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RESEARCH PAPER

Water and nitrogen conditions affect the relationships of $\Delta^{13}C$ and $\Delta^{18}O$ to gas exchange and growth in durum wheat

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Abstract

Whereas the effects of water and nitrogen (N) on plant $\Delta^{13}C$ have been reported previously, these factors have scarcely been studied for $\Delta^{18}O$. Here the combined effect of different water and N regimes on $\Delta^{13}C$, $\Delta^{18}O$, gas exchange, water-use efficiency (WUE), and growth of four genotypes of durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] cultured in pots was studied. Water and N supply significantly increased plant growth. However, a reduction in water supply did not lead to a significant decrease in gas exchange parameters, and consequently $\Delta^{13}C$ was only slightly modified by water input. Conversely, N fertilizer significantly decreased $\Delta^{13}C$. On the other hand, water supply decreased $\Delta^{18}O$ values, whereas N did not affect this parameter. $\Delta^{18}O$ variation was mainly determined by the amount of transpired water throughout plant growth (T_{cum}), whereas $\Delta^{13}C$ variation was explained in part by a combination of leaf N and stomatal conductance (g_s). Even though the four genotypes showed significant differences in cumulative transpiration rates and biomass, this was not translated into significant differences in $\Delta^{18}O_s$. However, genotypic differences in $\Delta^{13}C$ were observed. Moreover, ~80% of the variation in biomass across growing conditions and genotypes was explained by a combination of both isotopes, with $\Delta^{18}O$ alone accounting for ~50%. This illustrates the usefulness of combining $\Delta^{18}O$ and $\Delta^{13}C$ in order to assess differences in plant growth and total transpiration, and also to provide a time-integrated record of the photosynthetic and evaporative performance of the plant during the course of crop growth.

Key words: $\Delta^{13}C$ and $\Delta^{18}O$, leaf gas exchange, water and nitrogen limitation, wheat, WUE.

Introduction

In recent decades, stable carbon and oxygen isotopes have been shown to be powerful non-invasive probes for characterizing photosynthetic metabolism in plants. It has been known for some time that the carbon isotope composition of plant dry matter ($\delta^{13}C$), which is frequently expressed as the discrimination value ($\Delta^{13}C$), provides a time-integrated measurement of the plant's transpiration efficiency (i.e. the ratio of carbon gain to water transpired) over the period during which dry matter is assimilated. Indeed, it is >20 years since $\Delta^{13}C$ was first proposed as a potential tool for screening wheat genotypes with higher transpiration efficiency (Farquhar and Richards, 1984; Rebetzke *et al.*, 2002), and the first genotypes selected using this approach were subsequently released in Australia (Rebetzke *et al.*, 2002; Condon *et al.*, 2004). Whereas increased water input has been widely reported to have a positive effect on $\Delta^{13}C$ (Araus *et al.*, 2003; Condon *et al.*, 2004), there are contradictory reports as to how the amount of nitrogen (N) fertilization affects $\Delta^{13}C$. Thus, for wheat and other cereals, $\Delta^{13}C$ has been reported to increase (Shangguan *et al.*, 2000), decrease (Choi *et al.*, 2005; Cabrera-Bosquet *et al.*, 2007; Zhao *et al.*, 2007; Serret

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Abbreviations: AB, aboveground biomass; A_{sat}, light-saturated net CO_2 assimilation rate; C_i and C_a, intercellular and ambient CO_2 concentrations; $\delta^{13}C$, carbon isotope composition; $\Delta^{13}C$, carbon isotope discrimination; $\delta^{18}O$, oxygen isotope composition; $\Delta^{18}O$, oxygen isotope enrichment; E, transpiration rate; g_s, stomatal conductance; LDM, leaf dry mass per unit leaf area; RWC, relative water content; SB, spike biomass; $WUE_{biomass}$, time-integrated water-use efficiency; $WUE_{instantaneous}$, instantaneous water-use efficiency; $WUE_{intrinsic}$, intrinsic water-use efficiency.

et al., 2008), or not be affected (Hubick, 1990; White *et al.*, 1990) as a result of N supply.

The oxygen isotope composition ($\delta^{18}O$) of organic matter is known to reflect variation in: (i) the isotopic composition of source water; (ii) evaporative enrichment in leaves due to transpiration; and (iii) biochemical fractionation during synthesis of organic matter (Craig and Gordon, 1965; Dongmann *et al.*, 1974; Yakir, 1992; Farquhar and Lloyd, 1993). Hence, the oxygen isotope signature of plant matter, expressed either as composition ($\delta^{18}O$) or as enrichment above source water ($\Delta^{18}O$), has been used to assess the leaf evaporative conditions at the time the material was formed (Yakir *et al.*, 1990; Yakir, 1992; Saurer *et al.*, 1997; Barbour *et al.*, 2000*a*, *b*; Barbour, 2007). The leaf oxygen isotope signature has been negatively correlated with relative humidity (Saurer *et al.*, 1997; Barbour *et al.*, 2000*b*; Ferrio *et al.*, 2007) and also with transpiration rate (Barbour and Farquhar, 2000; Barbour *et al.*, 2000*a*). Conversely, Sheshshayee *et al.* (2005) reported a positive relationship between $\Delta^{18}O$ and the transpiration rate in groundnut and rice genotypes that contrasts with the present theory. Moreover, Barbour *et al.* (2000*a*) have proposed the use of the stable oxygen isotope signature (in their study expressed as $\delta^{18}O$) measured in plant matter as an integrative indicator of genetic differences in stomatal conductance (g_s), as well as a predictor of grain yield in field-grown wheat. Therefore, the ^{18}O signature of plant tissue is of interest in terms of breeding for improved water use, and genotypic variability for this trait has been identified in bread wheat (Barbour *et al.*, 2000*a*; Farquhar *et al.*, 2007; Ferrio *et al.*, 2007). As plant material has been shown to record leaf evaporative conditions, measurement of the ^{18}O signature might provide a powerful tool for plant breeders (Barbour, 2007), to track genotypic differences not only in stomatal conductance but also in yield. However, up to now, most studies dealing with the use of ^{18}O for breeding have been conducted under well-watered conditions (Barbour *et al.*, 2000 *a*, *b*; Bindumadhava *et al.*, 2005; Sheshshayee *et al.*, 2005), even if the plants in question have been grown under different evaporative demand (Barbour and Farquhar, 2000; Bindumadhava *et al.*, 2006). Research involving the ^{18}O signature under water-limited conditions is far scarcer (Yakir *et al.*, 1990; Ferrio *et al.*, 2007), although genotypic differences have been identified. Furthermore, the combined effect of N fertilizer and water regime on the $\delta^{18}O$ oxygen isotope composition of plant material has not been assessed, despite the fact that the amount of N fertilization may affect the photosynthetic (Lawlor, 2001) and transpiration rates (Claus *et al.*, 1993) in wheat and other cereals.

In terms of surface area, cereals are the main crops in the Mediterranean basin, occupying preferably the dry lands. Among cereals, durum wheat is the most widely grown in the South Mediterranean basin and is a very important crop in the European agro-food industry. In these environments, water limitation, which is frequently accompanied by low N availability, is the main constraint on wheat yield (Oweis *et al.*, 1998; Araus *et al.*, 2002; Passioura, 2002). Selecting genotypes better suited to the lack of water (Condon *et al.*, 2004) and N (Hirel *et al.*, 2007) is therefore one of the main targets for increasing yield in these environments. The use of physiological traits such as phenotypical criteria for selecting genotypes better adapted to such growing conditions might help plant breeders (Bänziger *et al.*, 2000; Araus *et al.*, 2002; Lafitte *et al.*, 2003). The best potential traits to use provide time-integrated (i.e. through the crop cycle) information on plant performance (Araus *et al.*, 2002). Among these traits the signature of the stable carbon and oxygen isotopes in plant matter reflects the photosynthetic (Farquhar *et al.*, 1989) and transpirative (Barbour 2007; Farquhar *et al.*, 2007) conditions in which the plants were grown (Araus *et al.*, 1998, 2003).

The aim of the present research was to study the combined effect of different water and N regimes on $\Delta^{13}C$, $\Delta^{18}O$, water-use efficiency (WUE), and growth of four contrasting genotypes of durum wheat. The usefulness of combining $\Delta^{13}C$ and $\Delta^{18}O$ measurements in shoot dry matter to track differences in WUE, leaf gas exchange, cumulative transpiration, and growth in these plants was also tested.

Materials and methods

Plant material and growth conditions

Three durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] genotypes (Bicrecham-1, Lahn/Haucan, and Omrabi-3) released by the CIMMYT/ICARDA durum wheat breeding programme, plus one of the most cultivated Spanish varieties (Mexa) were grown in a greenhouse at the University of Barcelona, from December 2004 to April 2005. These four genotypes were chosen on the basis of their contrasting yield performance under Mediterranean conditions (Villegas *et al.*, 2001; Ferrio *et al.*, 2005). Fourteen seeds of each genotype were planted per pot (5.0 L, filled with washed sand). After germination, they were thinned to seven plants per pot, representing a plant density of ~223 plants m^{-2}, and watered daily with deionized water for 2 weeks. After this, three different water regimes and two N levels were imposed, by applying nutrient solution every 2 d. The water levels were achieved by weighing the pots prior to watering, and then adding the amount of water needed to reach 40, 70, and 100% of their container capacity (CC). The N supply was controlled using two different nutrient solutions: complete Hoagland solution (Hoagland and Arnon, 1950) and the same solution with N diluted four times (i.e. 0.06725 g N L^{-1} and 0.27 g N L^{-1} for the low and high N, respectively). Pots with different treatments were displayed in a randomized complete block design. Each treatment was replicated four times and the whole experimental set-up accounted for a total of 96 pots. Plants were grown in the greenhouse under mean day/night temperatures of ~25/15 °C, a maximum photosynthetic photon flux density (PPFD) of ~1000 μmol m^{-2} s^{-1}, and a mean vapour pressure deficit (VPD) of 0.75 kPa.

Leaf gas exchange measurements

Gas exchange of the flag leaf blade was measured ~2 weeks after anthesis, just before harvesting, using an open IRGA LI-COR 6400 system (LI-COR Inc., Lincoln, NE, USA). Photosynthetic measurements were performed under light-saturated conditions (1000 μmol photon m^{-2} s^{-1} of PPFD), at 25 °C and 400 μmol mol^{-1} of CO_2. The measured gas exchange parameters were: light-saturated net CO_2 assimilation rate (A_{sat}); transpiration rate (E); and stomatal conductance (g_s). Then the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) was calculated according to Sharkey and Raschke (1981).

Determination of cumulative transpiration and water status

The amount of water evapotranspired was monitored throughout the experiment by weighing each pot just prior to watering. The pots were then adjusted to their water regime (40, 70, and 100% CC) by adding nutrient solution to maintain the experimental design. Simultaneously, empty pots without plants were also weighed to record direct evaporation from the soil. Then, the cumulative transpiration (T_{cum}) was calculated as the difference between evapotranspiration and evaporation.

Water status was determined by means of measurements on relative water content (RWC) in the flag leaf blades 1 d after the last watering and just before harvesting. Leaf blade segments were weighed (fresh weight; FW), floated on distilled water at 4 °C overnight, and weighed again (TW). They were then dried at 80 °C for 48 h. After this, the dry weight (DW) was determined. RWC was then calculated as:

$$RWC = [(FW - DW)/(TW - DW)] \times 100 \quad (1)$$

Water-use efficiency

WUE was calculated using both instantaneous and time-integrated measurements. The time-integrated WUE, $WUE_{biomass}$, was calculated as the ratio of total aboveground biomass (AB) to evapotranspired water throughout plant growth. Moreover, instantaneous WUE ($WUE_{instantaneous}$) and intrinsic WUE ($WUE_{intrinsic}$) were calculated as the ratio of A_{sat} to E and g_s, respectively.

Total nitrogen content and carbon isotope analyses

For each analysis, 1 mg of fine powdered shoot, spike, flag leaf, or root tissue was weighed in tin cups. The total N content of samples was analysed at the Colorado Plateau Stable Isotope Laboratory (CPSIL) using an Elemental Analyser (EA) (Carlo Erba 2100, Milan, Italy). The same EA interfaced with an isotope ratio mass spectrometer (IRMS) (Thermo-Finnigan Deltaplus Advantage, Bremen, Germany) was also used to analyse $^{13}C/^{12}C$ ratios (R) of plant material. Results were expressed as $\delta^{13}C$ values, using a secondary standard calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB), and the analytical precision was ~0.1‰.

$$\delta^{13}C(‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000 \quad (2)$$

The carbon isotope discrimination ($\Delta^{13}C$) of plant parts was then calculated from δ_a and δ_p (Farquhar *et al.*, 1989) as:

$$\Delta^{13}C = \frac{\delta_a - \delta_p}{\delta_p + 1} \quad (3)$$

where a and p refer to air and the plant, respectively. Air samples inside the greenhouse were taken and analysed by the gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) technique as previously described (Nogués *et al.*, 2008). This analysis was undertaken at the Scientific Facilities of the University of Barcelona. The carbon isotope composition of the air measured within the greenhouse was $\delta^{13}C$=–11.3‰.

Oxygen isotope analyses

The oxygen isotope composition was determined in shoots (including leaves and stems), since this plant part was considered as the most representative because it makes up a significant proportion of the dry weight in the plant. In addition, $\Delta^{13}C$ of shoots integrates the photosynthetic performance and the water status of the plant during their growth better than other plant parts (Tambussi *et al.*, 2007*a*).

The $^{18}O/^{16}O$ ratios of irrigation water were determined by the CO_2:H_2O equilibration technique and using an IRMS (Delta S Finnigan MAT, Bremen, Germany). The $^{18}O/^{16}O$ ratios of shoot dry matter were determined by an on-line pyrolysis technique using a thermo-chemical elemental analyser (TC/EA Thermo Quest Finnigan, Bremen, Germany) coupled with an IRMS (Delta C Finnigan MAT, Bremen, Germany). Results were expressed as $\delta^{18}O$ values, using a secondary standard calibrated against the Vienna standard mean oceanic water (VSMOW), and the analytical precision was ~0.2‰.

$$\delta^{18}O(‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000 \quad (4)$$

Then, following the same notation used for carbon isotope discrimination, the ^{18}O enrichment in shoot parts ($\Delta^{18}O_s$) was calculated as follows:

$$\Delta^{18}O_s = \frac{\delta^{18}O_s - \delta^{18}O_{iw}}{1 + \delta^{18}O_{iw}} \quad (5)$$

where $\delta^{18}O_s$ and $\delta^{18}O_{iw}$ refer to the oxygen isotope compositions of shoots and irrigation water, respectively ($\delta^{18}O_{iw}$ was approximately –6.5‰.). The $^{18}O/^{16}O$ ratios of both irrigation water and shoot dry matter were calculated at the Scientific Facilities of the University of Barcelona.

Biomass determination

Total biomass was collected ~2 weeks after anthesis. The spike, flag leaf, the rest of the shoot (including leaves and stems), and the root were separated and oven-dried at 80 °C for 48 h, before being weighed and powdered. The leaf dry mass per unit leaf area (LDM) of the flag leaf blades was calculated as the ratio between dry weight and leaf surface (g m^{-2}). Moreover, the specific leaf nitrogen (SLN) of the flag leaf blades was calculated as the ratio between dry N content in dry matter and the leaf surface (g m^{-2}).

Statistical analysis

Analysis of variance (ANOVA) was performed using the General Linear Model procedure to calculate the effects of water and genotype within each N treatment. Means were compared using a Tukey-b multiple comparison test (P <0.05). The 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. Linear stepwise models were built from water and genotype means within each N treatment. Data were analysed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

Results

Plant growth and N content

Large differences in total AB and root biomass, as well as in the different components of AB, were observed in response to the different levels of water supply and N fertilization studied (Table 1, Fig. 1). Accumulated transpiration (T_{cum}) also changed greatly in response to both N and water treatments (Table 1). Thus, AB, spike biomass (SB), and T_{cum} in control plants (i.e. high N and 100% CC) were five, three, and three times higher, respectively, than in plants grown under the most limiting conditions (i.e. low N and 40% CC, Table 1). However, the effect of water regime was greater in the low than in the high N regime; in fact, there were two-way significant interactions between water regime and N levels for total AB, SB, and T_{cum}. In addition, large genotypic differences in these three traits were also observed. Omrabi-3 was the genotype with highest AB and T_{cum} in both N treatments, whilst Mexa had the lowest values. Bicrecham-1 and Lahn/Haucan had intermediate values (Table 1). However, whereas interactions of genotypes with both N and water conditions were not significant for T_{cum}, these interactions did reach significance for total biomass, although only between genotypes and N regime. LDM values were affected by genotype but not by water

Table 1. Mean values for plant growth and gas exchange parameters of four wheat genotypes (G) subjected to different water regimes (WR) and grown under low and high nitrogen

Data are the mean of 16 (WR) or 12 (G) replicates. Within each nitrogen treatment and within each water regime (40, 70. and 100% CC) or genotype (Mexa, Bicrecham-1, Lahn/Haucan. and Omrabi-3); values with different letters are significantly different according to the Tukey-b test (P <0.05).

	AB	SB	RWC	LDM	SLN	LN	SN	T_{cum}	A_{sat}	E	g_s	C_i/C_a
Low N												
WR												
40%	7.9 c	3.6 c	85.1 b	31.7 a	1.1 b	3.6 b	1.8 a	2.58 c	16.2 b	3.1 a	0.32 a	0.72 a
70%	11.6 b	4.8 b	90.5 a	34.3 a	1.4 a	4.1 a	1.9 a	3.67 b	18.6 a	3.7 a	0.37 a	0.72 a
100%	32.2 a	11.2 a	91.3 a	33.2 a	1.4 a	4.1 a	1.9 a	8.21 a	17.4 a,b	3.0 a	0.30 a	0.68 a
G												
Mexa	14.9 b	7.5 a	87.7 a	30.8 b	1.0 c	3.4 c	1.9 a	4.48 b	15.4 b	2.9 a	0.30 a	0.72 a
Bic-1	16.1 b	6.4a	87.9 a	32.9 a.b	1.3 b	3.9 b	1.9 a	4.63 b	16.9 b	3.3 a	0.34 a	0.72 a
L/H	17.2 b	7.1 a	89.8 a	33.4 a,b	1.3 b	4.0 b	1.9 a	4.65 b	16.4 b	3.4 a	0.30 a	0.70 a
Omr-3	20.7 a	5.2 b	90.4 a	35.2 a	1.6 a	4.5 a	1.8 a	5.51 a	21.0 a	3.5 a	0.38 a	0.69 a
High N												
WR												
40%	14.8 c	5.6 c	87.5 a	32.1 a	1.4 b	4.3 a	2.8 c	3.25 c	14.1 b	2.1 b	0.19 b	0.62 a
70%	25.5 b	8.7 b	88.7 a	34.7 a	1.5 a,b	4.4 a	3.0 b	5.56 b	15.6 a,b	2.3 b	0.23 a,b	0.64 a
100%	38.3 a	11.8 a	90.7 a	34.9 a	1.6 a	4.5 a	3.2 a	7.85 a	18.1 a	2.9 a	0.29 a	0.66 a
G												
Mexa	20.1 c	10.0 a	86.0 b	31.1 b	1.2 c	4.0 c	2.5 b	5.03 b	16.1 a	2.8 a	0.28 a	0.69 a
Bic-1	26.9 b	9.7 a	91.2 a,b	36.2 a	1.6 a,b	4.4 b	3.2 a	5.80 a,b	15.8 a	2.4 a	0.24 a	0.64 a
L/H	24.5 b	8.3 b	88.5 a,b	33.5 a,b	1.4 b	4.3 b	3.0 a	5.29 a,b	15.4 a	2.3 a	0.22 a	0.63 a
Omr-3	33.3 a	6.9 c	90.2 a	34.7 a,b	1.7 a	4.9 a	3.2 a	6.10 a	16.4 a	2.2 a	0.20 a	0.58 b

AB, aboveground biomass (g dry weight); SB, spike biomass (g dry weight); RWC, relative water content (%); SLN, specific leaf N (g m^{-2}); LDM, leaf dry mass per unit leaf area (g m^{-2}); LN, flag leaf nitrogen content (%); SN, shoot nitrogen content (%); T_{cum}, cumulative transpiration (L); A_{sat}, light-saturated net CO_2 assimilation rate (μmol CO_2 m^{-2} s^{-1}); g_s, stomatal conductance (mol CO_2 m^{-2} s^{-1}); E, transpiration rate (mmol H_2O m^{-2} s^{-1}); C_i and C_a, intercellular and ambient CO_2 concentrations.

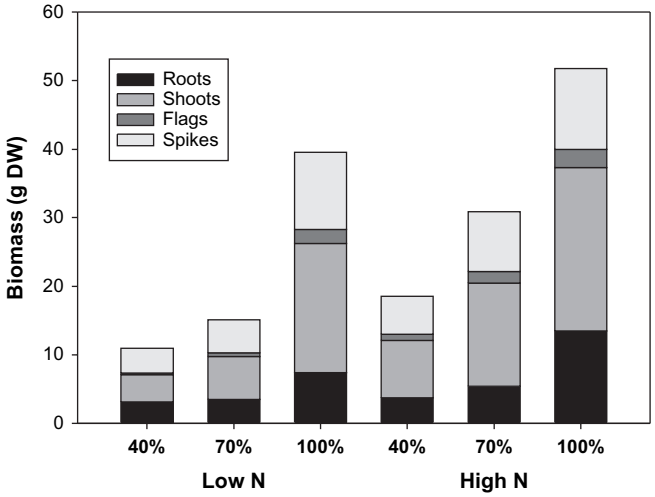

Fig. 1. Effect of water and nitrogen treatments on root, shoot, flag, and spike biomass. Values are the mean of 16 replicates.

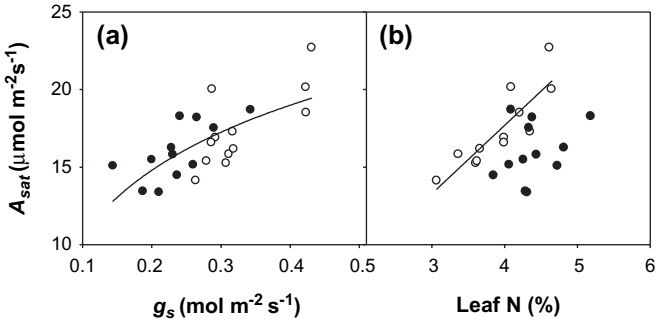

Fig. 2. (a) Relationship between A_{sat} and g_s, $y=21.3(1-e^{-6.03x})$; $r=0.76$, $P<0.0001$. (b) Relationship between A_{sat} and leaf N; low N treatment, $y=3.74x$LN–2.68; $r=0.66$, $P<0.01$. Open and filled circles represent, respectively, the low and high N treatment means for each genotype and water condition. Each value is the mean of four replicates.

regime and N, and the only significant interaction was between genotype and water regime.

The results also showed that the N content of shoots on a dry matter basis (SN), as well as that of the flag leaf per unit leaf area (SLN) and dry weight (LN), increased in response to N supply and, to a lesser extent, as water increased (Table 1). In addition, genotypic differences in the N content of shoots and the flag leaf were found. In both N treatments, Omrabi-3 was the genotype with the highest SLN and LN in the flag leaf, whereas Mexa had the lowest values. SN was also the highest and the lowest in Omrabi-3 and Mexa, respectively, but only under high N conditions. In fact, SN showed a genotypic interaction between genotype and both water and N treatments, whereas LN did not show any interactions between genotypes and growing conditions; for SLN, only interactions between genotype and water regime were observed (Table 1).

Leaf gas exchange

Water supply had a significant positive effect on light-saturated net CO_2 assimilation (A_{sat}) but not on stomatal conductance (g_s) or transpiration (E). However, for all three parameters there was a significant interaction between water regime and N levels. Thus, A_{sat}, g_s, and E increased (28, 52, and 38%, respectively) with water supply under high N treatments, whereas no differences were found in plants grown with a low N supply (Table 1). The C_i/C_a ratio did not change significantly in response to water treatment or according to the N regime. The N regime did have a significant effect on the four gas exchange parameters shown in Table 1. Hence, higher values of A_{sat}, g_s, C_i/C_a, and E were found in the low N compared with the high N treatment. Genotypic differences for A_{sat} and C_i/C_a were observed, but, whereas the interaction between genotype and water regime was not significant, that between genotype and N regime was. Thus, at low N, Omrabi-3 showed higher values of A_{sat} compared with the other three genotypes, while at high N no differences between the four genotypes were observed. Conversely, for C_i/C_a, Omrabi-3 showed lower values than the other genotypes at high N, while no differences were observed at low N. Differences across treatments and genotypes in net photosynthesis were mediated mostly by differences in stomatal conductance, as shown by the relationship between A_{sat} and g_s when all the treatments were plotted together (Fig. 2a). In contrast, A_{sat} and LN were only significantly correlated at low N (Fig. 2b).

Water status and WUE

Water supply increased the RWC values in both N treatments, although significant differences between water treatments were only found in plants grown at low N (Table 1). However, these values remained relatively high (>85%) even in the lower water regime. The N regime did not affect RWC. WUE, measured both gravimetrically ($WUE_{biomass}$) and using gas exchange measurements ($WUE_{intrinsic}$ and $WUE_{instantaneous}$), increased in response to N fertilization (Table 2). However, the effect of water deficit on these parameters was not clear. While at low N the three traits showed the same pattern of increase as the water supply increased, only the response of $WUE_{biomass}$ reached significance. At high N, an increase in water supply only led to a significant decrease in $WUE_{intrinsic}$, and, in fact, significant interactions between water and N conditions were observed for $WUE_{biomass}$ and $WUE_{intrinsic}$. Furthermore, significant differences between genotypes were also observed for all three WUEs, particularly in plants grown at high N. Nevertheless, Omrabi-3 was the most efficient genotype in terms of water use, regardless of the N regime.

Effects of water and nitrogen on $\Delta^{13}C$ and $\Delta^{18}O_s$

N conditions and genotype had a significant effect on $\Delta^{13}C$, whereas water had no effect (except for $\Delta^{13}C$ of flag leaves). When the two N treatments were compared, higher $\Delta^{13}C$ values in plant dry matter were found in low N plants (Table 2). Moreover, a slight but significant increase in $\Delta^{13}C$ of all plant parts was observed with increasing water

Table 2. Mean values for water-use efficiency (WUE): (i) measured from the biomass accumulated and total water transpired ($WUE_{biomass}$); and (ii) calculated from the gas exchange measurements ($WUE_{intrinsic}$ and $WUE_{instantaneous}$), carbon isotope discrimination ($\Delta^{13}C$) of the spike ($\Delta^{13}C_{sp}$), flag ($\Delta^{13}C_f$), root ($\Delta^{13}C_r$), and shoot ($\Delta^{13}C_s$), and the oxygen isotope enrichment above source water in shoots ($\Delta^{18}O_s$) of four wheat genotypes (G) subjected to different water regimes (WR) and grown under low and high N

	$WUE_{biomass}$	$WUE_{intrinsic}$	$WUE_{instantaneous}$	$\Delta^{13}C_{sp}$	$\Delta^{13}C_f$	$\Delta^{13}C_r$	$\Delta^{13}C_s$	$\Delta^{18}O_s$	$\Delta^{13}C_s/\Delta^{18}O_s$
Low N									
WR									
40%	2.2 b	53.9 a	5.3 a	18.0 a	20.4 a	18.9 a	19.6 a,b	35.8 a	0.55 a
70%	2.4 b	52.5 a	5.3 a	18.2 a	20.7 a	19.1 a	19.7 a	35.1 a,b	0.56 a
100%	2.9 a	60.3 a	5.9 a	17.5 b	19.9 b	18.9 a	19.2 c	34.5 b	0.56 a
G									
Mexa	2.3 b	54.5 a	5.4 a,b	18.5 a	21.2 a	19.5 a	20.0 a	35.2 a	0.57 a
Bic-1	2.3 b	52.7 a	5.1 b	17.9 b	20.2 b	19.0 b	19.5 b	35.2 a	0.56 a
L/H	2.5 a	57.0 a	5.1 b	17.6 b	20.0 b	18.5 c	19.1 b	35.0 a	0.55 a
Omr-3	2.6 a	58.0 a	6.4 a	17.6 b	19.9 b	18.8 a,b	19.3 b	35.3 a	0.55 a
High N									
WR									
40%	3.5 a	78.7 a	6.9 a	16.1 b	18.3 b	18.0 a,b	17.8 b	35.6 a	0.50 b
70%	3.6 a	74.2 a,b	6.9 a	16.1 b	18.4 b	17.9 b	17.8 b	34.9 a	0.52 b
100%	3.6 a	66.0 b	6.5 a	16.7 a	18.9 a	18.3 a	18.6 a	33.8 b	0.55 a
G									
Mexa	3.0 c	60.6 b	6.0 a	17.6 a	20.3 a	19.0 a	19.3 a	34.6 a	0.56 a
Bic-1	3.6 b	71.2 b,c	6.8 a	16.2 b	18.1 c	18.1 b	18.1 b	34.6 a	0.52 b
L/H	3.5 b	75.3 a,b	6.9 a	16.6 b	18.8 b	18.1 b	18.1 b	35.1 a	0.52 b
Omr-3	4.3 a	86.6 a	7.5 a	14.8 c	16.9 d	17.3 c	16.8 c	34.7 a	0.48 c

Data are the mean of 16 (WR) or 12 (G) replicates. Within each nitrogen treatment and within each water regime (40, 70, and 100% CC) or genotype (Mexa, Bicrecham-1, Lahn/Haucan, and Omrabi-3), values with different letters are significantly different according to the Tukey-b test (P <0.05).

supply from 70% to 100% CC in plants grown under high N, while, under low N, $\Delta^{13}C$ values from 70% to 100% CC followed the opposite pattern. In fact, the two-way interactions between growing conditions were significant for all the $\Delta^{13}C$. In addition, significant genotypic differences in $\Delta^{13}C$ were found in all plant organs within each N and water treatment. The Mexa genotype showed the highest values of $\Delta^{13}C$, regardless of the N regime and the tissue considered, whereas Omrabi-3 showed the lowest values but only at high N. In fact, there was a significant interaction between genotype and N regime for the four different $\Delta^{13}C$ analysed. In addition, differences in $\Delta^{13}C$ between plant parts were also found (Table 2).

The $\delta^{18}O$ of shoots was enriched compared with the $\delta^{18}O$ of irrigation water (–6.5‰) and, therefore, $\Delta^{18}O_s$ was positive in all cases (Table 2). The water regime had a significant effect on $\Delta^{18}O_s$, whilst no differences in $\Delta^{18}O_s$ values were found when comparing genotypes or N treatments. Thus, within each N treatment, $\Delta^{18}O_s$ decreased as the water supply increased. Moreover, two-way interactions were not significant in any case.

Relationships between different traits

When data for all the water regimes and genotypes grown together at high N were combined, $\Delta^{13}C$ of shoots, flag leaves, spikes, and roots correlated strongly and negatively with time-integrated WUE, and also negatively with instantaneous measurements of WUE (Table 3). Under low N conditions, correlations of different $\Delta^{13}C$ only reached significance against $WUE_{intrinsic}$, with $\Delta^{13}C$ of spikes being the best correlated. In contrast, the results showed no significant correlations between $\Delta^{18}O$ of shoots and either instantaneous or time-integrated measurements of WUE in either of the N treatments (Table 3). In addition, $\Delta^{18}O$ of shoots did not significantly correlate with $\Delta^{13}C$ of shoots, regardless of the N regime considered (data not shown).

Table 3. Correlation coefficients of the linear relationships between $\Delta^{13}C$ of the different plant organs and $\Delta^{18}O$ of shoots against WUEs for all the treatments and genotypes together

		$WUE_{instantaneous}$	$WUE_{intrinsic}$	$WUE_{biomass}$
Low N	$\Delta^{13}C_{sp}$	–0.406	–0.851**	–0.626*
	$\Delta^{13}C_f$	–0.378	–0.757**	–0.510
	$\Delta^{13}C_s$	–0.293	–0.778**	–0.491
	$\Delta^{13}C_r$	–0.123	–0.689*	–0.450
	$\Delta^{18}O_s$	–0.278	–0.291	–0.145
High N	$\Delta^{13}C_{sp}$	–0.736**	–0.708*	–0.958**
	$\Delta^{13}C_f$	–0.729**	–0.704*	–0.924**
	$\Delta^{13}C_s$	–0.734**	–0.746**	–0.928**
	$\Delta^{13}C_r$	–0.823**	–0.758**	–0.948**
	$\Delta^{18}O_s$	0.124	0.273	0.131

Linear correlations were calculated within each N treatment using water and genotype means (n=12), *P <0.05, **P <0.01.

Due to the water regime increasing biomass and decreasing $\Delta^{18}O_s$ values, significant negative correlations were found between $\Delta^{18}O_s$ and either AB, SB, or T_{cum} in both N

treatments (Table 4; Fig. 4). Conversely, $\Delta^{13}C_s$ did not correlate with either T_{cum} or AB (Table 4). However, it did correlate positively with SB, although only in the high N treatment.

Furthermore, whereas at high N $\Delta^{13}C_s$ correlated positively with g_s and E, $\Delta^{18}O_s$ correlated negatively with these same gas exchange parameters; these correlations were not found in the low N treatments. Moreover, in the high N treatments, strong correlations were found between the ratio of carbon and oxygen isotopes ($\Delta^{13}C_s/\Delta^{18}O_s$) and g_s and E (Table 4; Fig. 5).

In addition, a stepwise analysis was performed to explain the causes of variation of the different parameters studied (Table 5). Thus, the variation in the total AB was explained in both N treatments by the combination of $\Delta^{18}O_s$ and $\Delta^{13}C_s$. $\Delta^{18}O_s$ and $\Delta^{13}C$ explained, respectively, 59% and 17% of the variation in AB at low N, while under high N $\Delta^{18}O_s$ and $\Delta^{13}C$ explained 48% and 36% of AB variation, respectively. Moreover, in both N treatments, variation in $\Delta^{18}O_s$ was mainly explained by changes in T_{cum}. On the other hand, $\Delta^{13}C_s$ and A_{sat} variations were explained in both N treatments by a combination of g_s and leaf N (expressed as LN or SLN). Differences in the ratio C_i/C_a were explained in the high N treatments by changes in g_s and leaf N, whereas no explanation was found for low N treatments when these variables were entered into the model.

Table 4. Correlation coefficients of the stable isotopes with biomass and gas exchange parameters for all the treatments and genotypes together.

		AB	SB	g_s	E	T_{cum}
Low N	$\Delta^{18}O_s$	–0.79**	–0.89**	0.15	0.18	–0.84**
	$\Delta^{13}C_s$	–0.48	–0.13	0.29	–0.02	–0.38
	$\Delta^{13}C_s$ /$\Delta^{18}O_s$	–0.02	0.34	0.17	–0.11	0.09
High N	$\Delta^{18}O_s$	–0.72**	–0.80**	–0.58*	–0.71*	–0.80**
	$\Delta^{13}C_s$	0.11	0.63*	0.74**	0.67*	0.13
	$\Delta^{13}C_s$ /$\Delta^{18}O_s$	0.18	0.80**	0.80**	0.80**	0.40

Linear correlations were calculated within each N treatment using water and genotype means (n=12), *P<0.05, **P<0.01.
AB, aboveground biomass; SB, spike biomass; g_s, stomatal conductance; E, transpiration rate; T_{cum}, cumulative transpiration.

Discussion

Water and nitrogen effects on plant growth and water status

Although the different water regimes produced large differences in biomass in both N treatments (Fig. 1), the relatively small decreases in gas exchange parameters, $\Delta^{13}C$ and RWC, suggest that mild water stress was experienced even with the most water-limited treatments. This can be explained in part by a combination of different factors, including the fact that plant growth, more than gas exchange, is affected by moderate water stress (Hsiao, 1973; Jones, 1980; McCree, 1986; Kramer and Boyer, 1995). Hence, both the way in which water regimes were imposed (i.e. sustained water limitation during the course of crop growth) and the growing conditions in pots led plants to acclimatize, with an adjustment of the total leaf area to the available water conditions in the pot and, therefore, with water status (e.g. g_s and RWC) remaining at moderate or non-water stress levels. Moreover, the lack of differences in LDM between water treatments (and therefore the absence of differences in leaf structure) reinforces the idea that plants adjust their total leaf area to water availability.

In addition, when both N treatments were compared, a negative effect of N supply on gas exchange parameters was observed. High N supply clearly increased the total plant biomass (and therefore the total leaf area), thereby causing leaves to compete for the available water in the pot.

Table 5. Multiple linear regressions (stepwise) explaining biomass variation from stable isotopes ($\Delta^{13}C_s$ and $\Delta^{18}O_s$); $\Delta^{18}O_s$ and $\Delta^{13}C_s$ variations from gas exchange parameters (A_{sat}, E, g_s, C_i/C_a, T_{cum}) plus SLN, SN, and LN; and C_i/C_a and A_{sat} variations (g_s, SLN, SN, and LN) derived from water and genotype means within each N treatment.
Initial variable, first variable entering the model; initial r^2 and mean square error (MSE), adjusted coefficient of determination (r^2) and MSE after including the first variable in the model; final r^2 and MSE, adjusted r^2, and MSE obtained with the final stepwise model.

N treatment	Trait	Initial variable	Initial r^2	Initial MSE	Final stepwise model	Final r^2	Final MSE
Low N	AB	$\Delta^{18}O_s$	0.59**	7.36	AB= –15.1×$\Delta^{18}O_s$–8.5×$\Delta^{13}C_s$+716.8	0.76***	5.70
	$\Delta^{18}O_s$	T_{cum}	0.67**	0.33	$\Delta^{18}O_s$= –0.19×T_{cum}+36.3	0.67**	0.33
	$\Delta^{13}C_s$	SLN	0.37*	0.45	$\Delta^{13}C_s$= –1.8×SLN+5.6×g_s+20.0	0.68**	0.32
	C_i/C_a	–	–	–	–	–	–
	A_{sat}	LN	0.72***	1.32	A_{sat}=3.3×LN+17.4×g_s–1.4	0.85***	0.96
High N	AB	$\Delta^{18}O_s$	0.48**	8.4	AB= –11.6×$\Delta^{18}O_s$–5.8×$\Delta^{13}C_s$+538.5	0.84***	6.64
	$\Delta^{18}O_s$	T_{cum}	0.61**	0.58	$\Delta^{18}O_s$= –0.4×T_{cum}+36.8	0.61**	0.58
	$\Delta^{13}C_s$	LN	0.59**	0.65	$\Delta^{13}C_s$= –1.7×LN+10.7×g_s+23.1	0.87***	0.36
	C_i/C_a	g_s	0.59**	0.03	C_i/C_a=0.68×g_s–0.06×LN+0.74	0.74***	0.03
	A_{sat}	g_s	0.43**	1.39	A_{sat}=31.5×g_s+2.9×LN–4.5	0.74***	0.93

AB, aboveground biomass; $\Delta^{13}C_s$, shoot carbon isotope discrimination; $\Delta^{18}O_s$, ^{18}O enrichment in shoots; E, transpiration rate; g_s, stomatal conductance; C_i and C_a, intercellular and ambient CO_2 concentrations; *SLN*, specific leaf nitrogen; T_{cum}, cumulative transpiration.
*P <0.05; **P <0.01; ***P <0.001.

This resulted in a negative effect on gas exchange parameters (mainly in terms of decreasing g_s) in both well-watered and water-limited plants (in which the effect was greater, Table 1). Genotypic differences may also support this situation, as in the case of Omrabi-3, where the large increase in biomass reached in the high N treatment resulted in a clear decrease in g_s and E values. Similar results were found in field-grown rice in aerobic soils (Kondo *et al.*, 2004), where a large biomass production due to high N fertilization exacerbated water stress, which resulted in lower stomatal conductance and $\Delta^{13}C$.

Source of variation on plant isotope signatures

Carbon isotope discrimination ($\Delta^{13}C$) varied extensively between the different analysed plant parts (shoots, flags, spikes, and roots), as predicted by both theory (Hubick and Farquhar, 1989; Badeck *et al.*, 2005) and previous results reported in wheat grown under Mediterranean conditions (Araus *et al.*, 1997; Merah *et al.*, 2002). Lower $\Delta^{13}C$ values in spikes compared with the shoot or flag leaf may reflect changes in soil water availability, as well as the increase in evaporative demand occurring during the final stages of crop growth (Condon and Richards, 1992); however, the lower g_s of the spike compared with that of leaves (Araus *et al.*, 1993; Tambussi *et al.*, 2005, 2007*b*) may also be involved.

Moreover, when N treatments were compared, a significant decrease in $\Delta^{13}C$ values in plants grown under high N supply was found. According to Farquhar *et al.* (1989), a decrease in $\Delta^{13}C$ can be explained by a reduction in the ratio C_i/C_a (Table 1). This reduction in C_i/C_a may be due to either greater photosynthetic capacity or lower g_s, or to both factors (Farquhar and Richards, 1984; Condon *et al.*, 2004). Although many reports have suggested that N fertilization may decrease $\Delta^{13}C$ by lowering C_i/C_a, mainly as a result of improved carboxylation efficiency (Livingstone *et al.*, 1999; Ripullone *et al.*, 2004), a significant decrease in A_{sat} in response to N fertilization was found. This can be explained by the lower g_s values found in the high N treatments, which resulted in a significant decrease in both A_{sat} and $\Delta^{13}C$ as compared with the low N treatments.

However, within each N treatment, and regardless of differences found between N treatments, the increase in A_{sat} was associated with increases in g_s and LN (as revealed by stepwise analysis, Table 5). Nevertheless, the effect of LN was higher under low N treatments. Hence, at low N, where the effect of g_s was lower, a higher N content in leaves (SLN and LN) associated with more water input and/or N application was probably involved in lowering C_i/C_a and $\Delta^{13}C$. Conversely, at high N, differences in A_{sat} were mainly associated with changes in g_s. This is supported by the close relationships found between A_{sat} and g_s and LN (Fig. 2).

It was also observed that $\Delta^{18}O_s$ increased significantly in the water-limited treatments, while no differences in $\Delta^{18}O_s$ were found between N treatments. A similar pattern in response to differences in water status was found in bread wheat kernels (Ferrio *et al.*, 2007), where plants with the wettest conditions had the lower $\delta^{18}O$ values. Similarly, Saurer *et al.* (1997) reported a variation in $\delta^{18}O$ in the cellulose of trees grown under different soil moisture conditions, with the lowest values in those species growing with the highest moisture soil index.

Even though the four genotypes showed significant differences in cumulative transpiration rates, this was not translated into significant differences in $\Delta^{18}O_s$ between genotypes. Nevertheless, there are reports showing genotypic variability for this trait in bread wheat (Barbour *et al.*, 2000*a*; Ferrio *et al.*, 2007). However, a negative trend was observed, with higher ^{18}O enrichment in those genotypes with lower transpiration rates when grown at high N.

Stepwise analysis revealed that variation in $\Delta^{18}O_s$ was explained in both N treatments as a response to T_{cum}. In accordance with the theory (Barbour and Farquhar, 2000), where $\Delta^{18}O$ in plant organic material may provide an integrated measurement of water loss, it was found that under both N treatments $\Delta^{18}O_s$ was able to differentiate between water treatments, becoming enriched in ^{18}O as water supply decreased. This was also the case under low N levels, where differences between water regimes in terms of leaf gas exchange were less evident.

Although $\Delta^{18}O$ clearly showed differences between water treatments, the ^{18}O signature could be altered as shoots including leaves and stems were measured. It is well known that during cellulose formation in newly developing stems (sink tissues), the cleavage of the sucrose formed in leaves allows re-equilibration of some or all of the oxygen with the surrounding stem water (Barbour and Farquhar, 2000), altering the ^{18}O enrichment which has already occurred in the leaves (sucrose source). However, the shoot samples contained not only stems but also a large proportion of leaves. Nevertheless, a simplified Péclet model developed by Barbour and co-workers (http://www.ecophys.biology.utah.edu/) was used to validate the results. The model reasonably predicted the measured $\Delta^{18}O_s$ values (r=0.69 and slope 1.1) although the range of prediction was lower than that observed (Supplementary Fig. S1 available at *JXB* online).

Correlation between traits

The negative correlations observed between $\Delta^{18}O_s$ and both g_s and E are consistent with the strong relationships found between $\Delta^{18}O$ and g_s and E in bread wheat (Barbour *et al.*, 2000*a*) and in cotton (Barbour and Farquhar, 2000). However, these results contrast with the positive relationships between $\Delta^{18}O$ of leaf biomass and mean transpiration rate reported in groundnut and rice (Sheshshayee *et al.*, 2005) and, more recently, in the tropical tree, *Ficus insipida* (Cernusak *et al.*, 2007). Farquhar *et al.* (2007) have recently discussed the results of Sheshshayee *et al.* (2005) stating that when variation in E is caused by changes in the evaporative demand a positive relationship between E and isotopic enrichment can be maintained. However, when variation in E is driven by changes in g_s a negative relationship between E and isotope enrichment is expected. This is the case in the present study where, at a given VPD, a lower stomatal

conductance caused ^{18}O enrichment in plant dry matter, and a negative correlation between $\Delta^{18}O_s$ and E was observed (Table 4). A nice example is shown in Barbour and Farquhar (2000) where at a given relative humidity cotton leaves with lower g_s, and therefore lower E and higher leaf temperature, exhibited higher ^{18}O enrichment.

Regardless of the correlations between $\Delta^{18}O$ and gas exchange parameters, under the particular growing conditions of the present research, where water regimes were maintained at a constant and steady level through watering, it was found that cumulative transpiration (T_{cum}) played a pivotal role in $\Delta^{18}O$. Moreover, T_{cum} also strongly determined the amount of biomass accumulated, and this was supported by the strong positive correlations found between AB and T_{cum} (r=0.99, P <0.001 and r=0.99, P <0.001 for the low and high N treatments, respectively; Fig. 3). Therefore, a negative relationship between $\Delta^{18}O$ and AB in both N treatments was also found (Fig. 4b, Table 4).

In addition, and as suggested by Barbour *et al.* (2000*a*), under well-watered to mild water-limited conditions, and when grain yield and stomatal conductance are positively correlated, the ^{18}O signature would be a good predictor of yield. In agreement with this, a positive correlation between g_s and SB (r=0.87, P <0.01, data not shown) was found in the high N treatments, and therefore the expected correlation between SB and $\Delta^{18}O_s$ was also found (r= –0.80, P <0.01, Table 4).

Furthermore, Barbour *et al.* (2000*a*) reported that when variation in $\Delta^{13}C$ is mainly driven by changes in g_s, a negative correlation between $\Delta^{13}C$ and $\Delta^{18}O$ (or positive between $\delta^{13}C$ and $\delta^{18}O$) is expected. Saurer *et al.* (1997) also reported a linear positive relationship between $\delta^{13}C$ and $\delta^{18}O$ cellulose of trees grown under different soil moisture conditions, stating the linear dependence of the ratio C_i/C_a on the ratio e_a/e_i. In the present results, although a negative relationship was found between $\Delta^{13}C_s$ and $\Delta^{18}O_s$ (r= –0.42), this was not statistically significant. This can be explained by the fact that plants grown at high N supply displayed a $\Delta^{13}C$ variation that was not completely attributable to changes in g_s but was also influenced by intrinsic photosynthetic capacity (Table 5). As a consequence, the increase in C_i/C_a caused by increased g_s will be partially offset by a decrease in C_i/C_a associated with an increased photosynthetic capacity (Barbour *et al.*, 2000*a*), and therefore the expected correlation between $\Delta^{13}C$ and $\Delta^{18}O$ is reduced. In addition, at low N, no relationship was found between the two isotopes.

Bindumadhava *et al.* (2005) reported that the ratio C_i/g_s can be used as a rapid estimate of 'instantaneous' carbon assimilatory capacity, since at a given g_s the variation in C_i is mainly a function of photosynthetic capacity. Moreover, in their study, variation in $\Delta^{13}C$ and $\Delta^{18}O$ was mainly driven by changes in photosynthetic capacity and stomatal

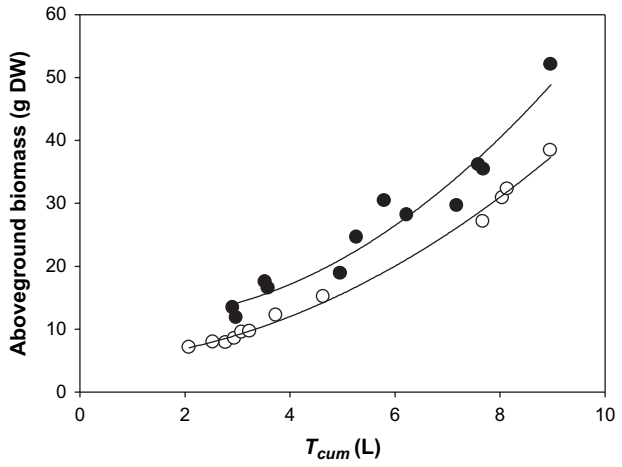

Fig. 3. Relationship between cumulative transpiration (T_{cum}) and aboveground biomass. Low N treatment y=4.35e$^{0.25x}$; r=0.99, P <0.001, n=12; high N treatment, y=7.34e$^{0.22x}$; r=0.99, P <0.001, n=12. Open and filled circles represent, respectively, the low and high N treatment means for each genotype and water condition. Each value is the mean of four replicates.

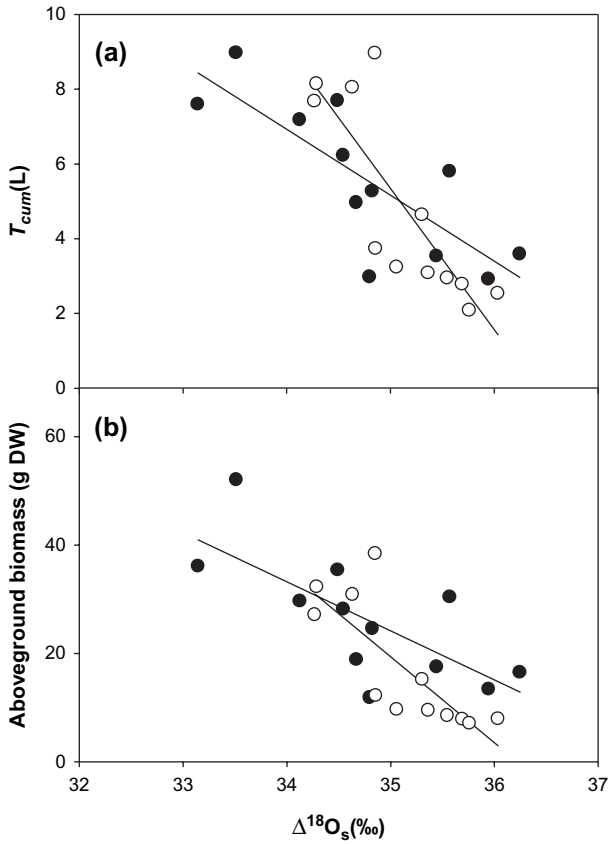

Fig. 4. (a) Relationship between the ^{18}O enrichment in shoots and cumulative transpiration, T_{cum}. Low N treatment, y= –3.78x+137.58; r=0.84, P <0.01, n=12; high N treatment, y= –1.76x+66.74; r=0.80, P <0.01, n=12; (b) Relationship between the ^{18}O enrichment in shoots and aboveground biomass. Low N treatment y= –15.87x+574.96; r=0.79, P <0.01, n=12; high N treatment, y= –9.05x+341.1; r=0.72, P <0.01, n=12. Open and filled circles represent, respectively, the low and high N treatment means for each genotype and water condition. Each value is the mean of four replicates.

conductance, respectively, and therefore they reported that the ratio $\Delta^{13}C/\Delta^{18}O$ would represent a time-averaged estimate of C_i/g_s, and hence an integrated estimation of the photosynthetic capacity during the course of crop growth. Conversely, the present results showed the inverse trend, with a negative correlation between the ratios C_i/g_s and $\Delta^{13}C/\Delta^{18}O$ ($r= -0.73$, $P<0.01$, Fig. 5b) in the high N treatments. These opposing results can be explained through consideration of the causes affecting variation in ^{13}C and ^{18}O signatures. Whereas the work of Bindumadhava *et al.* (2005) indicated that differences in $\Delta^{13}C$ were mainly driven by changes in photosynthetic capacity, the present results showed that $\Delta^{13}C_s$ was associated with changes in both g_s and intrinsic photosynthetic capacity. This could also explain why g_s and E correlated better with $\Delta^{13}C_s/\Delta^{18}O_s$ than with each isotope alone (Table 4).

In the present study, the differences in gas exchange parameters (g_s and E), as well as their correlation with stable isotopes found in the high N treatments, contrast with the lack of such results in the low N treatments. In the case of the correlation between $\Delta^{13}C$ and gas exchange parameters, this might be explained by $\Delta^{13}C$ dependence on C_i/C_a. Thus, at low N, changes in C_i/C_a across water regimes are not as dependent on g_s as at high N (see Table 5), and, in fact, increasing water input did not produce a parallel increase in g_s and E or $A_{sat.}$ In the case of $\Delta^{18}O$, instantaneous transpiration at low N is also less clearly affected by the water regime than at high N. Conversely, cumulative transpiration is highly dependent on the total leaf area, which in turn is strongly affected by the water regime in both N treatments. Thus, at high N, more water implies not just more photosynthesis and a better water status but, probably (and more importantly), higher levels of accumulated transpiration and, therefore, higher rates of plant growth. In contrast, at low N, the effect of higher quantities of irrigation leading to increased plant growth and increased cumulative transpiration is indirect, and this is mediated by greater availability of N with increasing fertirrigation. Hence, differences obtained in terms of biomass were not paralleled by significant differences in gas exchange parameters.

In addition, the strong correlation found between $\Delta^{18}O_s$ and AB in both N treatments, along with the lack of correlation of AB with $\Delta^{13}C_s$, emphazise the fact that plant growth (and thus total water transpired by the plant) more than gas exchange per unit leaf area is affected by moderate and steady water stress.

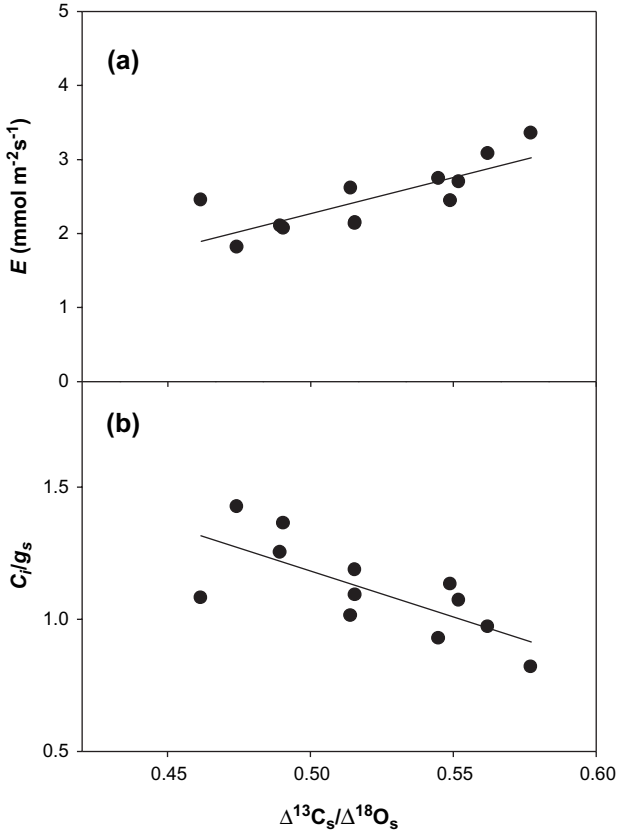

Fig. 5. Relationship between the ratio $\Delta^{13}C_s/\Delta^{18}O_s$ and transpiration rate, E (a), $y=9.77x-2.62$; $r=0.80$, $P<0.01$, $n=12$; and the ratio C_i/g_s (b), $y= -3.47x+2.92$; $r= -0.73$, $P<0.01$, $n=12$. Symbols represent the water and genotype means for the high N treatment. Each value is the mean of four replicates.

Conclusion

In accordance with the current theory of Barbour and Farquhar (2000), this study shows an increase in the ^{18}O enrichment of plant matter in water-limited plants as a consequence of a decrease in transpiration. Thus, under the particular growing conditions studied here, $\Delta^{18}O$ was strongly and negatively associated with AB regardless of the N regime. In fact, $\Delta^{18}O$ was the only trait among the stable isotope and gas exchange traits analysed that did not show an interaction between growing conditions and which was only affected by the water regime. This illustrates the usefulness of measuring $\Delta^{18}O$ to assess differences in plant growth and total transpiration. In addition, this study showed that the two isotopes, ^{13}C and ^{18}O, are not mutually exclusive and that the combined measurement of both at the plant matter level may provide a time-integrated record of the photosynthetic and evaporative performance of the plant during the course of crop growth, thus helping plant breeders to select genotypes that are better adapted to water limitation, regardless of the N status.

Supplementary data

Supplementary data are available at *JXB* online.

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