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How yield relates to ash content, $\Delta^{13}C$ and $\Delta^{18}O$ in maize grown under different water regimes

Llorenç Cabrera-Bosquet[1,2]**, Ciro Sánchez**[2] **and José Luis Araus**[2,*]

[1]*Unitat de Fisiologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, 08028 Barcelona, Spain and*
[2]*International Maize and Wheat Improvement Center (CIMMYT), El Batán, Texcoco, CP 56130, Mexico*

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• *Background and Aims* Stable isotopes have proved a valuable phenotyping tool when breeding for yield potential and drought adaptation; however, the cost and technical skills involved in isotope analysis limit its large-scale application in breeding programmes. This is particularly so for $\Delta^{18}O$ despite the potential relevance of this trait in C_4 crops. The accumulation of minerals (measured as ash content) has been proposed as an inexpensive way to evaluate drought adaptation and yield in C_3 cereals, but little is known of the usefulness of this measure in C_4 cereals such as maize (*Zea mays*). The present study investigates how yield relates to ash content, $\Delta^{13}C$ and $\Delta^{18}O$, and evaluates the use of ash content as an alternative or complementary criterion to stable isotopes in assessing yield potential and drought resistance in maize.

• *Methods* A set of tropical maize hybrids developed by CIMMYT were subjected to different water availabilities, in order to induce water stress during the reproductive stages under field conditions. Ash content and $\Delta^{13}C$ were determined in leaves and kernels. In addition, $\Delta^{18}O$ was measured in kernels.

• *Key Results* Water regime significantly affected yield, ash content and stable isotopes. The results revealed a close relationship between ash content in leaves and the traits informing about plant water status. Ash content in kernels appeared to reflect differences in sink–source balance. Genotypic variation in grain yield was mainly explained by the combination of ash content and $\Delta^{18}O$, whilst $\Delta^{13}C$ did not explain a significant percentage of such variation.

• *Conclusions* Ash content in leaves and kernels proved a useful alternative or complementary criterion to $\Delta^{18}O$ in kernels for assessing yield performance in maize grown under drought conditions.

Key words: Ash content, $\Delta^{13}C$, $\Delta^{18}O$, *Zea mays*, water stress, drought resistance, grain yield.

INTRODUCTION

Drought is the major factor constraining world maize (*Zea mays*) production (Bänziger and Araus, 2009), particularly in tropical areas (Edmeades *et al.*, 1989) where rain-fed maize is a staple food for millions of people. Thus, improving yield potential and drought adaptation are among the main targets in most maize breeding programmes. Yet the evaluation of appropriate phenotyping traits remains one of the bottlenecks in cereal breeding for yield potential and drought adaptation (Araus *et al.*, 2008). In the case of maize, a short anthesis-to-silking interval has proved a useful trait (Bänziger *et al.*, 2000), but recurrent use over breeding cycles leads to a reduction in their genetic variation and further advances in this regard remain uncertain (Monneveux *et al.*, 2008).

Carbon isotope discrimination ($\Delta^{13}C$) has been used in C_3 cereals as a surrogate method for screening genotypes with higher water-use efficiency (WUE; the ratio of biomass accumulation to water consumed) (Farquhar and Richards, 1984; Rebetzke *et al.*, 2002); and some drought-tolerant cultivars have been bred using low $\Delta^{13}C$ (i.e. high WUE) as a selecting trait (Rebetzke *et al.*, 2002). However, few reports exploring the use of $\Delta^{13}C$ in maize are available (but see Heng *et al.*, 2005; Dercon *et al.*, 2006; Monneveux *et al.*, 2007) and the results are not encouraging, mainly due to the C_4 metabolism of this species, given that C_4 plants are characterized by lower $\Delta^{13}C$ than C_3 plants (Farquhar, 1983; Bowman *et al.*, 1989). Nevertheless, $\Delta^{13}C$ has been successfully used in other species with C_4 metabolism, such as sorghum (*Sorghum bicolor*), to track genotypic differences in transpiration efficiency (i.e. instantaneous WUE at the leaf level) and yield (Hubick *et al.*, 1990; Hammer *et al.*, 1997; Henderson *et al.*, 1998). Therefore, further investigation is justified in maize plants grown under a wide range of growing conditions. Moreover, although much attention has focused on improving WUE when breeding for drought adaptation, it seems that, except for very severe drought conditions, water use (WU, i.e. the total water absorbed and further transpired by the plant) is a more important adaptive trait than WUE (Araus *et al.*, 2002*b*, 2008; Blum, 2005, 2009; Slafer and Araus, 2007). This is related to the genotypic capacity to use available water and therefore to sustain transpiration under unfavourable conditions (Blum, 1993; Morgan *et al.*, 1993; Slafer *et al.*, 1999). Thus, when analysed in kernels (or other organs developed late during the crop cycle) of C_3 cereals, high $\Delta^{13}C$ seems to reflect genotypic differences in WU (Araus *et al.*, 2003). This in turn may explain the positive relationship between high $\Delta^{13}C$ (and thus high WU) and yield in a wide range of growing conditions, ranging from fully

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* For correspondence. E-mail j.araus@cgiar.org

watered to drought stresses decreasing yield by up to approx. 70 % (Araus *et al.*, 2002*b*, 2003; Slafer and Araus, 2007).

Alternatively, oxygen isotope enrichment ($\Delta^{18}O$) measured in plant tissue can be used either in C_3 or in C_4 grasses (Helliker and Ehleringer, 2002; Ogée *et al.*, 2007; Araus *et al.*, 2008), owing to its independence of photosynthetic processes, and has therefore been proposed as an indirect measure of transpiration (Sheshshayee *et al.*, 2005; Cernusak *et al.*, 2007, 2008) and WU (Cabrera-Bosquet *et al.*, 2009a), as well as an integrative indicator of genetic differences in stomatal conductance (g_s) and yield in cereals such as wheat (Barbour *et al.*, 2000; Ferrio *et al.*, 2007; Cabrera-Bosquet *et al.*, 2009*a*) and maize (Cabrera-Bosquet *et al.*, 2009*b*). However, given the cost (over US $15 per sample, twice that for $\Delta^{13}C$) and technical skills and facilities involved in oxygen isotope analysis, its large-scale application to breeding programmes is at present unfeasible.

The accumulation of mineral or ash content in both vegetative tissues (Masle *et al.*, 1992; Mayland *et al.*, 1993; Araus *et al.*, 1998; Monneveux *et al.*, 2004) and kernels (Febrero *et al.*, 1994; Voltas *et al.*, 1998; Araus *et al.*, 1998; Merah *et al.*, 1999, 2001) have been proposed as inexpensive (approx. US $2 per sample) and simple ways to predict yield and genotypic adaptation to drought in different C_3 cereals. The mechanism of mineral accumulation in vegetative tissues appears to be explained through the passive transport of minerals via xylem driven by transpiration (Masle *et al.*, 1992; Mayland *et al.*, 1993; Araus *et al.*, 2002*a*, *b*). Conversely, mineral accumulation in mature kernels takes place via phloem (Nonogaki *et al.*, 2007). Thus, whereas ash content measured in vegetative tissues provides an indicator of transpirative gas-exchange activity and therefore of the total water transpired (Araus *et al.*, 1998, 2001), ash content in mature kernels could provide information on the integrated photosynthetic and retranslocation processes during grain filling (Febrero *et al.*, 1994; Voltas *et al.*, 1998; Araus *et al.*, 1998, 2001). In such a way, leaf and kernel ash content have been correlated with yield in barley (Febrero *et al.*, 1994; Voltas *et al.*, 1998) and wheat (Araus *et al.*, 1998; Merah *et al.*, 1999, 2001; Monneveux *et al.*, 2004) grown under different water regimes. Interestingly, such an approach can be used in both C_3 and C_4 crops (Masle *et al.*, 1992; Araus *et al.*, 2001). However, the only existing report in maize (Tanner and Beevers, 1990) investigated vegetative tissues in plants grown in both hydroculture and soil in pots, and the relationships between ash content and genotypic differences in grain yield were not explored.

In a recent study, the physiological basis of variation on $\Delta^{18}O$ measured in leaves and kernels was examined, as well as its feasibility to track differences in growth, yield and water status of a set of tropical maize hybrids grown under three contrasting water regimes (Cabrera-Bosquet *et al.*, 2009*b*). Following this research, as a first objective, the present investigation was developed to study the effect of inducing water stress during the reproductive stages on mineral accumulation in leaves and kernels (measured as ash content) and $\Delta^{13}C$ in comparison with $\Delta^{18}O$ in kernels. Secondly, the potential use of ash content and $\Delta^{13}C$ as alternative or complementary criteria to stable $\Delta^{18}O$ in the evaluation of differences in yield potential and drought resistance in maize were studied.

MATERIALS AND METHODS

Germplasm and growth conditions

A set of 16 maize (*Zea mays* L.) single hybrids were used, together with one commercial hybrid ('Puma') used as a check. Briefly, single hybrids were generated by crossing drought-tolerant lines derived from a *La Posta Sequía* (*LPS*) population with the tropical testers CML-449 and CML-495. The *LPS* population is a white dent (Tuxpeño-related synthetic) with improved drought tolerance (International Maize and Wheat Improvement Center, CIMMYT). Selection schemes are detailed elsewhere (see Pandey *et al.*, 1986; Monneveux *et al.*, 2008). Trials took place at the CIMMYT's experimental station in Tlaltizapán, Mexico (18°41′N, 99°07′W, 940 m asl) during the 2007 dry season. The soil of the experimental field is a calcareous vertisol (1·3–1·8 m deep), defined as Isothermic Udic Pellustert according to USDA soil taxonomy, with a pH of 7·6. Climate conditions during the maize-growing season are detailed in Fig. 1. Entries were planted on 14 December, 2006 in one-row plots (5-m rows with 0·25-m spacing within plants and 0·75 m between rows), with a final plant density of 6·67 m^{-2}. Full irrigation (well-watered, WW) and two different levels of water stress were assayed. Each of the three water regimes was set up as a randomized complete block design with three replications per genotype, resulting in a total of 153 rows. Sprinkler irrigation was applied in all treatments after sowing, to ensure homogeneous germination. The WW trials were also irrigated by furrow irrigation every 2 weeks during the entire cycle. Intermediate (IS) and severe (SS) water stress treatments were also irrigated every 2 weeks until water stress was imposed by deficit irrigation of the trials from 1 month after sowing and withholding irrigation around 2 and 4 weeks before anthesis (i.e. tassel flowering), respectively, and irrigating once again 1 week after anthesis. Trials under WW, IS and SS conditions received 1510·7, 685·7 and 335·7 mm of water input (i.e. irrigation plus precipitation), respectively, making their averaged ratio between total water input and reference evapotranspiration (WI/ETP) 1·5, 1·0 and 0·5, respectively. Nitrogen fertilizer was applied before and 35 d after sowing (V6 stage, Ritchie *et al.*, 1993), using a dose of 80 kg ha^{-1} of urea on both occasions. All trials received 80 kg ha^{-1} of phosphorous as a calcium superphosphate triple [$Ca\ (H_2PO_4)_2.H_2O$], applied prior to sowing. No potassium was applied, as previous tests showed no response to this element in these soils. Experiment plots were kept free of weeds, insect pests and diseases by recommended chemical measures as described in Monneveux *et al.* (2008).

Plant water status and yield

Leaf stomatal conductance (g_s) and leaf temperature were measured in the abaxial surface of sun-exposed leaves from the upper part of the plant by using a Decagon SC-1 Leaf Porometer (Decagon Device Inc., Pullman, WA, USA) during the period covering pre-anthesis and grain filling. Measurements were performed in the 4 h around solar noon on sunny and windless days. g_s measurements were expressed as crop mean values. Leaf evaporative conditions were further

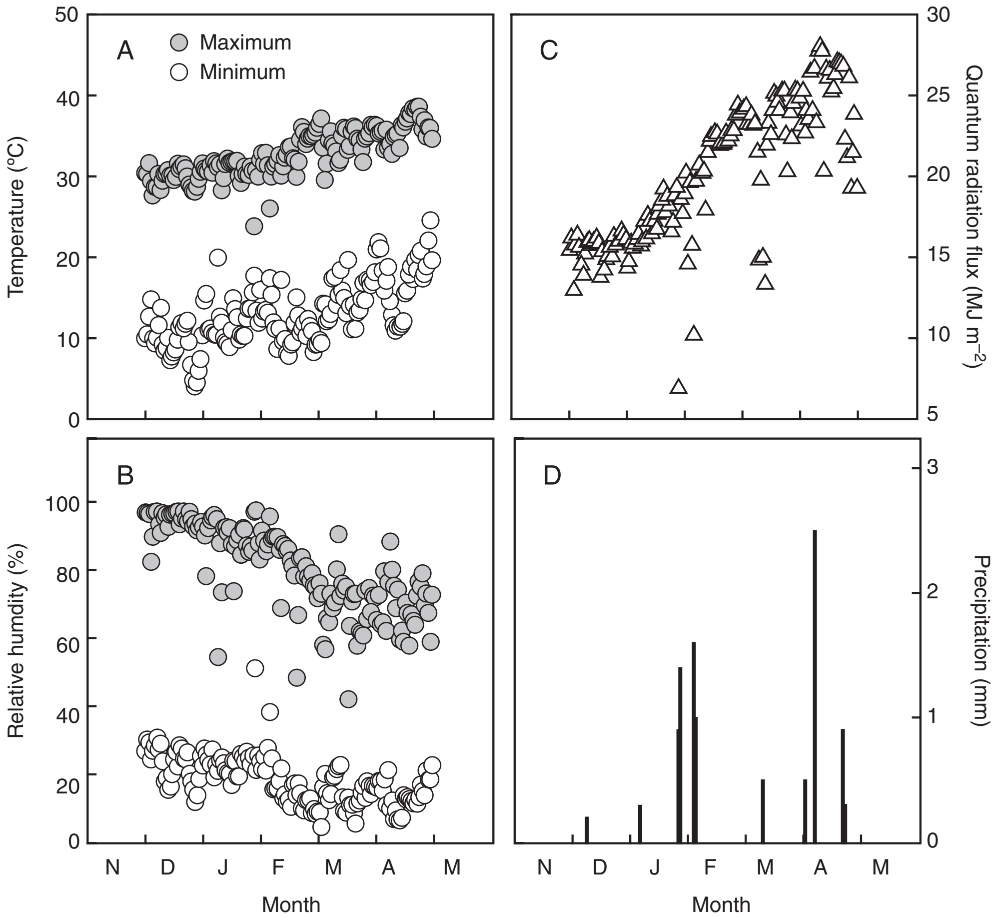

FIG. 1. Maximum and minimum daily air temperatures (A), maximum and minimum daily relative air humidity (B), total daily quantum radiation flux (C) and daily precipitation (D) during the maize-growing season at the Tlaltizapán CIMMYT's Experimental Station, Mexico (18°41′N, 99°07′W, 940 m asl) from December, 2006 to April, 2007. Total precipitation throughout the maize-growing season was 10·7 mm.

calculated. Thus, leaf and air temperatures and air relative humidity were used to calculate the ratios of atmospheric to intercellular space vapour pressure (e_a/e_i). In addition, green leaves from one entire plant per plot (sampled 2 weeks after anthesis) were used for measuring leaf water content (LWC) as LWC (%) = 100 × (f. wt − d. wt)/f. wt, where f. wt and d. wt refer to the fresh and dry weight of leaves, respectively. g_s, leaf temperature and LWC measurements were performed in three plants per genotype and water treatment. At maturity, grain yield (GY) and its main agronomical components, such as kernel number per hectare and kernel mass, were determined over the entire plots. Kernel mass was calculated as the average of 300 kernels randomly selected from each plot.

Leaf and kernel ash content and carbon and oxygen isotope analyses

The same leaves used for LWC determination and mature kernels were used for ash content analysis. Samples were oven-dried at 60 °C for 48 h and ground. Approximately 2 g of dry mass (either leaves or kernels) was placed in pre-weighed porcelain crucibles. Samples were burnt in a muffle furnace for 6–8 h at 600 °C. The mineral residue was then weighed. Results are expressed as percentage of dry mass.

The $^{13}C/^{12}C$ ratios (R) of leaves and kernels were analysed using an elemental analyser (Carlo Erba 2100, Milan, Italy) interfaced to an isotope ratio mass spectrometer (IRMS; Thermo-Finnigan Deltaplus Advantage, Bremen, Germany) at the Colorado Plateau Stable Isotope Laboratory (CPSIL). Results are expressed as $\delta^{13}C$ values, using a secondary standard calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB) and the analytical precision was about 0·1 ‰:

$$\delta^{13}C = (R_{\text{sample}}/R_{\text{standard}}) - 1. \qquad (1)$$

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

$$\Delta^{13}C = (\delta^{13}C_a - \delta^{13}C_p)/[1 + (\delta^{13}C_p/1000)] \qquad (2)$$

where $\delta^{13}C_a$ and $\delta^{13}C_p$ refer to air and plant carbon isotope compositions, respectively. $\delta^{13}C$ of free atmospheric CO_2 was taken as −8 ‰ (Farquhar *et al.*, 1989).

The $^{18}O/^{16}O$ ratios (R) of irrigation water were determined by the CO_2/H_2O equilibration technique and using an isotope ratio mass spectrometer (Delta S Finnigan MAT, Bremen, Germany) at the Scientific Facilities of the University of Barcelona. The $^{18}O/^{16}O$ ratios of kernel samples were analysed at the CPSIL via pyrolysis over glassy carbon at 1350 °C using a Thermo-Electron thermo-chemical elemental analyser interfaced via a CONFLO-II to a Thermo-Electron Delta Plus XL gas IRMS. Results were expressed as $\delta^{18}O$ values, using two secondary standards (IAEA 601 and IAEA 602) calibrated against to the Vienna Standard Mean Oceanic Water (VSMOW), and the analytical precision was about 0·3 ‰ for dry matter and 0·2 ‰ for irrigation water:

$$\delta^{18}\mathrm{O} = (R_{\mathrm{sample}}/R_{\mathrm{standard}}) - 1. \tag{3}$$

Then, the ^{18}O enrichment in kernels ($\Delta^{18}O$) was calculated as follows:

$$\Delta^{18}\mathrm{O} = (\delta^{18}\mathrm{O}_p - \delta^{18}\mathrm{O}_{\mathrm{iw}})/[1 + (\delta^{18}\mathrm{O}_{\mathrm{iw}}/1000)] \tag{4}$$

where $\delta^{18}O_p$ and $\delta^{18}O_{iw}$ refer to the oxygen isotope compositions of plant sample and irrigation water, respectively ($\delta^{18}O_{iw}$ was approx. −10·78 ‰).

Ash content as well as oxygen and carbon isotope analyses of leaves and kernels were performed for each plot individually.

Statistical analysis

Analysis of variance (ANOVA) was performed to calculate the effects of water treatment and genotype. Means were compared using a Duncan's multiple comparison test ($P < 0{\cdot}05$), and a bivariate correlation procedure was used to analyse the correlation between measured traits. Data were analysed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS

Effects of water regime and genotype on growth traits, ash content and stable isotopes

Water regime and genotype significantly affected yield, kernel number per hectare and kernel mass, with the water regime exerting the greater effect as revealed by ANOVA (Table 1). Among hybrids, yield ranged from 5294 to 8877, 580 to 2606 and 139 to 845 kg ha^{-1}, in the WW, IS and SS water treatments, respectively. Crop mean g_s was significantly different for the three water treatments. LWC values were the same for the WW and IS treatments, but significantly lower in the most water-stressed (SS) plants (Table 1). Furthermore, genotypic variation for these traits was also found. Leaf and kernel ash were greatly affected by water regime (82 and 78 % variability, respectively), but genotypic differences were also found (Table 1). Leaf ash content decreased significantly: by 26 and 33 % from WW to IS and from WW to SS water stress treatments, respectively. However, kernel ash content followed the opposite trend, with increases of 30 and 54 % in the IS and SS treatments over the WW plants. Kernel $\Delta^{18}O$ was also affected by water regime (71·7 %) and genotype (9·1 %), with significant increases of 1·1 and 2·3‰ in the IS and SS treatments, respectively, with respect to WW plants (Table 1). Furthermore, both leaf and kernel $\Delta^{13}C$ significantly increased with water stress: by 0·34 and 0·49‰ in leaves, and 0·77 and 0·81‰ in kernels in the IS and SS treatments compared with the WW plants, respectively. Genotypic differences in kernel and leaf $\Delta^{13}C$ were also found. However, in contrast to kernel $\Delta^{18}O$, leaf $\Delta^{13}C$ and ash contents, when the check (commercial 'Puma') was removed and only the 16 genotypes derived from the *LPS* population were considered, no genotypic differences in kernel $\Delta^{13}C$ were found (data not shown). In addition, regardless of the water treatment, higher $\Delta^{13}C$ values were found in leaves than in kernels.

Relationships between ash content, stable isotopes and water status

Significant negative correlations were observed between kernel $\Delta^{18}O$ and leaf ash content across genotypes within each water treatment (Fig. 2A), whereas no relationship was found between kernel and leaf ash content (Fig. 2B). No relationship was found between kernel $\Delta^{18}O$ and kernel ash content (data not shown). In addition, leaf ash content correlated positively with the ratio of atmospheric to intercellular space vapour pressure (e_a/e_i) ($r = 0{\cdot}91$, $P < 0{\cdot}001$) across genotypes and water treatments, whereas kernel $\Delta^{18}O$

TABLE 1. *Grain yield (GY) and related agronomical yield components, g_s, LWC, leaf and kernel ash content, leaf and kernel $\Delta^{13}C$, and kernel $\Delta^{18}O$ of a set of maize hybrids grown under three different water regimes (WW, IS and SS)*

				Source of variation			
	WW	IS	SS	E	G	G × E	Error
GY (kg ha^{-1})	7487·2 ± 208·1^{a}	1631·4 ± 95·6^{b}	434·2 ± 31·5^{c}	91·9***	2·1***	1·8$^{n.s.}$	4·1
Kernels per ha (×10^{5})	248·0 ± 6·0^{a}	107·9 ± 6·4^{b}	29·7 ± 2·3^{c}	84·8***	4·5***	3·8**	5·4
Kernel mass (mg)	301·3 ± 3·4^{a}	151·8 ± 3·1^{b}	148·9 ± 5·2^{b}	89·0***	2·1***	2·4$^{n.s.}$	6·3
g_s (mmol m^{-2} s^{-1})	201·4 ± 5·7^{a}	136·8 ± 4·3^{b}	116·7 ± 2·9^{c}	56·6***	9·7**	9·1$^{n.s.}$	24·6
LWC (%)	83·2 ± 0·1^{a}	82·7 ± 0·2^{a}	79·3 ± 0·3^{b}	45·5***	14·4***	12·7$^{n.s.}$	27·4
Leaf ash (%)	18·6 ± 0·2^{a}	13·7 ± 0·2^{b}	12·4 ± 0·2^{c}	82·0***	3·7**	4·2$^{n.s.}$	8·9
Kernel ash (%)	1·11 ± 0·01^{c}	1·44 ± 0·03^{b}	1·71 ± 0·01^{a}	78·4***	4·2*	3·1$^{n.s.}$	14·1
Leaf $\Delta^{13}C$ (‰)	5·29 ± 0·03^{c}	5·63 ± 0·03^{b}	5·78 ± 0·03^{a}	48·8***	11·2**	8·9$^{n.s.}$	31·1
Kernel $\Delta^{13}C$ (‰)	3·81 ± 0·02^{b}	4·58 ± 0·03^{a}	4·62 ± 0·04^{a}	84·2***	6·9***	2·8$^{n.s.}$	6·1
Kernel $\Delta^{18}O$ (‰)	40·4 ± 0·1^{c}	41·5 ± 0·1^{b}	42·7 ± 0·1^{a}	71·7***	9·1***	5·3$^{n.s.}$	14·2

WW, well-watered plants; IS, intermediate water stress; SS, severe water stress. n.s., not significant; *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Data are the mean ± s.e. of 51 values (corresponding to 17 genotypes and three replications per genotype). Values with different superscript letters are significantly different according to Duncan's multiple range test, $P < 0{\cdot}05$. Analysis of variance for the same variables is shown for the water regime environment (E), genotype (G) and interaction (G × E) effects. Results are expressed as the associated percentages of the sum of squares.

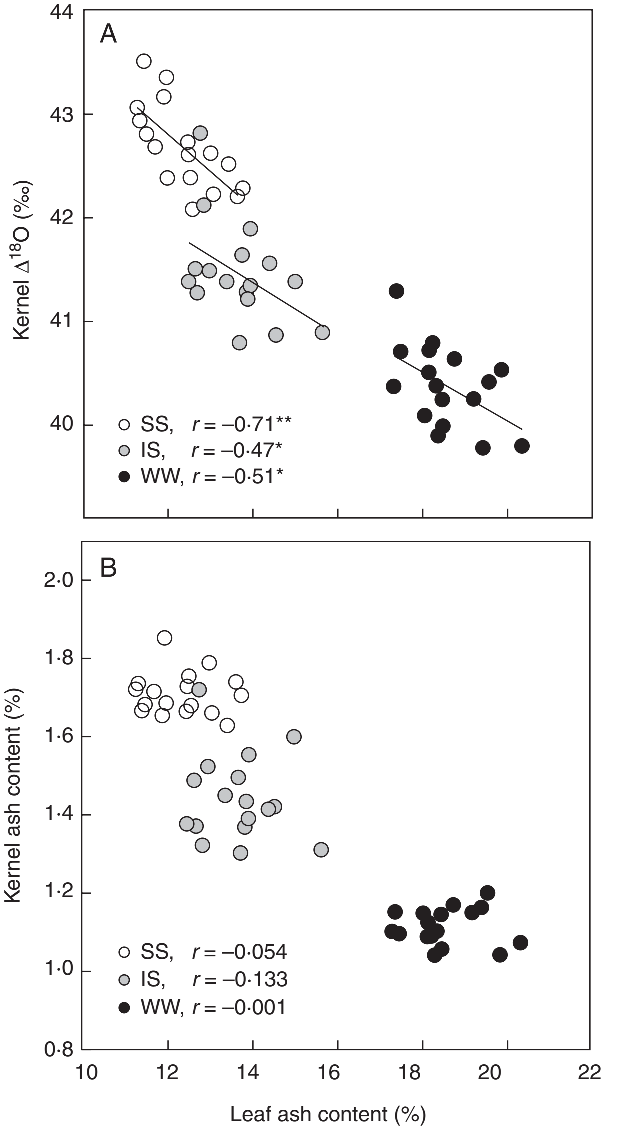

FIG. 2. The relationships between leaf ash content and (A) oxygen isotope enrichment ($\Delta^{18}O$) in kernels and (B) kernel ash. The three water treatments (WW, IS and SS) were plotted together, each point representing the mean of three replicates ($n = 51$).

correlated negatively with e_a/e_i ($r = -0{\cdot}87$, $P < 0{\cdot}001$; Fig. 3). Significant positive correlations were observed between leaf ash content and mean g_s ($r = 0{\cdot}81$, $P < 0{\cdot}001$), when all the genotypes and water treatments were plotted together (data not shown). However, no genotypic correlations (i.e. within each water treatment) were found between leaf ash and g_s measurements (Fig. 4). In addition, leaf ash correlated positively with LWC across water treatments ($r = 0{\cdot}60$, $P < 0{\cdot}001$, data not shown), and also weakly within the SS ($r = 0{\cdot}40$, $P < 0{\cdot}1$) and the IS ($r = 0{\cdot}53$, $P < 0{\cdot}05$) treatments (Fig. 4). No correlation was found in the WW treatment.

Relationships between grain yield, stable isotopes and ash content

GY strongly and positively correlated with kernel number per hectare in the WW ($r = 0{\cdot}88$, $P < 0{\cdot}001$), IS ($r = 0{\cdot}96$, $P < 0{\cdot}001$) and SS ($r = 0{\cdot}98$, $P < 0{\cdot}001$) treatments (Fig. 5A). GY also positively correlated with kernel mass in WW plants ($r = 0{\cdot}48$, $P < 0{\cdot}05$), but no correlations were found in the two other water regimes (Fig. 5B). Leaf ash correlated negatively with GY ($r = -0{\cdot}50$, $P < 0{\cdot}05$) and kernel number per hectare ($r = -0{\cdot}51$, $P < 0{\cdot}05$) in the SS treatment, whereas no correlations were found in plants grown under IS and WW conditions (Table 2). Kernel ash correlated negatively with GY in the IS ($r = -0{\cdot}73$, $P < 0{\cdot}001$) and SS ($r = -0{\cdot}49$, $P < 0{\cdot}05$) treatments (Table 3, Fig. 5C), and also correlated negatively with kernel number per hectare ($r = -0{\cdot}64$, $P < 0{\cdot}01$ and $r = -0{\cdot}52$, $P < 0{\cdot}05$ for the IS and SS treatments, respectively), whereas no correlations were found in WW plants (Table 2). No correlations were found between either leaf or kernel ash content

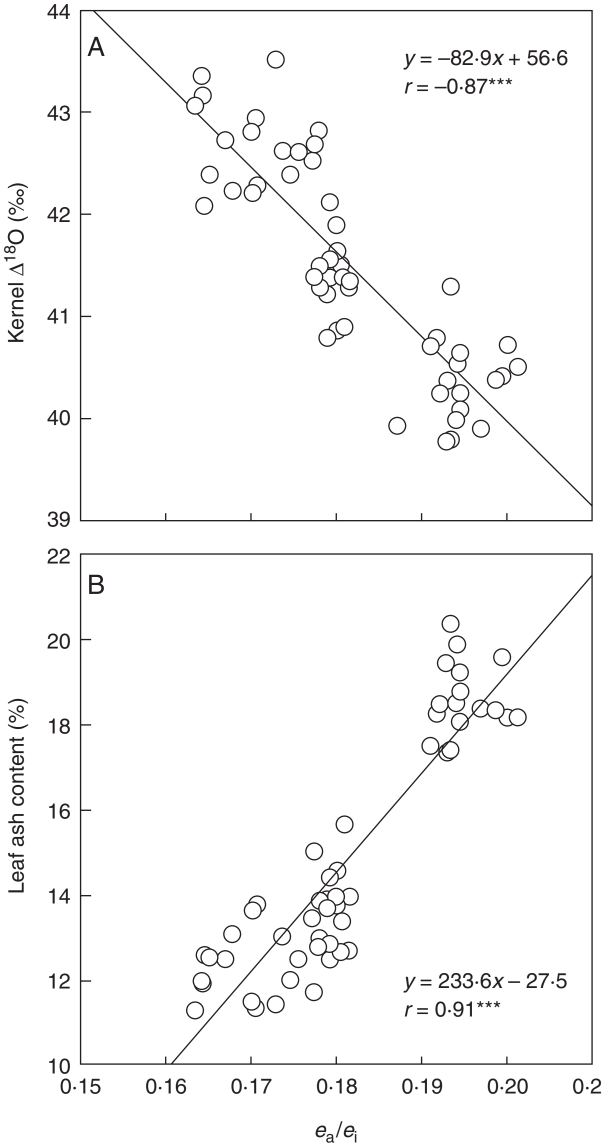

FIG. 3. The relationships between the ratio of atmospheric to intercellular space vapour pressure (e_a/e_i) and (A) oxygen isotope enrichment ($\Delta^{18}O$) in kernels, and (B) leaf ash content. The three water treatments (WW, IS and SS) were plotted together, each point representing the mean of three replicates ($n = 51$).

and kernel mass in any of the three water treatments. Kernel and leaf Δ^{13}C did not correlate with any of the studied yield parameters within any of the three water treatments assayed (Table 2). Kernel Δ^{18}O correlated negatively with GY ($r = -0{\cdot}48$, $P < 0{\cdot}05$) and kernel mass ($r = -0{\cdot}53$, $P < 0{\cdot}05$) in the WW treatments, and with GY ($r = -0{\cdot}58$, $P < 0{\cdot}01$) and kernel number per hectare ($r = -0{\cdot}58$, $P < 0{\cdot}01$) in the IS plants. Conversely, a positive but not significant trend was observed between kernel Δ^{18}O with GY and kernel number per hectare in the SS treatment (Table 2). In addition, highly significant correlations between leaf ash, kernel ash, leaf and kernel Δ^{13}C and kernel Δ^{18}O in relation to GY, kernel per hectare and kernel mass were found when the three water treatment were combined (Table 2).

The percentage of variation in GY explained by the combination of leaf ash, kernel ash and kernel Δ^{18}O was studied with a multiple correlation analysis (Table 3). Different results were found, depending on the water treatment. In the IS and SS treatments, over 50 % of GY variability was explained by the combination of leaf and kernel ash content. Alternatively, the combination of leaf ash plus kernel Δ^{18}O explained about 30 % of GY variability in both IS and SS plants, while the combination of kernel ash plus kernel Δ^{18}O explained 60 and 35 % in IS and SS treatments, respectively. The combination of the three parameters (leaf ash, kernel ash plus kernel Δ^{18}O) slightly increased the percentage of variation in GY over the best combination of two traits. Conversely, in the WW plants, only 15 % of the variation in GY was explained by the combination of leaf and kernel ash, while the combination of Δ^{18}O with any of the other two traits explained about 26 %. Further addition of leaf or kernel Δ^{13}C in combination with ash content and kernel Δ^{18}O values did not improve the percentage of GY variability explained (data not shown).

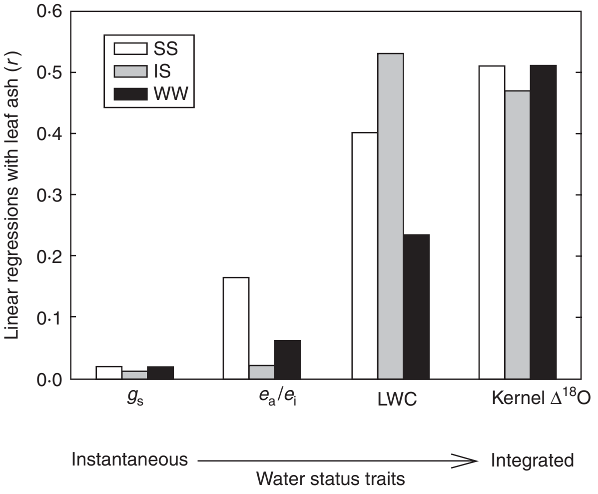

FIG. 4. Correlation coefficients (r) of the linear regressions across genotypes within each water treatment between leaf ash and the water status traits g_s, e_a/e_i, LWC and kernel Δ^{18}O. The x-axis shows the water status traits evaluated, with the more instantaneous (g_s and e_a/e_i) on the left and the more integrative in time and plant level (LWC and kernel Δ^{18}O) on the right. For the 15 degrees of freedom available, Pearson correlation coefficients greater than 0·33, 0·41 and 0·56 are statistically significant at the 10, 5 and 1 % levels, respectively. Correlation coefficients are shown in absolute values.

DISCUSSION

Although the use of secondary traits such as mineral accumulation and stable isotopes when breeding for yield potential and drought adaptation in C_3 cereals has been tackled, little is known about its application in C_4 cereals such as maize. We suggest mineral accumulation (measured as ash content) and Δ^{13}C (despite limitations in maize owing to its C_4 photosynthetic metabolism) in combination with or alternative to Δ^{18}O as potential secondary phenotypic traits for maize breeding under drought conditions.

Source of variation in ash content and plant isotope signatures

Increasing the level of water stress significantly decreased leaf ash content, as previously reported in wheat and barley plants exposed to different water regimes (Araus *et al.*, 1998; Voltas *et al.*, 1998). As mineral accumulation in vegetative tissues appears to take place via xylem through the transpirational stream, it is closely related to the amount of water transpired by the plant (Masle *et al.*, 1992). Thus, differences in mineral accumulation between water treatments could probably be explained by differences in total water input. In addition, within each water treatment, genotypes able to

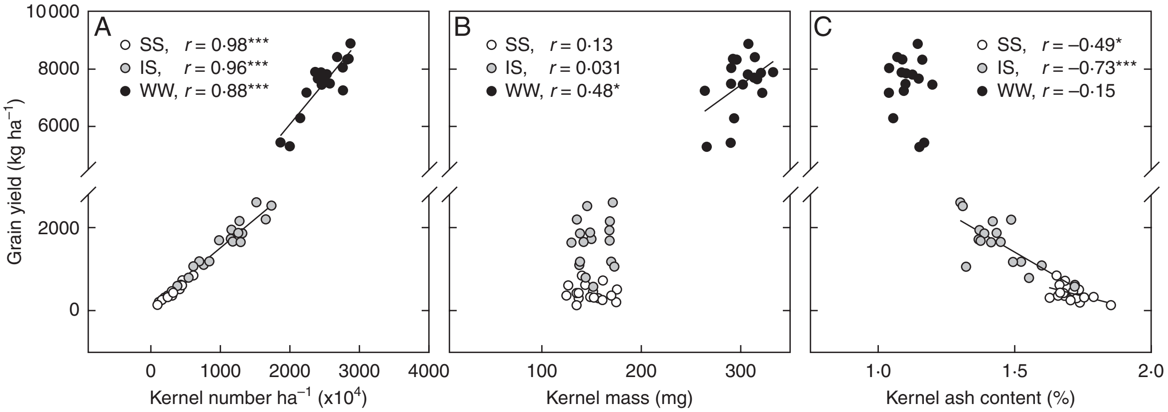

FIG. 5. The relationships between grain yield and (A) kernel number per hectare, (B) kernel mass and (C) kernel ash content. The three water treatments (WW, IS and SS) were plotted together, each point representing the mean of three replicates ($n = 51$).

TABLE 2. *Correlation coefficients of the linear regressions between leaf and kernel ash content, leaf and kernel $\Delta^{13}C$, and kernel $\Delta^{18}O$ in relation to GY, kernel number per hecare and kernel mass across genotypes within each water treatment (WW, IS and SS) and across water treatments*

	GY				Kernels per hectare				Kernel mass			
	WW	IS	SS	Across	WW	IS	SS	Across	WW	IS	SS	Across
Leaf ash	0·36$^{\text{n.s.}}$	0·27$^{\text{n.s.}}$	−0·50*	0·95***	0·23$^{\text{n.s.}}$	0·34$^{\text{n.s.}}$	−0·51*	0·95***	0·31$^{\text{n.s.}}$	0·40$^{\text{n.s.}}$	0·05$^{\text{n.s.}}$	0·92***
Kernel ash	−0·15$^{\text{n.s.}}$	−0·73***	−0·49*	−0·92***	−0·14$^{\text{n.s.}}$	−0·64**	−0·52*	−0·91***	−0·12$^{\text{n.s.}}$	−0·33$^{\text{n.s.}}$	0·24$^{\text{n.s.}}$	−0·85***
Leaf Δ^{13}C	−0·13$^{\text{n.s.}}$	0·01$^{\text{n.s.}}$	0·02$^{\text{n.s.}}$	−0·80***	−0·07$^{\text{n.s.}}$	0·07$^{\text{n.s.}}$	−0·02$^{\text{n.s.}}$	−0·78***	−0·10$^{\text{n.s.}}$	−0·17$^{\text{n.s.}}$	0·01$^{\text{n.s.}}$	−0·78***
Kernel Δ^{13}C	0·26$^{\text{n.s.}}$	−0·30$^{\text{n.s.}}$	0·17$^{\text{n.s.}}$	−0·90***	0·31$^{\text{n.s.}}$	−0·23$^{\text{n.s.}}$	0·16$^{\text{n.s.}}$	−0·82***	0·02$^{\text{n.s.}}$	−0·18$^{\text{n.s.}}$	−0·40$^{\text{n.s.}}$	−0·92***
Kernel Δ^{18}O	−0·48*	−0·58**	0·23$^{\text{n.s.}}$	−0·86***	−0·28$^{\text{n.s.}}$	−0·58**	0·26$^{\text{n.s.}}$	−0·89***	−0·53*	0·12$^{\text{n.s.}}$	−0·17$^{\text{n.s.}}$	−0·78***

WW, well-watered plants; IS, intermediate water stress; SS, severe water stress. n.s., not significant; *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

TABLE 3. *Percentage of grain yield (GY) variation across genotypes within each water treatment (WW, IS and SS) explained by the combination of kernel $\Delta^{18}O$, leaf and kernel ash content*

Combination of traits	WW (% GY)	IS (% GY)	SS (% GY)
Leaf ash + kernel ash	15·2	56·3	52·2
Leaf ash + kernel Δ^{18}O	25·0	33·5	28·2
kernel ash + kernel Δ^{18}O	24·4	60·4	35·0
Leaf ash + kernel ash + kernel Δ^{18}O	26·3	60·8	52·6

maintain a better water status through higher stomatal conductance and transpiration rates (i.e. higher WU) will accumulate more minerals in vegetative parts. The results here support such a hypothesis, as leaf ash content was found to be positively correlated with g_s and e_a/e_i ratio when all treatments were plotted together. However, despite the relationships found between leaf ash and 'time-instantaneous' traits such as g_s and e_a/e_i, leaf ash content should be more closely related to time-integrated traits such as LWC (performed at the whole plant level) and kernel Δ^{18}O than to instantaneous measurements performed in single leaves. In such a way, there was a clear increase in the correlations between leaf ash and traits informing about plant water status (g_s, e_a/e_i, LWC and Δ^{18}O) when moving from instantaneous (g_s and e_a/e_i) to time-integrated (LWC and Δ^{18}O) measurements.

The negative relationships observed between leaf ash and kernel Δ^{18}O support the fact that mineral accumulation in vegetative tissues may reflect differences in transpiration and then water use. In fact, Δ^{18}O in dry matter is known to integrate leaf evaporative conditions throughout the crop cycle (Barbour *et al.*, 2000; Barbour, 2007; Cabrera-Bosquet *et al.*, 2009a). Thus, Δ^{18}O has been proposed as a selection criterion to track genotypic differences in stomatal conductance in bread wheat grown under well-watered conditions (Barbour *et al.*, 2000), as well as an indirect measurement of transpiration and WU (Sheshshayee *et al.*, 2005; Cernusak *et al.*, 2007, 2008; Cabrera-Bosquet *et al.*, 2009a,b). Increases in kernel Δ^{18}O with decreasing water input have been reported in wheat plants grown under different water regimes (Ferrio *et al.*, 2007; Cabrera-Bosquet *et al.*, 2009a). In addition, kernel Δ^{18}O correlated negatively with the ratio of atmospheric to intercellular space vapour pressure (e_a/e_i), so reflecting the close relationship between Δ^{18}O and leaf evaporative conditions.

By contrast, the significant increase in kernel ash content from WW to SS plants is consistent with previous studies in C_3 cereals (Febrero *et al.*, 1994; Araus *et al.*, 1998; Voltas *et al.*, 1998). Such an increase is explained by the mechanisms involved in mineral accumulation in kernels. In maize and other cereals, the main proportions of phosphorous and other cations in the grain are found together in the form of phytin (i.e. *myo*-inositol hexaphosphoric acid) (Lott, 1984). In addition, in maize nearly 90 % of the total grain phytin is in the embryo, constituting from 1 % to several per cent of the seed's total dry weight (O'Dell *et al.*, 1972). Minerals are retranslocated to the grains from the photosynthetic tissues during plant senescence (Rajcan and Tollenaar, 1999).

However, increasing the proportion of carbohydrates in the starchy endosperm, due to an enhanced supply of photo-assimilates during grain filling (such as in the WW treatments), lowers the proportion of minerals in the grain. Therefore, whereas leaf ash reflects differences in WU or transpiratory gas exchange, kernel ash reflects photosynthetic and translocation processes during grain filling. This may explain the lack of genotypic correlations between kernel ash in relation to both leaf ash content and $\Delta^{18}O$ in all three water treatments.

Relationship of ash content and of stable isotopes to GY

The negative correlations between leaf ash in relation to GY and kernel number per hectare in the SS treatment, and the positive correlations (although not reaching statistical significance) in the WW and IS treatments, are comparable with the results of Voltas *et al.* (1998) in barley grown under different water regimes. They reported negative relationships between ash content in straw and GY in plants growing in the poorest yield conditions. This is explained by the fact that under severe water stress conditions (here, plants showed a decrease of 94 % in GY compared with WW plants), those genotypes that are more able to save water (i.e. with reduced WU) are not penalized in terms of productivity. This hypothesis was supported by the significant positive correlations of kernel $\Delta^{18}O$ to GY and kernels per hectare in the SS treatments (when removing the check, 'Puma'), and the negative correlations between kernel $\Delta^{18}O$ and GY in the WW and IS treatments. $\Delta^{18}O$ may record the transpirative status (and then WU) during the weeks before flowering, when the total number of kernels (the main agronomic component) is defined. Therefore, under WW to IS conditions, those genotypes showing high leaf ash or low kernel $\Delta^{18}O$ (i.e. increased ability to sustain transpiration) are the most productive. Conversely, under severe water stress conditions, the highest yields were achieved by those genotypes that have the lowest leaf ash and the highest kernel $\Delta^{18}O$ values. Such a change in the sign of the correlation between leaf ash or between kernel $\Delta^{18}O$ and GY is comparable with that reported when using $\Delta^{13}C$ for breeding in C_3 cereals under Mediterranean conditions, where drought develops steadily from flowering onwards. Under such conditions, the genotypic relationship between $\Delta^{13}C$ and GY moves from positive, under moderate water stress and well-watered conditions, to negative (or absent) under severe water stress conditions (Voltas *et al.*, 1999; Condon *et al.*, 2004; Royo *et al.*, 2005; Araus *et al.*, 2003, 2008). A highly significant negative correlation of either leaf or kernel $\Delta^{13}C$ with GY and the yield components studied was observed when all treatments were combined. However, no significant relationships across genotypes within each water treatment were found between either leaf or kernel $\Delta^{13}C$ and GY, kernel per hectare or kernel mass. The few previous studies that report the relationship between yield and $\Delta^{13}C$ in maize have shown contradictory results. Whereas Monneveux *et al.* (2007) reported positive correlations between $\Delta^{13}C$ measured in leaves, ears and silks in relation to ear dry weight measured at female flowering, but not to yield, Heng *et al.* (2005) reported positive, negative or non-existent relationships between $\Delta^{13}C$ measured in different plant parts and yield. The lack of correlation between either leaf or kernel $\Delta^{13}C$ and GY in the present study can be explained by the very low range of variation in $\Delta^{13}C$ between genotypes within each water treatment. Thus, $\Delta^{13}C$ only ranged from 4·98 to 5·53, from 5·37 to 5·83 and from 5·53 to 5·95 ‰ in leaves and from 3·59 to 4·01, from 4·22 to 4·74 and from 4·07 to 4·78 ‰ in kernels for the WW, IS and SS treatments, respectively. The low genotypic and even environmental variation in $\Delta^{13}C$ observed here is far lower than that reported in C_3 plants such as wheat grown under different water conditions (e.g. 13·0–17·7 and 14·59–18·36 ‰, see Araus *et al.*, 1997, 2003; 14·21–17·64 ‰, see Ferrio *et al.*, 2007; 13·68–18·58 ‰, see Cabrera-Bosquet *et al.*, 2007), because in C_4 plants the potentially large effect of fractionation by Rubisco is suppressed in the semi-closed bundle sheath (Bowman *et al.*, 1989). Nevertheless, the increases in $\Delta^{13}C$ under drought conditions, and the higher $\Delta^{13}C$ values in vegetative tissues (i.e. leaves) than in reproductive organs (i.e. kernels) are consistent with those reported previously in maize hybrids and lines grown under different water regimes (Heng *et al.*, 2005; Dercon *et al.*, 2006; Monneveux *et al.*, 2007). Monneveux *et al.* (2007) reported the usefulness of $\Delta^{13}C$ for preliminary screening of maize lines and hybrids that have a high contrast for drought tolerance. However, overall, the lack of genotypic differences in kernel $\Delta^{13}C$ between the set of genotypes derived from the *LPS* population, together with the lack of correlation of either kernel or leaf $\Delta^{13}C$ with yield, prevents the use of $\Delta^{13}C$ in maize for breeding purposes.

The negative correlation of kernel ash with GY under drought conditions and the lack of relationship under WW conditions is in line with previous studies in barley (Febrero *et al.*, 2004; Voltas *et al.*, 1998). In addition, no relationship was found between kernel ash and kernel mass in any of the three water treatments. Therefore, differences in kernel ash seem to be sustained by factors other than differences in kernel mass (Araus *et al.*, 1998). The negative relationships between kernel ash and kernel number per hectare in the IS and SS treatments support such a hypothesis. In fact, within each water treatment, and particularly in the IS and SS treatments, differences in GY were largely explained by increases in the kernel number per unit area, rather than an increase in kernel mass (Bolaños and Edmeades, 1996; Bruce *et al.*, 2002). However, in plants grown under WW conditions, differences in GY were also determined by changes in kernel mass. Such differences could also explain the lack of correlation of kernel ash in relation to GY and kernel number per hectare in the WW treatments. Therefore, kernel ash seems to reflect the balance between the reproductive sink and the photosynthetic source. Thus, as minerals in maize accumulate mainly in the embryo rather than in the endosperm (O'Dell *et al.*, 1972), ash concentration in kernels indirectly combines information on the number of kernels per plant, the main agronomic component determining grain yield under drought (Bruce *et al.*, 2002). This corroborates the negative relationship between ash content in kernels and the number of kernels per hectare, and the lack of a correlation with kernel mass.

Implications for plant breeding

Under drought conditions the combination of leaf and kernel ash accounted for more than 50 % of the genotypic variability in GY, far higher than Δ^{18}O alone. However, under WW conditions, leaf ash combined with kernel ash explained only 15 % of the variation in GY, and the addition of kernel Δ^{18}O explained up to 26 % of GY variation. In fact, under WW conditions, the only trait that correlated significantly with GY was Δ^{18}O. Araus *et al.* (1998) reported that in durum wheat growing under contrasting water conditions, about 40 % of the variation in GY was explained by the combination of leaf and kernel ash. In the present study, the combination of ash content and Δ^{18}O in kernels provided a reasonable prediction of GY, regardless of the water regime under consideration. In conclusion, there is a close relationship between mineral accumulation in vegetative tissues (leaves) and the plant's water status (g_s, e_a/e_i, LWC, Δ^{18}O), together with a correlation between yield and ash content in leaves and kernels. This supports the combined use of mineral content in leaves and kernels as a useful alternative to Δ^{18}O in kernels for assessing yield performance and selecting maize genotypes that are better suited to drought conditions. In addition, when complemented with Δ^{18}O in kernels, combined leaf and kernel mineral content also explained differences in yield under irrigated conditions. This approach could be particularly interesting if it were coupled with techniques such as near-infrared reflectance spectroscopy, which would allow a fast, cheap (US $0·5 per sample) and reliable estimation of ash content (see Araus, 1996), to be routinely applied in grain quality tests.

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