19,43]. As in the case of water stress under Mediterranean conditions [28], the genotypic tolerance of Tunisian durum wheat was associated with a lower (i.e. more negative) $\delta^{13}C$ in grains. The more productive genotypes are those with lower $\delta^{13}C$ in grains, which suggests they exhibit higher stomatal conductance (because a better water status) and lower intrinsic water use efficiency than the more susceptible genotypes [53]. However, the more productive genotypes also exhibited the highest WUE_{yield}.

Moreover, genotypic differences in plant $\delta^{15}N$ seem to reflect the extent to which plants acquire and retain N in their tissues [21]. Thus the positive relationship between the $\delta^{15}N$ of grains and both GY and the N concentration of grains across treatments might be due to differences in $\delta^{15}N$ being associated with the effect of assimilation capacity and N demand on this isotopic signature [54–57]. Alternatively, it has been suggested that salt stress decreases the $\delta^{15}N$ and N concentration compared with control conditions due to down-regulation of assimilating enzymes [23] such as nitrate reductase or ammonium assimilation by glutamine synthetase [58–60] as response to salinity in wheat [61]. However, within a given salinity level $\delta^{15}N$ did not correlate (13 dS m^{-1}) or correlated negatively (6 dS m^{-1}) with GY across genotypes. Moreover, the N concentration in kernels had a stronger relation with GY than $\delta^{13}C$ or $\delta^{15}N$. Such a strong negative relationship with GY may be the consequence of a low N concentration in kernels being the result of a "dilution" effect caused by a high kernel weight per spike [17,62,63].

4.3. Differences between landraces and improved varieties

The greater crop duration, plant height and leaf area and lower grain yield of landraces compared to improved (post Green-Revolution) varieties has been extensively reported for durum wheat [49,64]. Moreover, the TKW tended to decrease in the improved varieties, which also agrees with previous studies [49] and does not support the higher N concentration of kernels of landraces being the consequence of smaller kernels. The higher GY of modern varieties is due to a larger number of kernels per m^2 compared with the landraces and occurs regardless of the salinity conditions. It has been stated that the wheat landraces are better adapted than modern cultivars to changing climate conditions and to stress environments due to their population genetic structure, buffering capacity, and a combination of morpho-physiological traits conferring adaptability to stress environments [65]. However, our study clearly shows that modern varieties outyielded the landraces irrespective of the salinity conditions. On the other hand, total aerial biomass at maturity (including weight of grains) was comparable in landraces and advanced lines, which agrees with the majority of reports indicating that total biomass has not (or has only marginally) increased in the improved varieties compared to the landraces [64]. Moreover, the modern genotypes clearly showed higher WUE_{yield} than landraces in spite their lower $\delta^{13}C$ in kernels. The higher WUE_{yield} was mainly the consequence of a higher grain yield, whereas the effect of a shorter cycle duration (and thus less water evapotranspirated) appears as minor. Overall, our results do not support the landraces as being, in general, more salinity tolerant than the improved varieties, which has been the conventional view. Moreover, landraces also exhibited higher $\delta^{13}C$ compared with improved varieties, which has been reported in the past for durum wheat under different water regimes [17,66,67]. It has been reported that the genotypic tolerance of Tunisian durum wheat to different water regimes was associated with a lower (i.e. more negative) $\delta^{13}C$ in the flag leaves and the grains [28]. These results suggest that the most salinity tolerant genotypes are those that maintain more open stomata [8], which is the case for the improved varieties. However, whether the lower $\delta^{13}C$ of improved genotypes compared to the landraces is the consequence of a constitutive higher stomatal conductance, the capacity to keep higher stomatal conductance under osmotic stress conditions or just the consequence of differences in phenology (a shorter cycle in modern varieties), remains to be elucidated. Even so, in our study a shorter duration seems to be the main factor responsible for the lower grain $\delta^{13}C$ of improved genotypes because days to flowering correlated positively with $\delta^{13}C$ and this phenological trait was the first chosen in the stepwise analysis to explain differences in GY. Nevertheless, phenology is not the only factor involved as suggested from the stronger negative relationship between $\delta^{13}C$ and GY of modern cultivars compared with the landraces. Concerning $\delta^{15}N$, a higher shoot $\delta^{15}N$ has been reported as a favourable trait in terms of genotypic differences in biomass under different salinity levels and hydroponics [8,10]. However, it does not translate necessarily to a higher grain yield. This is the case of our study under field conditions, where the improved varieties exhibited lower flag leaf $\delta^{15}N$ than the landraces.

4.4. Flag leaves or mature grains, which is the most informative organ?

Our results showed that both the $\delta^{13}C$ and N of mature grains were highly correlated with GY within each salinity level, whereas correlations between the same traits in the flag leaves against GY were absent. The $\delta^{13}C_{grain}$ and N_{grain} were also the traits that best separated the landraces from the improved cultivars under 6 dS m^{-1} and 13 dS m^{-1}. In addition, $\delta^{13}C_{grain}$ was the only variable chosen by the model in the stepwise analysis and explained 60% of the GY variability within improved cultivars, while the N of mature grains was the first variable chosen by the model to explain the GY variability within the landraces. Previous work on durum wheat subjected to different water regimes under Mediterranean conditions [17,28,68] has shown that grains perform better than leaves when using the $\delta^{13}C$ and the N concentration as traits to assess the genotypic performance in terms of GY. However, to the best of our knowledge this is the first study showing that samples from kernels perform better than those from leaves when assessing genotypic variability under salinity. The progressive effect of salinity in terms of increasing water stress and ion toxicity of the crop may account for the better performance of traits analysed in the kernels, these being the last parts of the plant to develop. Moreover, $\delta^{13}C$ of mature grains also was well correlated with WUE_{yield} but opposite (i.e. negatively) to the predicted relationship between $\delta^{13}C$ and WUE.

4.5. Implications for breeding

This study highlights the importance of phenology in adaptation of durum wheat to salinity conditions, whereas it does not support any advantages of landraces compared with improved varieties under this form of stress. Moreover, leaf chlorophyll content has not proven to be an adequate trait for selection, at least under moderate-medium salinity conditions. On the other hand, the study shows the suitability of the stable C isotope signature together with N concentration, particularly when analysed in mature kernels, for assessing the genotypic performance of landraces and improved cultivars under salinity. However, the use of $\delta^{13}C$ (or $\Delta^{13}C$) as a

selection proxy for water use efficiency have to take into consideration what is the nature of WUE targeted. Whereas $\delta^{13}C$ in plants has been proved for long as an indicator of "intrinsic or physiological" water use efficiency in terms of photosynthetis per transpiration it may actually correlate in an opposite direction with "agronomical" WUE evaluated as GY or biomass per unit of water received, since water losses include, despite water transpired by the plant, the direct evaporation from the soil. Thus, the estimation of WUE in experiments carried out in pots (where soil evaporation is commonly eliminated) may be substantially different with respect to plants grown in the field [20,39]. In fact, most of the studies on the positive relationship between $\delta^{13}C$ and WUE based on plant growth or grain yield versus water used have been performed in pots or in conditions where soil evaporation has been prevented.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2016.07.005.

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