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Basis of the relationship between ash content in the flag leaf and carbon isotope discrimination in kernels of durum wheat

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Abstract

The relationship between ash content and carbon isotope discrimination (Δ) was studied in durum wheat (*Triticum durum* Desf.) grown in a Mediterranean region (Northwest Syria) under three different water regimes (hereafter referred to as environments). In two of these environments, 144 genotypes were cultivated under rain-fed conditions. In the third environment, 125 genotypes were cultivated under irrigation. Ash content was measured in the flag leaf about 3 weeks after anthesis, whereas Δ was analysed in mature kernels. Total transpiration of the photosynthetic tissues of the culm contributing, from heading to maturity, to the filling of kernels was also estimated. Leaf ash content, expressed either on dry matter or leaf area basis or as total ash per blade, correlated positively ($p< 0.001$) with Δ in the three environments. However, this relationship was not the result of a positive correlation across genotypes between Δ and tissue water content. Moreover, only a small part of the variation in Δ across genotypes was explained by concomitant changes in ash content. When all genotypes across the three environments were plotted, Δ and ash content followed a non-linear relationship (r^2 = 74), with Δ tending to a plateau as the ash content increased. However, for the set of genotypes and environments combined, total ash content per leaf blade was positively and linearly related ($r^2 = 0.76$) with the accumulated culm transpiration. The non-linear nature of the relationship between ash content and Δ is sustained by the fact that culm transpiration also showed a non-linear relationship with kernel Δ. Therefore, differences in leaf ash content between environments, and to a lesser extent between genotypes, seem to be brought about by variations in accumulated transpiration during grain formation.

Additional key words: $^{13}C/^{12}C$ ratios; dry and fresh mass; grain filling; minerals; transpiration efficiency; *Triticum durum*.

Introduction

Carbon isotope discrimination (Δ) may provide a useful indirect measure of genetic variation in transpiration efficiency (TE) in C_3 species (Farquhar *et al.* 1982, 1989, Farquhar and Richards 1984). Moreover, when measured in plant dry mass, Δ integrates TE over the period during which the dry mass was laid down. Indeed, Δ might be usefully applied in breeding programs to modify the TE and hence the yield of water-limited C_3 crop species such as wheat (Condon *et al.* 1987, Condon and Richards 1992, 1993). However, given the cost involved in and technical skills needed for carbon isotope analysis, several surrogates have been proposed for the measurement of Δ, such as the accumulation of either potassium, silicon, total mineral, or ash content in the vegetative tissues of cereals, forages, and soybean (Walker and Lance 1991, Masle *et al.* 1992, Mayland *et al.* 1993, Mian *et al.* 1996). In principle, total mineral and ash contents seem to be better surrogates of Δ than the content of just one single mineral. These measures show a higher positive correlation (and lower coefficient of variation) with Δ or the transpiration ratio (1/TE) than silicon or potassium contents (Masle *et al.* 1992, Mayland *et al.* 1993). Therefore, the estimation of plant mineral content and, in particular, that of ash content, which only requires a muffle furnace, might become attractive alternatives to Δ for a preliminary screening of large, genetically diverse populations. Subsequent selections, however, could be based on the more precise and accurate, yet costly, Δ analysis (Mayland *et al.* 1993).

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Abbreviations: Δ – carbon isotope discrimination; SFM/DM – saturated fresh to dry mass ratio; TE – transpiration efficiency; THI - Tel Hadya irrigation; THR -Tel Hadya rain-fed.
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However, before considering ash content in vegetative organs (principally leaf ash) as a good alternative to Δ, certain aspects need to be clarified, such as the mechanisms underlying the physiological association between mineral accumulation and Δ (Walker and Lance 1991, Masle *et al.* 1992; see also Rekika *et al.* 1998). This seems particularly evident when comparison is performed across environments (Masle *et al.* 1992, Mayland *et al.* 1993). In this regard Masle *et al.* (1992) indicate that ash content cannot be linearly related to transpiration rate. They suggest that other stronger mechanisms related, for example, to the control of leaf water content could explain the relationship between ash content and Δ. Another major requirement, which needs to be clarified, is the definition of the best growing environment (if such an environment exists) in which to use this alternative. From the literature on wheat and other plants (Masle *et al.* 1992, Mayland *et al.* 1993), the relation between Δ and leaf ash was not only much weaker (at times non-existent) but also a negative correlation was found for water-stressed plants.

This study was performed on a large set of durum wheat genotypes, cultivated in Mediterranean environments differing in drought level. The aim was to study the physiological association between the ash content of the flag leaf and the carbon isotope discrimination of mature kernels. In this context, the role of transpiration flux during grain filling in ash accumulation of the flag leaf was evaluated.

Materials and methods

Plants and growth conditions: A set of 144 genotypes of durum wheat (*Triticum durum* Desf.) was cultivated under rain-fed conditions in two sites (Breda and Tel Hadya) in NW Syria, where there are consistent differences in rainfall and evaporative demand. A third trial with only 125 genotypes was planted at Tel Hadya under support irrigation. Plant material and growth conditions are described in detail in a previous paper (Araus *et al.* 1997*a*). Sampling and field measurements in the rain-fed trials were performed on the four central rows of each plot. Thousand grain mass was recorded at maturity. In addition, the number of grains per spike was measured in a set of 20 stems per plot collected at random. The dates of heading and maturity were recorded in the three trials (hereafter referred to as environments). The date of heading was recorded when about half the culms showed emerging spikes. Physiological maturity was defined as the time when spike peduncles changed colour. Values were analysed using *NCSS*, version *5.03 9/91* (Dr. J.L. Hintze, Kaysville, Utah, USA).

Ash content and carbon-isotope analysis: Ash content was determined in the flag leaf blades. For each plot, a set of 20 flag leaves was sampled about 3 weeks after anthesis (before the onset of leaf senescence). Leaves were placed in a plastic tube with their cut ends immersed in distilled water. The tube was then placed inside a portable icebox, transported to the laboratory, and stored in a refrigerator (4 °C) for at least 12 h (overnight) to achieve saturation of leaves. Leaves were removed from the tube, the saturated fresh mass (SFM) was determined, and their area was measured using a leaf meter (*LI-3000*, *Li-Cor*, Lincoln, Nebraska, USA). Leaves were then dried in a forced oven at 60 °C for at least 48 h, weighed to determine the dry mass (DM), and ground. Approximately 1.3 g of dry mass was placed into a pre-weighed porcelain crucible (empty crucible), the crucible was weighed (filled crucible), and then the sample was burnt in a furnace at 450 °C for 12 h. The crucible containing the mineral residue (burnt crucible) was weighed again. For each plot, the value was the mean of either 1-2 replicates. The ash content was expressed on sample dry mass basis [%] as the ratio [(burnt crucible mass – empty crucible mass)/(filled crucible mass – empty crucible mass)] × 100. Further, total ash content per blade was calculated by multiplying the above value by the DM of the blade. Ash content was also expressed on a leaf area basis, by multiplying ash content on dry mass basis by the ratio of DM to leaf area of the same leaf, and as a percentage of saturated fresh mass by multiplying ash content by the ratio DM/SFM.

For stable carbon isotope analysis, the $^{13}C/^{12}C$ ratios of finely ground oven-dried kernels were determined by mass spectrometric analysis, as reported before (Araus *et al.* 1997*a*) at *Isotope Services* (Los Alamos, NM, USA). Results are expressed as $\delta^{13}C$ values (Farquhar *et al.* 1989), where $\delta^{13}C$ [‰] = [(R sample/R standard) – 1] × 1 000, and R is the $^{13}C/^{12}C$ ratio. A secondary standard calibrated against Peedee belemnite (PDB) carbonate was used for comparison. Samples of 5-10 mg were used. Replicate samples differed by less than 0.10 ‰. Carbon isotope discrimination (Δ, ‰) was then calculated after Farquhar *et al.* (1989) from δ_a and δ_p, where a and p refer to air and plant, respectively: $\Delta = (\delta_a - \delta_p)/(1 + \delta_p)$. On the PDB scale, free atmospheric CO_2 has a current deviation, δ_a, of approximately –8.0 ‰ (Farquhar *et al.* 1989). Total carbon content [%] per unit dry mass of the same kernels used for stable carbon isotope analysis was determined also at *Isotope Services* using an elemental analyser.

Evaluation of accumulated transpiration: The amount of water [g(H_2O)] transpired (E) by the photosynthetic organs of the culm responsible for grain filling was estimated using the relationship:

$$E = (A/\mathrm{TE})\,(18/1\,000) \qquad (1)$$

where A is net assimilation and TE the transpiration efficiency.

A of the culm [μmol(CO_2)] was calculated from the total grain mass [mg] per spike, since grain filling is the main sink of photosynthates (including those coming from pre-anthesis reserves) during that period:

$$A = (\text{kernel spike}^{-1})\ (\text{kernel mass})\ (\%\ C/100)\ (1\ 000/12) \quad (2)$$

where %C is the percentage of carbon content in kernels. We assumed for the sake of simplicity, for all the different genotypes within the three environments, constancy in the percentages of dark respiration losses as well as the amount of assimilates partitioned to growing kernels, and therefore these two parameters were ignored in the calculations.

TE [μmol(CO_2) $mmol^{-1}$(H_2O)] was estimated using Δ of mature kernels, based on the model of Farquhar *et al.* (1982):

$$TE = [p_a]\ \{1 - (\Delta - 4.4)/22.6\}/(\text{vpd}\ 1.6) \quad (3)$$

where p_a is the atmospheric partial pressure of CO_2 (35.5 Pa), and vpd is the (leaf-air) water vapour pressure difference [kPa]. Leaf temperature was assumed to be close to that of the atmosphere. Although isotope fractionation of plant carbon may occur during respiration, this effect seems to be close to zero (Hubick and Farquhar 1989).

As vpd was considered the driving force for evapotranspiration (ETP), it was estimated through:

$$\text{vpd} = \text{ETP}/g_b\ P_{atm} \quad (4)$$

where ETP_i is the instantaneous evapotranspiration [mmol(H_2O) m^{-2} s^{-1}], g_b is the boundary layer conductance of the pan evaporimeter (in the same units as ETP_i), and P_{atm} is the atmospheric pressure (101.3 kPa). For the sake of simplicity, g_b was assumed to have a constant value of 1 000 mmol(H_2O) m^{-2} s^{-1} for all the environments. It is unlikely that there would have been significant differences in g_b between environments and thus, though its value does affect the absolute estimates of E, it does not affect the relative differences of E between environments. Instantaneous ETP was determined for each genotype from the measured total pan ETP from heading to maturity [kg(H_2O) m^{-2}] divided by the duration of this period in seconds:

$$ETP_i = (\text{pan ETP} \times 10^6)/(18 \times 24 \times 3\ 600 \times \text{DGF}) \quad (5)$$

where DGF is the duration of grain filling in days for each genotype. Since the contribution from pre-anthesis reserves to total grain mass per spike needs to be included, DGF was considered as the number of days from heading (when the culm has almost completed the growth of its vegetative parts) to maturity, instead of the number of days from anthesis to maturity. Therefore, in our calculation, E can be assumed as an estimation of the total water transpired by the culm from heading to maturity.

Results and discussion

Ash content, Δ, and yield components were affected by the environment. Indeed the three environments assayed in this work provide a broad range of variability for all these parameters (Table 1). Thus, in Tel Hadya Irrigation (THI) ash content (expressed either on dry mass basis or leaf area) in the flag leaf blade around 3 weeks after anthesis was nearly 3 times that recorded in Breda and nearly 7 times when ash content was expressed in terms

Table 1. Different parameters of mineral accumulation of the flag leaf blade, the carbon isotope discrimintion of kernels (kernel Δ), the kernel mass, and the number of kernels per spike in durum wheat cultivated in three different environments of Northwest Syria. Two of them were rain-fed trials located at Breda and Tel Hadya (THR), whereas the other was a supplementary irrigation trial placed at Tel Hadya (THI). Flag leaves were sampled about 3 weeks after anthesis and the following parameters were measured: ratio of saturated fresh-mass-to-dry-mass (SFM/DM) and ash content. Leaf ash content was expressed on DM, SFM, and leaf area basis as well as per total flag leaf blade. Kernels were sampled at maturity. Values presented are means ± standard deviation of the 144 genotypes (125 from the Durum Core Collection of ICARDA) assayed in the rain-fed trials, or the same 125 genotypes of the DCC cultivated under support irrigation. Means bearing different letter are significantly different ($p < 0.05$) by Duncan's comparison test. Range of genotype values within each trial is also included in the table.

		Breda				THR				THI			
		Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
SFM/DM		3.29^A	0.27	2.85	4.14					3.57^B	0.22	3.00	4.31
Leaf ash:	% DM	5.00^A	0.60	3.60	6.40	8.2^B	1.0	5.4	10.8	13.5^C	1.5	9.9	16.6
	% SFM	1.89^A	0.22	1.40	2.46					4.27^B	0.48	2.91	5.29
	[mg m^{-2}]	302^A	38	201	407	442^B	60	281	624	757^C	122	1148	447
	[mg $blade^{-1}$]	3.52^A	0.78	1.78	5.59	6.78^B	1.46	2.59	10.30	24.58^C	6.65	9.69	45.25
Kernel Δ	[%]	14.0^A	0.4	13.0	15.2	15.3^B	0.4	13.8	16.2	16.7^C	0.5	15.2	17.7
Kernel mass	[mg]	41.2^A	4.6	28.3	56.0	45.2^B	4.9	31.5	59.0	45.1^B	6.1	28.0	59.3
Kernel	per spike	22.0^A	6.3	3.4	38.0	26.2^B	5.4	9.5	41.5	43.1^C	8.5	23.4	63.1

of total blade. The number of kernels per spike was about 20 and 95 % greater in Tel Hadya rainfed (THR) and THI, respectively, than in Breda, whereas kernel mass was only 10 % higher in THR and THI than in Breda. Highly significant differences in Δ were also found between the three environments (Table 1). Differences in plant water status, with a progressive improvement from Breda to THI (Araus *et al.* 1997*a*), appear to explain the above differences between environments in ash content, Δ and yield components.

Relationships across genotypes between ash content, Δ, and transpiration: Ash content, expressed either on dry mass or leaf area basis or per total blade area, was positively correlated with kernel Δ across genotypes. This relationship was present not only within the support irrigation environment, which coincides with previous reports on wheat, barley, and other species (Walker and Lance 1991, Masle *et al.* 1992, Mayland *et al.* 1993), but also in the two rain-fed environments where correlation, although lower, was still significant ($p < 0.001$). The positive correlation across environments between leaf ash and kernel Δ might indicate that plants that are able to maintain higher stomatal conductance and *E* during grain filling (and therefore show higher kernel Δ), accumulate more ash in a transpiring organ such as the flag leaf blade, provided the transport and accumulation of minerals in the plant is (at least in part) through the transpiration stream (Walker and Lance 1991). However, in our results only a small part of the variation in Δ within environments could be explained by concomitant changes in the ash content, which is consistent with previous reports (Masle *et al.* 1992, Mayland *et al.* 1993). Thus, the determination coefficient (r^2) of this relationship was about 0.25 within THI and around 0.20 in the rain-fed environments. Furthermore, Masle *et al.* (1992) point out that whereas a linear relationship between mineral content and Δ would arise if minerals were passively transported into the shoot *via* the xylem, this does not seem to fit some of the available data. Alternatively, they suggest that the positive correlation between mineral content and Δ could be brought about by a positive correlation across genotypes between Δ and tissue water content (expressed as fresh-to-dry-mass ratio), with the variations in this trait largely overriding those in mineral concentration in fresh tissue (Masle *et al.* 1992). If this were the case, the variation across genotypes in mineral content expressed on a fresh-mass basis would be much smaller than that expressed on a dry mass basis (Masle *et al.* 1992). Indeed, there are several examples in the literature where differences in mineral concentrations in dry mass, across genotypes or environments, disappear when expressed on a fresh-mass basis (Pitman 1988, Tanner and Beevers 1990, Clarkson *et al.* 1992). However, our results show that the variability in ash content of the flag leaf, either across genotypes (*i.e.* within environments) or between environments, was similar when values were expressed on a dry mass or saturated fresh mass basis (Table 1). In addition, variations in the saturated fresh-to-dry-mass-ratio (SFM/DM) across genotypes, and particularly between environments, were lower than those observed in ash concentration on a dry mass basis (Table 1). Moreover, positive correlations within environments between kernel Δ and SFM/DM were, at least for THI, much lower than those reported between kernel Δ and leaf ash on a fresh-mass basis ($r^2 = 0.01$, n.s. compared with $r^2 = 0.23$, $p < 0.001$, respectively).

Additionally, we examined the relationship between the total ash accumulated in the flag leaf blade and the estimated amount of water transpired (from heading to maturity) by the photosynthetic organs of the culm providing assimilates to the filling of grains. Within environments, linear relationships between the ash accumulated and the water transpired were positive, significant ($p < 0.001$), and somewhat higher than those between ash and Δ (mean r^2 of around 0.25 for the rain-fed environments and $r^2 = 0.36$ for THI). The positive linear pattern of the relationships between water transpired and the ash accumulated support an accumulation of minerals driven by mass flow transport and transpiration, which is associated with the assimilation activity of the flag leaf and the other photosynthetic organs providing assimilates to growing kernels. The absence of stronger relationships within environments between ash and transpiration could be due to genotype differences in the relative photosynthetic and transpiration contribution of the flag leaf blade to grain filling. Indeed, whereas this plant part is one of the photosynthetic organs that is active during the filling of grains, frequently it is not the main contributor to this process (see Araus *et al.* 1993 for durum wheat). Differences between genotypes in other processes, for example in active accumulation of minerals such as potassium (and perhaps silicon), the degree of osmotic adjustment driven by mineral solutes and ion uptake from soil, or just the pattern of mineral retranslocation during grain filling to the reproductive sink, among others, may also be responsible for differences in leaf ash between genotypes.

Relationships across environments between ash content, Δ, and transpiration: When all the genotypes and environments were combined, a strong non-linear relationship between ash content and kernel Δ was observed. Thus, Δ tended to a plateau, with a progressive decline in the growth rate of Δ as the ash content increased. Thus, for the relationship between Δ and the logarithm (on a decimal basis) of total ash content of the flag leaf blade, r^2 was 0.74 (Fig. 1*A*). However, when considering the set of genotypes and environments together a strong positive linear relationship ($r^2 = 0.76$) was observed between total

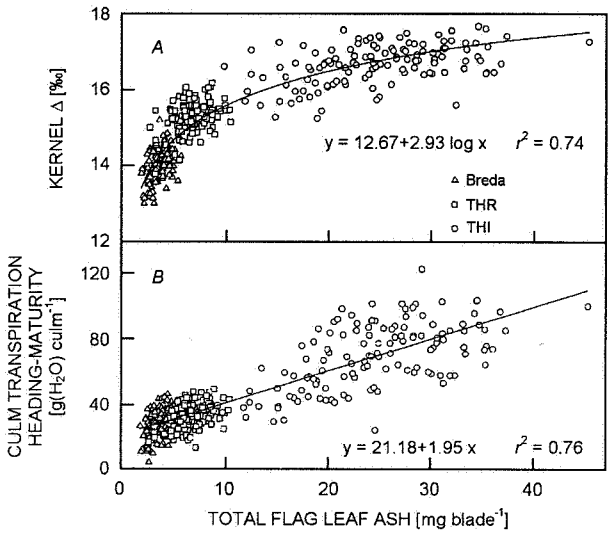

Fig. 1. Relationship between the total ash content of flag leaf blade around 3 weeks after anthesis and either the carbon isotope discrimination (Δ) of mature kernels (*A*) or the accumulated transpiration from heading to maturity of the photosynthetic organs of the culm contributing to the filling of grains (*B*). Plants were cultivated in three trials of different water status: Breda, Tel Hadya rainfed (THR), and Tel Hadya with supplementary irrigation (THI). Within a given trial each point represents an individual genotype. The line of fit, equation, and coefficient of determination (r^2) of the relationship for all the genotypes and trials considered together are also shown.

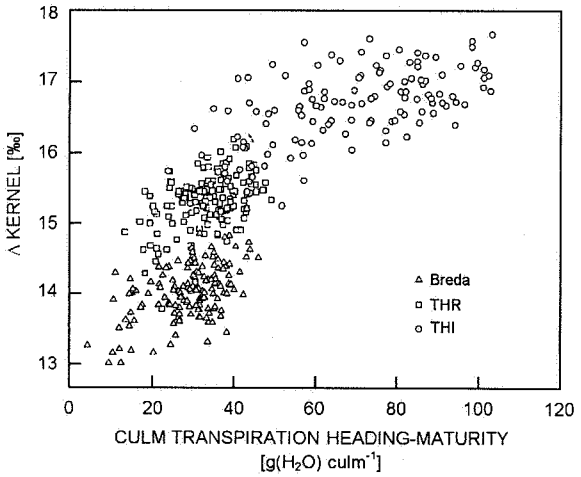

Fig. 2. Relationship between the estimated accumulated transpiration (from heading to maturity) of the photosynthetic organs of the culm contributing to grain filling and the carbon isotope discrimination (Δ) of mature kernels. For details on the estimation of culm transpiration see Materials and methods. Plants were cultivated in three trials of different water status: Breda, Tel Hadya rainfed (THR), and Tel Hadya with supplementary irrigation (THI). Within a given trial each point represents an individual genotype.

ash content of the flag leaf blade and the accumulated transpiration of the photosynthetic organs of the culm from heading to maturity (Fig. 1*B*). The positive, linear relationship seems to indicate that differences across environments in leaf ash are due basically to variations in transpiration sustaining a passive mineral transport and accumulation. The non-linear nature of the relationship between the total ash content of the flag leaf and kernel Δ could be sustained by recent reports on durum wheat (Araus *et al.* 1999) and barley (Araus *et al.* 1997*b*) showing that kernel Δ is strongly correlated with the logarithm of the total water input from heading to maturity. Consistently our results also showed a non-linear relationship of the calculated culm transpiration with kernel Δ (Fig. 2). In contrast, another report (though not in cereals or within just a single species) shows that Δ and water input are linearly correlated (Stewart *et al.* 1995). Nevertheless, a non-linear relationship between transpiration and Δ can be supported by the fact that in photosynthetic tissues the assimilation-weighed ratio of intercellular to atmospheric concentration of CO_2 (on which Δ directly depends) does not follow a linear pattern of increase with increasing stomatal conductance (Nobel 1991).

The strong linear relationship between ash content and culm transpiration (Fig. 1*B*) does not agree with a previous report where ash (on dry mass basis) was not consistently related to Δ across environments (Mayland *et al.* 1993). These authors concluded that mineral accumulation in leaves from water stressed plants seems to be greater than expected through a pasive mineral transport and accumulation driven by transpiration. Moreover, Masle *et al.* (1992) reported that the correlation between transpiration and ash content was absent when variations in transpiration were induced by changes in atmospheric humidity or CO_2 concentration. Nevertheless, the experimental conditions in this study, with strong differences in soil water availability across environments (see climatic conditions in Araus *et al.* 1997*a*), differed from those in Masle *et al.* (1992).

In conclusion, the present study shows that in durum wheat the differences in flag leaf ash between environments, and to a lesser extent between genotypes, are brought about by variations in accumulated transpiration during grain filling. This would be the basis of the non-linear relationship between leaf ash and kernel Δ across the environments. Within each environment, even when only a small part of those differences between genotypes in kernel Δ may be explained by differences in the amount of minerals accumulated in the flag leaf, the ash content is still a potentially useful indicator of Δ in kernels, and can be used, for example, as an indirect trait to culm genotypes with contrasting Δ. Thus, kernel Δ was analysed within each environment in two subsets of 15 genotypes each, representing the highest and lowest values of ash content in the flag leaf. The subset with the highest values of leaf ash consistently showed significantly higher kernel Δ ($p < 0.001$), regardless of the environment in which it was tested.

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