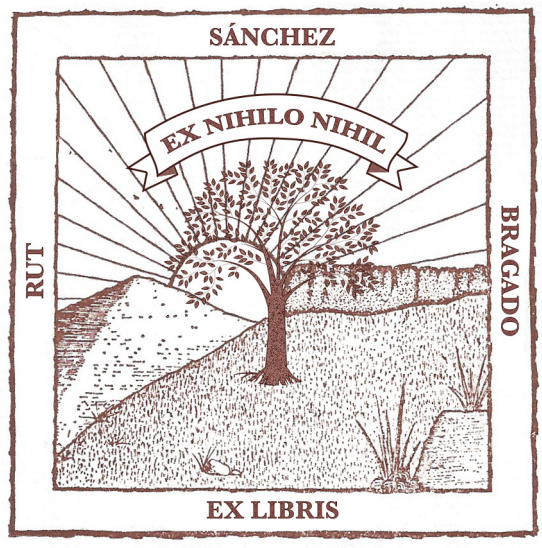
UNIVERSITAT DE BARCELONA

Ús d'isòtops estables d'O, H, C com eines de selecció de rendiment potencial i adaptació a la sequera i deficiència de nitrogen en cereals C_3 i C_4

Rut Sánchez Bragado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ÚS D'ISÒTOPS ESTABLES D'O, H, C COM EINES DE SELECCIÓ DE RENDIMENT POTENCIAL I ADAPTACIÓ A LA SEQUERA I DEFICIÈNCIA DE NITROGEN EN CEREALS C_3 I C_4

Memòria presentada per Rut Sanchez Bragado per optar al títol de Doctor per la Universitat de Barcelona. Aquest treball s'enmarca dins del programa de doctorat de Biologia Vegetal de la Facultat de Biologia de la Universitat de Barcelona. Aquest treball s'ha realitzat al Departament de Biologia Vegetal, Unitat de Fisiologia Vegetal de la Facultad de Biologia de la Universitat de Barcelona sota la direcció del Dr. Josep Lluis Araus i la Dra. Maria Dolors Serret Molins.

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UNIVERSITAT DE BARCELONA

Al Kiko,

el meu sentit.

Als meus pares,

el meus pilars.

Tanca els ulls, encara que sigui només per després tornar-los a obrir. Mou, amb la ment en blanc, les pupil·les i només les pupil·les. Veuràs el tros d'univers que correspon a la teva posició: una mica de sòl, aigua, un tros de selva i una resta de cel. Mira les fulles, les arrels i els troncs, els arbres i els núvols. Troba, si pots, una repetició, només una.

La perfecció existeix (perquè és imaginable) però no és perfecte (perquè és inassolible)

La realitat pot ser invisible per gran: és la astrofísica, la cosmologia... o el jaguar per l'ameba. La realitat pot ser invisible per petita: és la microbiologia, la física quàntica... o el jaguar per un observador instal·lat a la lluna. La realitat pot ser invisible per complexa: és la política, la psicologia... o el jaguar per l'ecòleg intrigat per l'intrincat joc de relacions d'aquell amb la resta de les criatures. Investigar, en ciència, significa buscar camins que parteixen de tals invisibilitats i que acaben passant per certes representacions finites i intel·ligibles.

Jorge Wagensberg

AGRAÏMENTS

Encara recordo el primers dies que vaig arribar a la universitat de Biologia. Des que vaig fer el màster a la universitat de Göttingen que m'havia proposat fer un doctorat. En aquella època un doctorat quedava molt llunyà, semblava una meta molt difícil d'aconseguir, i en aquell moment no sabia que significava, bé, doncs ara si que ho sé. Quasi sense adonar-me'n em trobo escrivint els agraïments de la meva pròpia tesi. De fet, penso que els agraïments són una part molt important, ja que dona l'oportunitat d'agrair a tota la gent la seva contribució durant el transcurs d'aquest viatge que ha suposat fer la tesi. Val a dir que un doctorat no és una meta que s'aconsegueixi en solitari, aquesta tesi s'ha aconseguit gràcies a moltes persones que han estat al meu costat durant aquests quatre anys i que sense elles acabar-la hagués sigut impossible. La ciència no és un camí fàcil, i si alguna cosa he après durant aquests anys a part de com funcionen els isòtops, és que aquest camí no s'ha de fer en solitari. La ciència és un esforç comú on tots i cadascun de nosaltres aportem el nostre petit gra de sorra per construir un món millor.

Començant pel principi, voldria agrair a la persona que realment em va introduir en el món de la ciència i que ara mateix crec que he fet un doctorat gràcies a ella, per la seva passió per la ciència, que va saber transmetre'm tant bé i per la confiança que sempre va tindre en mi. Gràcies a la meva directora de la tesi del màster a Göttingen, Dra. Luitgard Schwendenman. Realment vaig aprendre molt durant la meva estada a Göttingen i són anys que mai oblidaré. Vielen dank Luitgard!

Després sense cap mena de dubte agrair a les persones que en el seu dia van confiar en mi i em van donar l'oportunitat de fer un doctorat i són el Josep Lluis i la Dolors. Durant aquests anys he après moltes coses que m'han fet créixer tant a nivell personal com a nivell professional i tot això no hagués estat possible sense el vostre suport. Dolors,

sempre recordaré els moments de 'petar' la xerrada que en principi eren per 5 minuts i acabaven sent una hora, aquells moments sempre m'han agradat molt. Josep Lluis, he de dir que he après moltíssimes coses gràcies als teus amplis coneixements. Gràcies també als membres de l'equip, Jordi Bort, Omar, Susan, Fadia, Bang, Shawn perquè també han participat en la realització d'aquesta tesi, sobretot durant els mostrejos, i per estar sempre disposats a ajudar-me a recollir les meves mostres, encara que això suposés no dinar. Salima, moltes gràcies per compartir aquells cafès amb xocolata, per estar sempre quan ho necessitava, m'ha agradat molt coincidissim en el mateix equip. Sempre recordaré aquells bombons que vas portar durant un mostreig d'Aranjuez, ens van salvar la vida.

Gràcies a tots els companys dels departament, a la Marteta, a la Melanie per les nostres xerrades existencials quan anaven a agafar el tren, als 'Munnis' per sempre acompanyar-me en els dinars de la quarta planta, i per tenir sempre de tot en els vostres despatxos i en el laboratori, m'heu salvat en més d'una ocasió.

Jordi, Isis, Mireia i Iker. He de dir que els dinars a la 'gespings' van ser molt divertits, i sempre eren un manera ideal de desconnectar per uns moments del món de vegades tortuós de la tesi. Heu format part d'aquesta tesi, l'heu patit i celebrat amb mi, sense vosaltres això no hagués sigut possible tampoc. Mai oblidaré la nostra ineludible cita dels divendres a la tarda amb la 'birra' de les 5pm. Realment he rigut molt amb tots vosaltres, aquest serà el record que se'm quedarà, el riure. Mireia, gràcies en especial pel teu suport en els moments difícils i per suposat i he de fer referència en aquesta tesi, per venir-me a ajudar en els experiments que vaig fer a les cambres de creixement, vas salvar-me moltes vegades de no quedar-me a 'dormir' a la universitat.

Gemma, que puc dir si no és que m'ha encantat treballar i conviure amb tu el temps

que vaig estar a Obregón. He d'agrair-te de veritat la confiança que vas depositar en mi, i la gran implicació i el positivisme que has tingut en aquesta tesi. A Obregón vam treballar molt dur, hores i hores sota el sol Mexicà abrasador, però ens ho passàvem tant bé i rèiem tant que per mi va ser una experiència que espero repetir alguna dia i que no oblidaré mai. El teu suport ha sigut fonamental, gràcies.

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Agrair de tot cor el suport incondicional de la meva família. Sense el vostre suport aquesta tesi hagués sigut impossible de fer. A l'Isaac i la Jossy, a la tia Marion i l'oncle Francesc, als cosinets Jordi i Roger, als meus oncles i cosines de Madrid, a la meva àvia, en especial gràcies al tio Àngel per donar-nos l'oportunitat de sortir a la televisió, va ser molt emocionant, a la familia del Kiko, sense vosaltres saber-ho tots i cadascun heu format part de la realització d'aquesta tesi.

Gràcies als meus pares. En especial gràcies al meu pare per ajudar-me en els experiments, he de dir que ha sigut el millor ajudant que he tingut mai. Gràcies a la meva mare per donar-me el seu suport incondicional i per creure sempre en mi. Gràcies als dos per sempre estar disposats a ajudar, per estar sempre tant orgullosos de mi, per escoltar-me, i un sense fi de gratituds que serien més llargues que la pròpia tesi. Gracies sou els millors pares del món.

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Moltes gracies a tots per acompanyar-me en aquest viatge de la ciència que mai oblidaré.

Com diria el meu avi

Ex nihilo nihil

Res ve de res

Parmènides

ABREVIATURES

α, coeficient de discriminació durant la difusió de l'aire (‰)

ANOVA, anàlisi de la variança

ASI, Anthesis-to-Silking Interval, és l'interval entre la floració masculina (antesis) i femenina (aparició de les "sedes")

β, es la proporció del CO_2 fixat via PEPC

b, fraccionament net causat per la carboxilació (‰)

$\mathbf{b_3}$, coeficient de discriminació durant la carboxilació de la Rubisco (‰)

$\mathbf{b_4}$, coeficient de discriminació de la (‰)

C, la concentració molar de l'aigua (mol m^{-3})

$\mathbf{C_a}$, la concentració del CO_2 ambient (mol mol^{-1})

$\mathbf{C_i}$, la concentració del CO_2 intercel·lular (mol mol^{-1})

CIMMYT Centro Internacional de Mejora del Maiz i el Trigo

D, l'auto-difusivitat de l'aigua ($H_2{}^{18}O$) ($m^2\ s^{-1}$)

E, las taxa de transpiració (mmol $m^{-2}\ s^{-1}$)

$\mathbf{e_a}$, pressió de vapor atmosfèric (kPa)

EA, analitzador elemental

$\mathbf{e_i}$, pressió de vapor de l'espai intercel·lular (kPa)

ET, l'evapotranspiració (mm)

ETP, l'evapotranspiració potencial (mm)

$\mathbf{g_s}$, conductància estomàtica (mmol $m^{-2}\ s^{-1}$)

GY, el rendiment de gra ($Mg{\cdot}ha^{-1}$)

HI, índex de collita

HKW, pes de cent grans (g)

HN, condicions d'adobat nitrogenat (fertilitzat)

IRMS, Isòtops Relació Espectròmetre de Masses

L, escala eficaç de la longitud de la trajectòria del moviment de l'aigua des de les venes als llocs de evaporació (m)

LN, condicions sense fertilitzar

PAR, radiació fotosintèticament activa (mμ mol m^{-2} s^{-1})

PDB, Pee-Dee Belemite (calcària)

PEPC, fosfoenolpiruvat carboxilasa

PPFD, fotosintètica densitat de flux de fotons (mμ mol m^{-2} s^{-1})

Rubisco, carboxilasa ribulosa-1,5-bisfosfat / oxigenasa

TKW, pes de mil grans (g)

VPD, dèficit de pressió de vapor (kPa)

VSMOW, Viena Standard Mean Oceànica Aigua

WW, condicions de reg suplementati

WS, estrès hídric

WSF, fracció soluble de l'aigua

EUA, l'eficiència de l'ús de l'aigua

Z, nombre atòmic

$\delta^{13}C$, composició d'isòtops de carboni (‰)

$\Delta^{13}C$, la discriminació d'isòtops de carboni (‰)

$\delta^{18}O$, la composició isotòpica d'oxigen (‰)

$\delta^{2}H$, la composició isotòpica de l'hidrogen (‰)

$\Delta^{18}O$, oxigen enriquiment isotòpic en relació amb les fonts d'aigua (‰)

$\Delta^{18}O_L$, enriquiment mitjà del ^{18}O de l'aigua de la fulla sobre la longitud efectiva (‰)

$\Delta^{18}O_p$, enriquiment del ^{18}O de matèria orgànica vegetal en relació amb la font d'aigua (‰)

$\Delta^{18}O_{sacarosa}$, ^{18}O enriquiment de sacarosa en relació amb les fonts d'aigua (‰)

$\Delta^{18}O_v$, enriquiment del ^{18}O de l'atmosfera del vapor d'aigua pel que fa a la font d'aigua (‰)

ε^+ , Equilibri del fraccionament del ^{18}O entre l'aigua líquida i vapor (‰)

ε_k, fraccionament cinètic per a la difusió del $H_2{}^{18}O$ desde els espais intracelulars de la fulla a l'atmosfera (‰)

ε_{wc}, factor de fraccionament entre l'aigua i l'oxigen carbonil (‰)

Φ, fracció de CO_2 fixat per PEPC que s'escapa fora del feix de la beina

Introducció

INTRODUCCIÓ GENERAL

1. Importància dels cereals a l'agricultura i de les eines de fenotipatge en cereals

El blat (Triticum spp), el blat de moro (Zea mays L.) i l'arròs són dels cultius més importants a nivell mundial pel que fa a la seguretat alimentària global (FAOSTAT 2013). Concretament el blat dur (Triticum turgidum var. Durum L.) i el blat fariner (Triticum aestivum L.) són dels principals cultius a la conca mediterrània (FAOSTAT 2012). El blat de moro per altra banda és un cultiu molt diversificat arreu del món on el seu volum de producció a nivell mundial ha arribat a superar inclús el del blat i el de l'arròs (FAOSTAT 2012).

A partir de mitjans del segle XX, gràcies a la Revolució Verda en el cas del blat i de la introducció dels híbrids pel blat de moro, es va incrementar substancialment la productivitat d'aquests conreus (Evenson i Gollin 2003). En el procés de millora de blat, la identificació d'al·lels de nanisme i la seva generació de varietats semi-nanes (juntament amb la utilització de fertilitzants) representà un salt històric en els rendiments dels cultius. Però, no obstant aquest gran descobriment, el repte d'alimentar una estimació creixent de població de 9 mil milions prevista pel 2050 (Reynolds et al., 2011) està cada dia més present. Així, per bregar amb aquest augment de la crisi dels aliments juntament amb els canvis socials en curs i l'amenaça del canvi climàtic (en essència, la calor i la sequera), la productivitat agrícola haurà d'augmentar (Parry et al., 2005). Tanmateix, és necessari un increment del rendiment per satisfer l'esmentada demanda mundial dels cereals (Edgerton, 2009). No obstant això, la falta d'expansió o inclús en alguns casos de reducció de la terra cultivable, emfatitza la importància de les pràctiques agronòmiques i de la millora dels cultius adreçades a incrementar el rendiment potencial (Richards et al., 2002; Edgerton, 2009) i l'adaptabilitat dels cereals (Slafer et al., 1999; Araus et

al., 2002, 2008; Reynolds et al., 2009). A més, els països situats a latituds baixes, com els de la conca mediterrània, sovint estan exposats simultàniament a una alta evapotranspiració (Lobell et al., 2008) i a la baixa fertilitat del nitrogen (Oweis et al., 1998; Sadras, 2004). Malauradament, la combinació de la sequera i la baixa fertilitat poden afectar substancialment el creixement de la planta i el rendiment de gra (Nilsen i Orcutt, 1996). A més a més, en les regions mediterrànies l'efecte del canvi climàtic, juntament amb les condicions agro-ecològiques pot provocar una avançament de l'aridesa. L'increment general de la demanda de cereals per l'alimentació (tant per sustentar l'augment de població com per la ramaderia), i de manera més recent per al combustible, són una amenaça per la seguretat alimentària (Edgerton, 2009). Per tant, tenint en compte el context esmentat, un dels principals reptes pels milloradors, els agrònoms i els agricultors és aconseguir abastir la futura demanda dels cereals a nivell mundial.

Com ja s'ha comentat, l'èxit de la millora de cultius durant la segona meitat del segle passat, ha sigut sense precedents gràcies a la introducció dels gens de nanisme i el consegüent increment de l'índex de collita (HI) (Richards, 1996, 2000). Tanmateix, els beneficis obtinguts per la millora de cultius han sigut notables, sobretot en condicions de creixement més favorables (Araus et al., 2002). No obstant, el progrés atribuït a la millora no ha sigut tant satisfactori en condicions de creixement desfavorables (Richards, 1996). L'adaptació de les plantes sotmeses a l'estrès (p.e. estrès hídric) és un fidel testimoni de la remarcable història evolutiva de moltes espècies. Això ens demostra la presència d'una variació genètica en condicions ambientals més extremes (Richards, 1996). Tanmateix, aquesta adaptació de les plantes a condicions d'estrès, ens dona la possibilitat d'utilitzar la variació genètica per millorar certes varietats que ens ajudin a superar alguns dels devastadors efectes causats als nostres cultius més importants (Richards, 1996). Això no és trivial davant de les

futures condicions ambientals pronosticades pel canvi climàtic. Altres factors com ara el coneixement de la resposta de les plantes davant de condicions d'estrès, o la quantificació d'aquesta resposta, ens han ajudat a desenvolupar instruments per millorar l'adaptació d'aquestes plantes davant de condicions d'estrès. La gran quantitat de variació genètica davant dels estressos, ja sigui per mitjans moleculars o per hibridació amb els seus parentals, són factors addicionals que augmenten les nostres esperances de millorar varietats tolerants a les condicions d'estrès ambiental imposades (Richards, 1996). En aquest context es necessari desenvolupar eines precises que ens ajudin a avaluar les característiques genètiques de les varietats més adaptades a condicions d'estrès per així poder incorporar-les en programes de millora de cultius (Araus et al., 2002). Però això és un repte, ja que la millora genètica encara haurà de ser més eficient que en el passat, perquè s'haurà d'aplicar en cultius que ja han estat prèviament millorats per tenir major increment del rendiment (Slafer et al., 1999).

2. Reptes de les eines de selecció de genotips en cereals

Fins fa uns anys la millora de cereals en ambients Mediterranis s'havia basat principalment en seleccions empíriques (millora empírica) del rendiment (Loss i Siddique, 1994). No obstant això, aquesta aproximació està lluny del seu òptim, ja que el rendiment està caracteritzat per una baixa heretabilitat degut una alta interacció genotip x ambient (G X E) (Jackson et al., 1996).

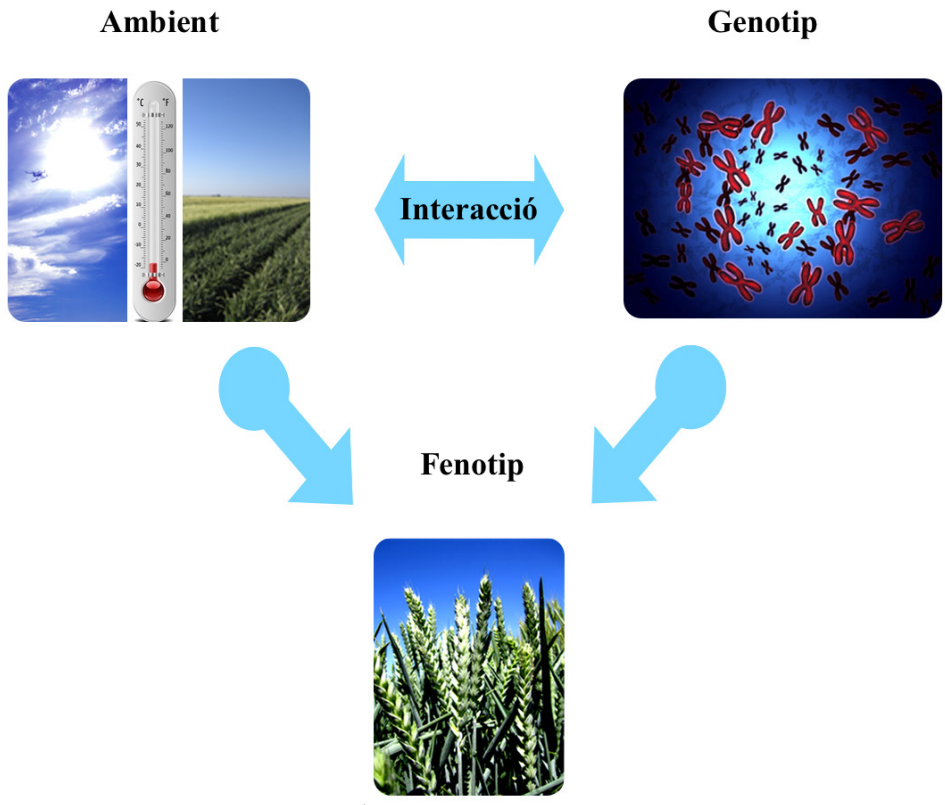

Figura 1. *Interacció entre l'ambient i el genotip, factors que bàsicament determinen el fenotip.*

Així l'adaptació de la planta a l'ambient (Figura 1) és el factor clau que determinarà la futura severitat dels efectes del canvi climàtic en la producció d'aliments. Durant les últimes dècades, el trets fisiològics no s'han pogut utilitzar satisfactòriament com a criteri de selecció (Slafer et al., 1994), ja que no eren molt pràctics alhora de mesurar-los en programes de millora. Des de la segona meitat del segle passat, el rendiment de gra (GY) en el blat i altres cereals ha augmentat de manera constant gràcies al gran èxit de la millora de cultius, combinat amb pràctiques agronòmiques intensives (Calderini i Slafer, 1998). No obstant això, els increments de la millora han estat menors sota condicions menys favorables de creixement, on l'estrès abiòtic com la sequera limiten fortament el GY i les millores genètiques associades als

cultius (Araus et al., 2002). En aquestes condicions, la millora genètica es basa en un delicat equilibri entre GY potencial i les diferents estratègies fisiològiques de resposta de la planta davant l'estrès. En aquest context, s'han proposat enfocs indirectes alternatius (o complementaris) que impliquin la utilització de trets que no siguin el GY, però que s'utilitzin com a selecció empírica dirigida a augmentar el GY sota sequera (Araus et al., 2002). Com el GY és per naturalesa un tret integratiu, els trets més prometedors per la millora són aquells capaços de condensar la informació durant tot el cicle de cultiu (Araus et al., 2002).

3. Eines de selecció per a un major rendiment

Al llarg de les últimes dècades s'han descrit nombrosos mètodes bioquímics i fisiològics per estudiar la resposta de les plantes envers els canvis produïts al seu entorn (Reigosa, 2001). No obstant això, la majoria de mètodes per estudiar la resposta de les plantes es veuen forçats a teoritzar sobre el comportament global de la planta a partir de mesures o experiments puntuals. Degut a això, els mètodes capaços d'integrar els processos fisiològics en una escala més amplia en el temps i en l'espai són especialment valorats (Craig, 1954).

Com a trets integratius, s'ha suggerit la utilització d'altres trets secundaris que no pas el propi rendiment (Araus et al., 2002). Entre les nombroses categories de trets secundaris, els més satisfactoris com s'ha comentat, han sigut trets que proporcionen informació a llarg termini de la resposta que ha tingut la planta als factors ambientals durant el seu creixement (Araus et al., 2002). De tal manera els isòtops estables de carboni i oxigen analitzats en els teixits de la planta (descrits més avall) semblen ser unes eines no invasives potents ja que per la seva naturalesa integradora poden avaluar el rendiment fotosintètic i el transpiratiu de les plantes durant tot el cicle del cultiu (Farquhar i Richards, 1984a; Farquhar, 1989; Condon i Richards, 1992;

Richards et al., 1993; Farquhar et al., 1998). Així aquests trets podrien ajudar en les tasques de millora a seleccionar noves varietats amb més rendiment genètic potencial per aconseguir augmentar la productivitat dels cultius.

3.1 Isòtops estables en ecofisiologia vegetal

La majoria d'elements presents a la terra contenen el que s'anomenen isòtops estables. Els isòtops estables contenen un isòtop més pesat que normalment és troba en una concentració molt menor comparat amb l'isòtop lleuger (en general d'un 1% o menor). L'isòtop més pesat per una diferència atòmica, es difon o respon a una menor velocitat que l'isòtop lleuger davant de processos de transformació biogeoquímica a la biosfera. Aquests processos poden provocar empobriments o enriquiments relatius d'un isòtop respecte de l'altre. La velocitat d'aquests empobriments o enriquiments depèn de les condicions fisicoquímiques i biològiques del medi en el que es desenvolupen. Així, l'abundància isotòpica relativa d'un teixit vegetal, serà un recull de les condicions ecofisiològiques en les que la planta s'ha desenvolupant durant un període de temps concret (Mateo et al., 2003; Sulzman, 2007).

Els isòtops són àtoms d'un element idèntic que contenen el mateix número de protons però diferent número de neutrons, d'ambdós mantenint les mateixes propietats químiques. Així, els isòtops (Figura 2) difereixen en la seva massa atòmica (número de neutrons) però no en el seu número atòmic (número protons).

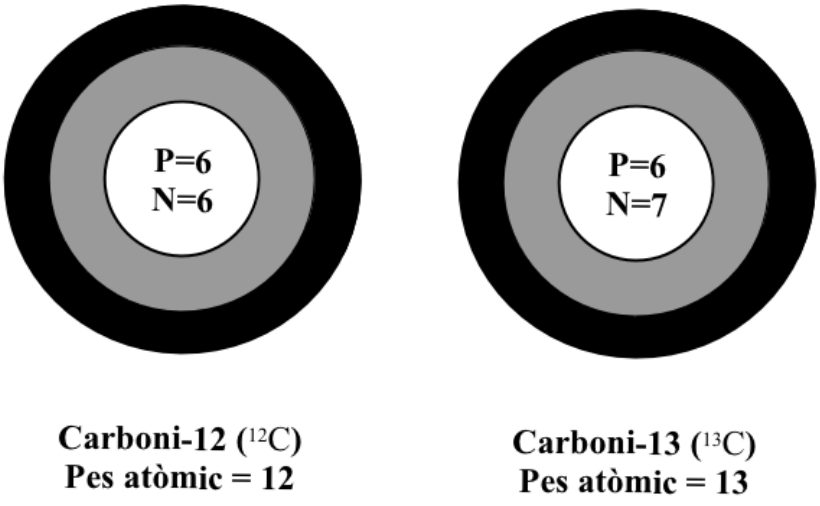

Figura 2. *Representació gràfica de l'isòtop de carboni*

Per cada element es poden trobar més d'una categoria d'isòtops: els isòtops estables (o no radioactius) i els isòtops inestables (o radioactius). Els isòtops radioactius es deterioren al llarg del temps per convertir-se en productes més estables, mentre que els isòtops estables no semblen patir tal deteriorioramen amb el pas del temps. Els elements més nombrosos a la naturalesa són C, H, O i N, i per tant els seus respectius isòtops estables, són els que desperten més interès en el camp de l'ecofisiologia (Craig, 1954; Gonfiantini et al., 1965; Epstein et al., 1977; Kohl i Shearer, 1980; Francey i Farquhar, 1982; Farquhar, 1989; Barbour, 2007; Araus et al., 2013a).

Durant les últimes dècades, l'ús de l'abundància natural dels isòtops estables dels quatre elements més abundants a la biosfera C, H, O i N, amb els seus isòtops pesats corresponents ^{13}C, ^{2}H, ^{18}O i ^{15}N (Taula 1) s'han considerat com a eines potencials ja sigui en el camp de la ecofisiologia com en altres àrees científiques dins la biologia, la bioquímica, la nutrició o la paleobotànica. També des de mitjans del segle passat s'estan utilitzant les tècniques d'isòtops estables com a traçadors fisiològics, de reconstrucció ambiental i biogeoquímics (Mateo et al. 2003).

***Taula 1**. Abundància relativa dels isòtops estables més comuns en estudis ecològics (elaborada a partir de Dawson et al. 2002; Mateo et al. 2003; Sulzman 2007) i dades pròpies. El rang observat està expressat en δ, on 'l'error analític de literatura' està extret de referències bibliogràfiques (Mateo et al., 2003), l'error analític patró primari es va calcular a partir de la desviació estàndard entre els patrons primaris analitzats a la Universitat de Barcelona, 'l'error analític de les mostres' es va calcular a partir de la desviació estàndard de mostres de fulles de matèria seca de blat analitzades a la Universitat de Barcelona i els 'patrons primaris' són els patrons utilitzats en els anàlisis isotòpics.*

Element	Isòtop	Abundància (%)	Nomenclatura	Rang observat δ (‰)	Error analític literatura (‰)	Error analític patró primari (‰)	Error analític mostres (‰)	Patró primari
Hidrogen	^{1}H	99.985						
	^{2}H	0.0155	$\delta^{2}H$	-300 to +20	4-7	1.73	4.85	SMOW[a]
Carboni	^{12}C	98.892						
	^{13}C	1.108	$\delta^{13}C$	-35 to -5	0.1	0.13	0.14	PDB[b]
Oxigen	^{16}O	99.759						
	^{18}O	0.204	$\delta^{18}O$	-30 to +5	0.05-0.3	0.22	0.28	SMOW[a]

[a] Standard Mean Oceanic Water

[b] Pee-Dee Belemnite (calcària)

La composició isotòpica d'una substancia es calcula normalment per espectrometria de masses de relació isotòpica i la notació diferencial és la forma d'expressar-la (Coplen 2008):

$$\delta^{n}X(‰) = (R_{mostra} / R_{estàndar} - 1) * 1000 \quad (1)$$

On, $\delta^{n}X$ és la composició isotòpica referida a un estàndard i Rmostra i Restàndar són els radis molars entre l'isòtop pesat i lleuger de la mostra i de l'estàndard, respectivament (Taula 1). Degut a que la diferència entre la composició isotòpica de l'estàndard i de la mostra és molt petita, els valors de δ s'expressen en parts per mil (‰).

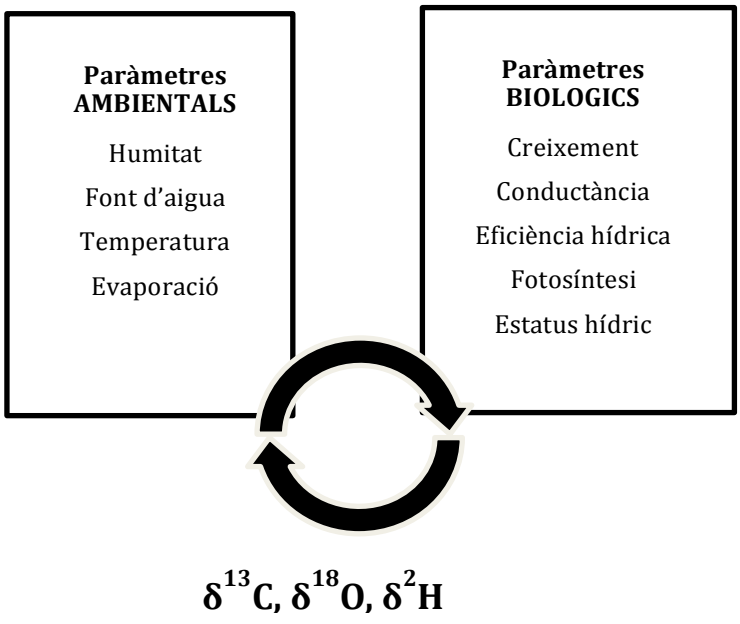

***Figura 3**. Esquema de la utilitat dels isòtops estables en la ecofisiologia vegetal. La variabilitat ambiental modifica els processos i les velocitats de reacció dins la planta. Els àtoms involucrats durant els processos i les reaccions es veuen sotmesos a canvis característics en la proporció dels seus isòtops estables. D'aquesta manera la composició isotòpica del carboni, oxigen i hidrogen a les plantes pot ser un indicador de la interacció d'aquestes amb el seu entorn.*

Existeixen dos tipus d'efectes isotòpics: termodinàmic (provocat per diferències entre la constant d'equilibri) i cinètic (degut a que entre diferents isòtops hi ha diferències entre velocitat de reacció). L'efecte isotòpic termodinàmic es produeix durant reaccions d'intercanvi d'isòtops quan hi ha un canvi d'estat de la matèria (ex.

líquid) a una altra fase (ex. vapor). Per altra banda, l'efecte cinètic es produeix quan la reacció és unidireccional i les velocitats de reacció són massa-depenents (Dawson et al. 2002). Així, per un mateix element, quan s'exposa a reaccions físiques, químiques i biològiques (Figura 3), els seus isòtops lleugers, (ex. ^{12}C) tenen la tendència a reaccionar més ràpidament en comparació als isòtops pesats (ex. ^{13}C) els quals reaccionen més lentament. Aquests efectes isotòpics es denominen a través del fraccionament isotòpic (α), el qual porta associat un enriquiment o empobriment de l'isòtop pesat respecte al isòtop lleuger. Així, la magnitud d'un efecte isotòpic és descrit pel factor de fraccionament:

$$\alpha = R_p / R_r \tag{2}$$

on R_p i R_r són els radis molars dels productes i dels reactants, respectivament. Per altra banda, una altra manera d'expressar el fraccionament isotòpic és a través de la discriminació isotòpica (Δ) respecte al substrat de la reacció (com ara per exemple la $\delta^{13}C$ de l'aire o la $\delta^{15}N$ de la font de nitrogen emprat per la planta), ja que aquesta té en compte els efectes isotòpics (que normalment són propers a la unitat), i s'expressa com:

$$\Delta(‰) = \alpha - 1 = (\delta_r - \delta_p) / [\, 1 + (\delta_p / 1000)] \tag{3}$$

On δ_r és la composició isotòpica dels reactius i δ_p és la composició isotòpica dels productes, i α el factor de fraccionament de l'equació 2. L'avantatge d'utilitzar la Δ d'un producte de reacció es basa en la independència d'aquest càlcul sobre l'estàndard utilitzat a les mesures i sobre la composició isotòpica dels reactius (ex. el carboni del CO_2 de la fotosíntesi). A més, la Δ permet comparar d'una manera més directa els resultats obtinguts per diferents equips d'investigació.

3.2 Isòtops de carboni en plantes

El carboni, a part de ser l'element més abundant a la biosfera, intervé en la majoria de reaccions biològiques que s'hi produeixen a la biosfera. Com a conseqüència d'aquestes reaccions es produiran fraccionaments isotòpics que quedaran impresos en els teixits de les plantes. La família isotòpica del carboni conté dos isòtops estables (^{12}C i ^{13}C) i també altres isòtops radioactius. La signatura isotòpica del ^{13}C ($\delta^{13}C$) o també expressada com a discriminació del ^{13}C ($\Delta^{13}C$) del material vegetal, pot aportar molta informació sobre una gran quantitat de processos fisiològics a més de ser una mesura integradora de la resposta de les plantes a les condicions ambientals. El fet d'utilitzar els isòtops de C com a índex ecològic del funcionament de la planta resideix en l'habilitat d'aquest isòtop d'integrar la relació entre les condicions ambientals i la discriminació bioquímica durant l'assimilació del C (Dawson et al., 2002). De fet, l'abundància del ^{13}C relatiu al ^{12}C en els teixits de la planta és en general menor que el C del CO_2 atmosfèric, indicant l'existència d'una discriminació a l'hora d'incorporar aquest CO_2 a la biomassa de la planta. Així, la $\delta^{13}C$ de la matèria orgànica es pot veure afectada per diferents factors com són: la $\delta^{13}C$ de l'aire, les condicions ambientals en que la planta es va desenvolupar, els processos de fraccionament durant l'assimilació del CO_2 així com les reaccions post-fotosintètiques durant la biosíntesi de matèria orgànica (Farquhar, 1989). En conseqüència, la $\delta^{13}C$ reflexa la relació entre les condicions ambientals en que creixen les plantes i l'assimilació de carboni de la planta (fraccionament isotòpic de la Rubisco) així com les relacions d'eficiència de l'ús de l'aigua (EUA) de la planta (Dawson et al., 2002). De fet, l'isòtop de C com a indicador ecofisiològic, ha sigut una eina potencial molt utilitzada per a la caracterització de la resposta fotosintètica de les plantes (Farquhar i Richards, 1984). A més a més la $\delta^{13}C$ s'ha utilitzat per diferenciar entre metabolismes fotosintètics (Figura 4), on s'ha observat que la $\delta^{13}C$ en plantes C_4 és més alta en comparació amb la $\delta^{13}C$ de plantes amb metabolisme

C3 mentre que les plantes CAM exhibien valors entremitjos (Farquhar, 1983; Winter i Holtum, 2002).

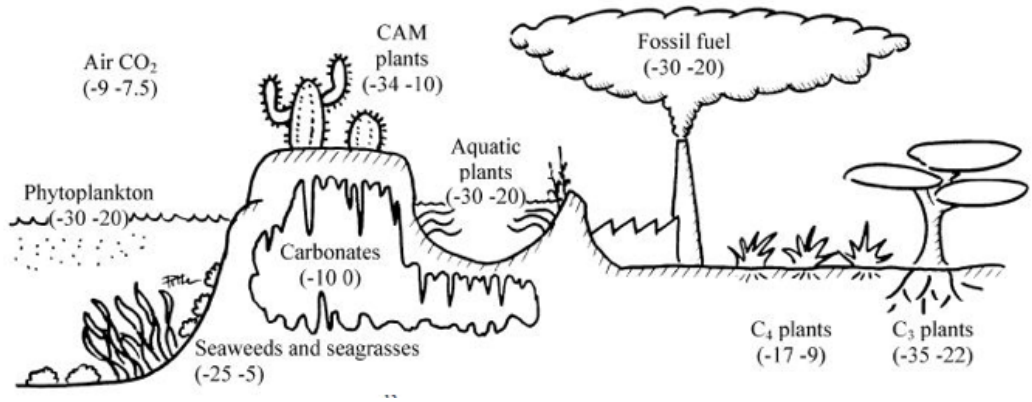

***Figura 4**. Composició isotòpica del carboni en diferents parts de la biosfera (Ehleringer i Rundel, 1988).*

3.2.1. Composició i discriminació isotòpica de carboni en plantes C_3 C_4 i CAM

El fraccionament dels isòtops de carboni ^{13}C i ^{12}C durant la fotosíntesi de les plantes s'ha estudiat i modelitzat àmpliament (Vogel, 1980; O'Leary, 1981; Farquhar i Richards, 1984b; Farquhar, 1989; Dawson et al., 2002). Tots els models són similars ja que atribueixen el major fraccionament isotòpic del ^{13}C respecte del ^{12}C a la diferent difusivitat a través dels estomes i de la carboxilació del CO_2 del aire que conté ^{12}C i ^{13}C. En plantes C_3 la carboxilació depèn principalment de la ribulosa 1,5-bifosfat carboxilasa-oxigenasa (RuBisCo) (Farquhar, 1989). En conseqüència, degut a la discriminació tant de la Rubisco com dels estomes, les plantes en general contenen en els seus teixits menys ^{13}C, comparat amb el ^{13}C que hi ha al CO_2 de l'aire ($\delta^{13}C_{aire}$ = -8‰) (Figura 4, 5A). A més, el fraccionament isotòpic en les plantes també pot variar depenent de la limitació relativa imposada per pressió parcial de CO_2 a l'interior de la fulla (C_i). D'aquest manera, el model que explicaria la Δ a les plantes C_3 seria el següent (Farquhar, 1989):

$$\Delta(‰) = a + (b_3 - a)\frac{c_i}{c_a} \quad (4)$$

on a és el factor de fraccionament degut a la difusió de l'aire (4,4‰), b_3 és el fraccionament net causat per la carboxilació de la Rubisco (30‰), i C_i i C_a són la pressió parcial del CO_2 de l'aire intercel·lular i de l'ambient, respectivament. De manera que si els estomes estan oberts, el CO_2 es difondrà fàcilment a la cap a la càmera subestomàtica (Figura 5A) el que farà que el CO_2 intercel·lular (Ci) sigui més proper al CO_2 ambiental (C_a). Si pel contrari els estomes estan tancats, el CO_2 es difondrà menys fàcilment cap a la càmera subestomàtica (Figura 5B) el que farà que el CO_2 intercel·lular (C_i) sigui marcadament menor al CO_2 ambiental (Ca).

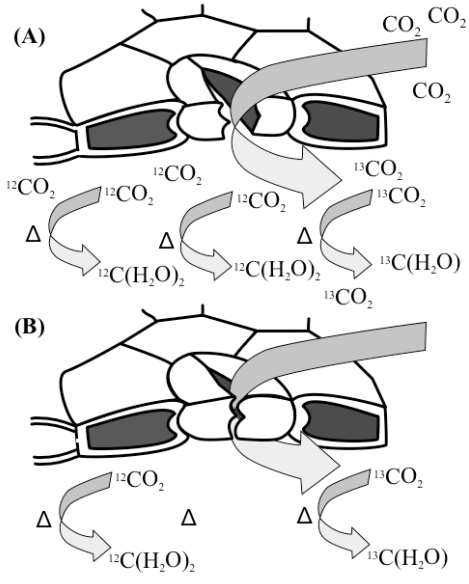

***Figura 5**. Adaptat de Mateo et al. (2003). Il·lustració de la difusió del CO_2 a la càmera subestomàtica i en conseqüència de la discriminació de la composició isotòpica del carboni $\delta^{13}C$ a través dels estomes a les plantes C_3: amb els estomes oberts (A) on la difusió del CO_2 a la càmera subestomàtica es troba menys limitada, permetent discriminar a la Rubisco el ^{13}C i, amb els estomes tancats (B) on degut a la reducció de flux del CO_2 la discriminació de la Rubisco davant el ^{13}C disminueix, provocant que la Rubisco utilitzi major proporció de ^{13}C.*

A diferència de les plantes C_3, en les plantes C_4 la carboxilació depèn d'una reacció prèvia a la Rubisco. L'enzim responsable d'aquesta reacció prèvia és el Fosfoenol piruvat carboxilasa (PEPC). El CO_2 que es troba dins la cavitat subestomàtica s'hidrata a HCO3- (bicarbonat) per l'anhidrasa carbònica, la qual reacciona amb el fosfoenol piruvat (PEP) a través de la PEPC per produir oxalacetat en el mesòfil. L'oxalacetat es converteix en altres àcids C_4 (malat, aspartat o alanina) els quals es difonen a les cèl·lules de la beina on són descarboxilats alliberant CO_2 que serà posteriorment fixat per la Rubisco i la resta del cicle C_3. El fraccionament isotòpic del carboni produït pels dos enzims difereix d'una manera molt contrastada: -29‰ i -5.7‰, per la Rubisco i PEPC, respectivament. Així, les diferents $\delta^{13}C$ en plantes C_3 i C_4 s'expliquen en part pel fraccionament de l'isòtop de carboni produït pels dos enzims diferents (Rubisco i PEPC). En aquest sentit, Farquhar (1983) elaborà el següent model per les plantes de metabolisme C_4.

$$\Delta(‰) = a + (b_4 + \phi b_3 - a)\frac{C_i}{C_a} \quad (5)$$

on la a, b_3 Ci i Ca són els mateixos paràmetres descrits a l'equació (4) b_4 és el fraccionament degut a la PEPC i Φ la fracció del CO_2alliberat a la beina que es difon cap al mesòfil. En conseqüència la Rubisco té limitada la discriminació davant del ^{13}C. En condicions on la capa límit de la beina es troba semi tancada, el terme (b_4 + Φ b_3) és molt menor que b_3 el que no afavoreix a la Rubisco a la hora de dur a terme un gran fraccionament (Bowman et al., 1989) (Figura 4).

Les plantes CAM (crassulacean acid metabolsim) capten el CO_2 a través de la PEPC d'una manera similar a les plantes C_4. La diferència amb les C_4, és que les plantes CAM fixen el CO_2 durant la nit on el fosfoenol piruvat és carboxilat i reduït a malat que s'acumula a la vacuola. Després es descarboxila durant el següent període de

llum i la Rubisco fixa aquest CO_2 (O'Leary, 1981). Aquest mecanisme els hi permet mantenir els estomes tancats durant el dia, en cas de que les condicions diürnes siguin desfavorables. Per contra, si les condicions són favorables, les plantes CAM poden obrir els seus estomes durant el dia i fixar CO_2 a través de la Rubisco (com una C_3). Així, la $\delta^{13}C$ d'una planta CAM presenta una gran variabilitat ja que la $\delta^{13}C$ pot fluctuar entre el rang de valors marcats per les plantes de metabolisme C_3 i C_4 (Vogel, 1980).

3.2.2 Fraccionament i variació de la $\delta^{13}C$ dins de la planta

En termes generals, la importància de la $\delta^{13}C$ es basa en el fet que aquest isòtop és capaç d'integrar la interrelació entre les discriminacions bioquímiques durant assimilació de CO_2 i les condicions ambientals (Dawson et al., 2002). En un sentit ampli podriem dir que hi ha tres factors principalment que poden provocar l'alteració de la $\delta^{13}C$ en els diferents teixits de la planta; (i) les diferències constitutives o morfològiques específiques de cada teixit de la planta (Araus et al., 1992, 1993), (ii) el metabolisme fotosintètic (Cerling et al., 1997) i (iii) les reaccions post fotosintètiques (Badeck et al., 2005). Tot i que la majoria d'estudis amb isòtops estables, es duen a terme a les fulles (especialment en el cas del carboni), la variabilitat ambiental (o genotípica) també pot quedar reflectida en altres teixits autòtrofs o inclús heteròtrofs de la planta. Però, s'ha de tenir cura alhora d'interpretar la $\delta^{13}C$ en els diferents teixits de planta, ja que els processos de fraccionament post-fotosintètic com serien la respiració o el transport de foto-assimilats podrien estar alterant la composició isotòpica original del teixit (Badeck et al. 2005). El fraccionament de la $\delta^{13}C$ dels compostos assimilats recentment en els òrgans fotosintètics i la seva subseqüent remobilització a òrgans heterotròfics, s'ha demostrat majoritàriament en espècies arbòries (Damesin i Lelarge, 2003; Scartazza et al., 2004; Badeck et al., 2005; Brandes et al., 2006; Gessler et al., 2009a) i espècies arbustives (Tcherkez et al., 2004;

Badeck et al., 2005). Per contra, en espècies herbàcies aquest fraccionament post-fotosintètic no acostuma ser tant evident i de fet no s'ha pogut confirmar (Yoneyama et al., 1997; Ghashghaie et al., 2001; Gessler et al., 2009b; Kodama et al., 2011).

També, el període de formació dels diferents òrgans de la planta (el que implica una possible formació dels òrgans sota condicions ambientals diferenciades) s'ha de tenir en compte alhora de realitzar estudis on s'utilitza simultàniament la $\delta^{13}C$ d'òrgans autòtrofs i heteròtrofs (Condon i Richards 1992). No obstant això, hi ha un ampli rang d'estudis on s'han trobat relacions significatives entre la $\delta^{13}C$ del gra madur i les condicions hídriques durant el període reproductiu en cereals (Araus et al., 1997, 2003, 2013b; Cabrera-Bosquet et al., 2009a).

3.2.3 Alteració de la $\delta^{13}C$ degut a l'efecte de factors ambientals

El principal factor que pot provocar una gran alteració en la $\delta^{13}C$ dels diferents teixits d'una mateixa espècie són les condicions ambientals. Concretament, la disponibilitat d'aigua n'és un dels més importants i àmpliament estudiats. En condicions de sequera per poder mantenir el potencial i el contingut hídric als teixits, en les plantes C3 els estomes es tanquen (Farquhar, 1989) limitant la fotosíntesi. Aquest tancament estomàtic degut a les condicions de sequera fa decréixer la $\Delta^{13}C$ (o augmentar la $\delta^{13}C$) (Araus et al., 1997) degut a la disminució del C_i (equació 4). Així, la $\Delta^{13}C$ (o altrament $\delta^{13}C$) s'ha proposat com una mesura integradora en el temps de la resposta de les plantes a l'estrès hídric ja que és una variable depenent del radi C_i/C_a.

Per altra banda, la fotosíntesi C_4 és una adaptació de la via C_3 que supera la limitació de la fotorespiració, millorant l'eficiència fotosintètica i minimitzant la pèrdua d'aigua en ambients càlids i secs (Edwards i Walker 1983). Generalment, les espècies C4 viuen en climes més càlids que les espècies C_3 (Sage i Monson 1999). No obstant això, aquesta variabilitat ambiental no és tant evident en les plantes C_4 comparat

amb les plantes C_3, pel que fa a la variació de la $\Delta^{13}C$. De fet, en les plantes C_4 la $\Delta^{13}C$ no només depèn de la Rubisco si no també està relacionada amb la Φ (equació 5). L'alteració de la $\delta^{13}C$ en condicions de sequera és molt menor en les plantes C_4 que en les C_3, degut a que el CO_2 alliberat a les cèl·lules de la capa límit de la beina dona poques oportunitats a la Rubisco de discriminar l'isòtop més lleuger ^{12}C.

D'altra banda, un altre factor que pot provocar una gran alteració en la $\delta^{13}C$ dels diferents teixits d'una mateixa espècie és el nitrogen. Però, l'efecte de nitrogen (N) en la $\delta^{13}C$ segueix sent poc clar. Hi ha estudis contradictoris que reporten l'efecte del N en la $\delta^{13}C$ (Zhao et al., 2007; Serret et al., 2008). Així, l'augment del subministrament de N, s'ha observat que pot disminuir la $\delta^{13}C$ (Shangguan et al., 2000), augmenta-la (Cabrera-Bosquet et al., 2007) o bé no tenir cap efecte (Hubick, 1990).

3.2.4 Isòtop estable de carboni com a eina de fenotipejat

La discriminació de l'isòtop del carboni, $\Delta^{13}C$ (o composició isotòpica del carboni, $\delta^{13}C$) s'ha utilitzat com una eina per seleccionar genotips amb alta eficiència transpiratòria (ET) durant el període en el qual la matèria seca s'assimila (Farquhar i Richards, 1984b; Araus et al., 2002, 2008; Richards et al., 2002). S'ha demostrat a més que amb l'increment de la disponibilitat hídrica, la $\delta^{13}C$ disminueix (o altrament augmenta $\Delta^{13}C$) (Araus et al., 2003; Condon et al., 2004). A més, la $\delta^{13}C$ s'ha correlacionat raonablement amb la ET (Hall et al., 1990) i amb una elevada eficiència de l'ús de l'aigua (EUA) (Condon et al., 2002).

Per altra banda, està molt ben documentat que les plantes tanquen els estomes com a resposta a l'estrès per sequera (Farquhar, 1989), però s'ha observat una major conductància estomàtica (g_s), transpiració i en conseqüència rendiment en genotips de blat sotmesos a sequera (Araus et al., 2002). Això es pot explicar

pel fet que uns valors alts de g_s al llarg del temps permeten una major fixació de CO_2 i en conseqüència un augment de la producció de les plantes (Blum, 2009). Malauradament, l'ús de la $\delta^{13}C$ com a eina de selecció de genotips amb un major rendiment potencial (GY) no és sempre constant. Això és degut a que la relació entre la $\delta^{13}C$ i GY depèn de les condicions d'aigua experimentades pel cultiu. Així, s'han vist relacions negatives entre la $\delta^{13}C$ i GY en condicions d'aigua òptimes i d'estrès moderat tant en blat com en altres cereals C3 (Araus et al. 1998; Fischer et al. 1998; Voltas et al. 1999; Araus et al. 2003; Monneveux et al. 2005; Lopes i Reynolds 2010). Per contra, en condicions on el dèficit hídric és molt gran, s'ha observat una relació positiva entre la $\delta^{13}C$ i el GY (Voltas et al. 1999; Rebetzke et al. 2002; Condon et al. 2002), sent els genotips amb l'estratègia més conservadora (associat a una alta EUA i $\delta^{13}C$) els més productius (Voltas et al. 1999; Condon et al. 2004).

3.3 Isòtops d'hidrogen i d'oxigen a les plantes

Durant la dècada dels anys 50 es va descobrir que la composició isotòpica de l'aigua fresca depenia dels diferents orígens de l'aigua oceànica (Dansgaard, 1954). La precipitació registrada en diferents habitats proporcionava així un primer nivell de control de la composició isotòpica de l'oxigen i l'hidrogen incorporats als carbohidrats de la planta. En aquest rang hi havia una forta covariació en l'abundància dels isòtops de l'hidrogen i oxigen. Posteriorment es va demostrar que la $\delta^{2}H$ i $\delta^{18}O$ estaven directament correlacionades amb la temperatura mitja anual (Dansgaard, 1964). Aquesta correlació apareixia perquè la temperatura de condensació de l'aigua de la pluja té una gran influència sobre la composició isotòpica de $\delta^{2}H$ i $\delta^{18}O$. Més tard es va estudiar aquestes característiques des d'un punt de vista més global i es demostrà que la composició isotòpica de l'aigua del sòl era molt similar a la de l'aigua de la pluja (Gat, 1971). Seguidament, Epstein et al. (1977) van demostrar que hi havia una relació directa entre les variacions temporals o geogràfiques de la composició

isotòpica de la planta i les variacions de la temperatura anual mitjana. Així, aquests treballs tant pioners van suposar un gran incentiu per explorar la utilitat dels quocients isotòpics de l'oxigen i de l'hidrogen a les plantes com a indicadors i integradors de paràmetres ambientals. Així, es va assumir que els efectes isotòpics associats a processos fisiològics dins la planta, per exemple a la síntesi de carbohidrats eren constants. A més a més, a través d'un estudi dut a terme per Deniro i Epstein (1979) es va demostrar que tot l'oxigen lligat a la cel·lulosa durant la seva producció durant el procés de la fotosíntesi aparentment provenia de l'aigua. Malauradament, estudis subsegüents van demostrar que l'efecte isotòpic entre l'aigua del sòl i la cel·lulosa no era constant (Sternberg i Deniro 1983a). La inconstància d'aquests resultats es va explicar pel fet de que les composicions isotòpiques d'oxigen i d'hidrogen de la cel·lulosa es veien afectades per l'aigua implicada en la seva síntesi; és a dir per l'aigua metabòlica (Sternberg et al., 1986). Així l'estudi de la composició isotòpica de l'oxigen i l'hidrogen es va complicar ja que es va veure que diversos factors podien afectar la composició isotòpica primigènia de la cel·lulosa.

Poc a poc les relacions isotòpiques entre l'aigua metabòlica i l'aigua del sòl s'han anat entenent una mica més, tot i que encara avui en dia són complicades degut als efectes isotòpics associats amb l'evaporació i amb el transport de l'aigua a les fulles. A més a més la composició isotòpica primigènia de l'aigua metabòlica de les fulles, impresa en els carbohidrats de la planta durant la fotosíntesi, es podria modificar posteriorment degut a processos d'intercanvi post-fotosintètic que ocorren en fraccions d'aigua metabòlica isotòpicament diferents (Barbour, 2007). A part d'això, les característiques fisiològiques de la planta també poden influenciar de manera molt marcada ja sigui l'efecte isotòpic metabòlic com l'efecte de la composició isotòpica de l'aigua on es desenvolupen les reaccions metabòliques (Yakir i Deniro, 1990). Així doncs, la composició isotòpica d'oxigen i d'hidrogen final de la cel·lulosa, serà en definitiva un recull de la suma de tots aquests factors (Yakir, 1992).

3.3.1 Isòtops d'oxigen en plantes

L'oxigen conté una amplia família d'isòtops, tres dels quals són estables (^{18}O, ^{17}O, ^{16}O), tot i que en el camp de la fisiologia l'única relació que s'utilitza és la $^{18}O/^{16}O$. La importància de la $\delta^{18}O$ es deu bàsicament a la capacitat d'aquest isòtop d'integrar les condicions evaporatives durant tot desenvolupament de la planta (Barbour et al., 2000a; Barbour, 2007). La conductància estomàtica afecta essencialment a la $\delta^{18}O$, a diferència de la $\delta^{13}C$, que varia simultàniament en funció de la g_s i de la fotosíntesi (Barbour et al., 2000a; Farquhar et al., 2007b). Tot i així, l'ús de la $\delta^{18}O$ no és senzill ja que hi ha diversos factors que poden afectar aquest isòtop. Bàsicament, són tres els factors poden influenciar d'una manera molt marcada la signatura isotòpica de l'oxigen ($\delta^{18}O$) de la planta (Barbour et al., 2000). El primer factor és (i) la composició isotòpica de la font d'aigua que absorbeix la planta (Yakir i Deniro, 1990; Roden i Ehleringer, 1999; Roden et al., 2000; Williams et al., 2005), en segon lloc (ii) l'evaporació de la fulla durant la transpiració provoca un enriquiment en ^{18}O respecte al ^{16}O (Gonfiantini et al., 1965; Pande et al., 1995; Barbour, 2007) i en tercer lloc (iii) el fraccionament de les reaccions bioquímiques durant la síntesi de matèria orgànica (Yakir, 1992; Farquhar et al., 1993). La composició isotòpica de l'oxigen tant de la matèria orgànica com de l'aigua de la planta es pot expressar com enriquiment envers a l'aigua font $\Delta^{18}O$:

$$\Delta(‰) = \frac{\delta^{18}O_m - \delta^{18}O_r}{1+(\frac{\delta^{18}O_r}{1000})} \quad (6)$$

on $\delta^{18}O_m$ i $\delta^{18}O_r$ es refereix a la composició isotòpica de l'oxigen de la mostra de la planta i de l'aigua font, respectivament.

3.3.1.1 Composició isotòpica de l'oxigen de l'aigua font

La humitat del sòl és bàsicament l'aigua font que utilitzen la majoria de plantes terrestres, i està fortament lligada a la precipitació. No obstant, la $\delta^{18}O$ de l'aigua captada per les plantes pot variar considerablement comparada amb la $\delta^{18}O$ de l'aigua de la precipitació. L'origen d'aquesta variació pot ser una conseqüència de l'evaporació experimentada en les capes superficials (provocant un enriquiment) i de la separació temporal de la precipitació i l'absorció d'aigua subterrània (Dawson i Ehleringer, 1993). A més a més, la $\delta^{18}O$ de la precipitació també depèn de l'estacionalitat, la quantitat de pluja, la continentalitat (distància de la costa), la latitud, la altitud i la temperatura (Farquhar et al., 1993; Barbour et al., 2001).

Tanmateix, tot i les esmentades etapes de fraccionament que poden donar-se abans de que l'aigua del sòl sigui absorbida per la planta, no s'ha demostrat un fraccionament isotòpic de l'oxigen quan la planta capta aigua del sòl (Dawson i Ehleringer, 1991). Així, en espècies no llenyoses, el fraccionament isotòpic de l'oxigen de l'aigua associat a la captació d'aigua del sòl i el subseqüent transport pel xilema no s'ha demostrat (ja que l'anatomia de la tija herbàcia sovint dificulta el mostreig independent del xilema i del floema). Diversos estudis realitzats en plantes herbàcies confirmen l'absència de fraccionament entre la $\delta^{18}O$ de l'aigua extreta del sòl (aigua font) i la $\delta^{18}O$ de l'aigua basal de la tija (Williams et al., 2005; Barnard et al., 2006), així com l'absència de fraccionament durant la captació d'aigua per les arrels (Dawson i Ehleringer, 1991). Ara bé, en espècies arbòries si que s'ha observat un cert desacoblament temporal entre la $\delta^{18}O$ de l'aigua del xilema i l'aigua del sòl (Brandes et al., 2007; Offermann et al., 2011; Treydte et al., 2014). No obstant això, en condicions de camp també s'ha observat que en funció de la disponibilitat d'aigua en el sòl (i el nivell de competència), les arrels són capaces de canviar el patró de captació d'aigua, tant en espècies herbàcies (Zhang et al., 2011) com en espècies arbòries (Schwendenmann et al., 2015).

Per això és important estudiar la $\delta^{18}O$ de l'aigua font ja que aquesta pot influir d'una manera molt marcada en la $\delta^{18}O$ de la matèria orgànica de la planta (Barbour, 2007). En primer lloc l'aigua font determina la $\delta^{18}O$ de l'aigua que arriba a la fulla (Gonfiantini et al., 1965) i a més és la que posteriorment participa en moltes de les reaccions de la fotosíntesi. En segon lloc, degut a que l'aigua del floema i del xilema solen estar en equilibri (Gessler et al. 2014), l'aigua font és el medi on tenen lloc les transformacions post-fotosintètiques durant el transport floemàtic al llarg de la tija.

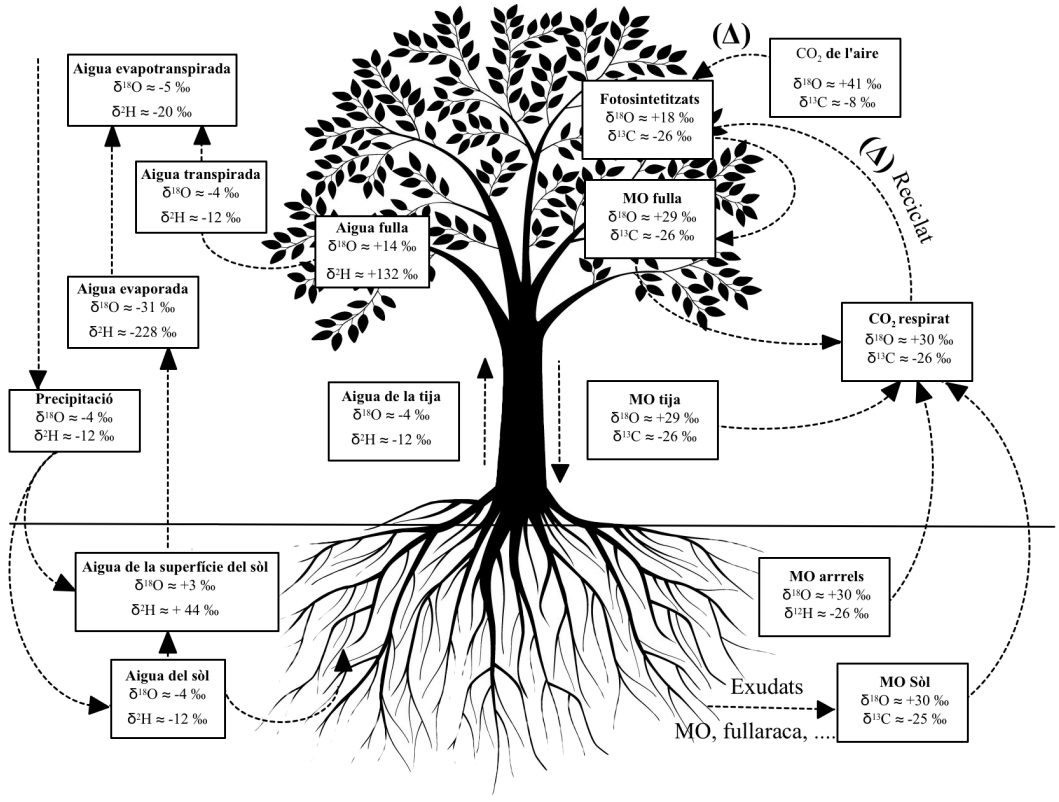

***Figura 6**. (Adaptat de Dawson et al. 2002). "Pools" de la composició isotòpica del C, O i H en el cicle del carboni i de l'aigua a través la planta . Les fletxes representen els processos de fraccionament i les caixes els pools. Els valors es van extreure de dades experimentals obtingudes a Israel i les dades meteòriques provenen de la mateixa regió (Gat i Carmi 1970). Els valors es troben en les escales SMOW (per ^{18}O) i PBD (per ^{13}C) i són aproximats ja que poden variar en funció de la localització geogràfica i les condicions ambientals. MO es refereix a la matèria orgànica.*

3.3.1.2 Enriquiment evaporatiu en la fulla

Durant la transpiració es produeix un enriquiment de la $\delta^{18}O$ de l'aigua del xilema (aigua font) degut a l'evaporació (Gonfiantini et al., 1965; Farquhar et al., 1993). Aquest aspecte bàsicament es regeix per la difusió diferencial entre $H_2{}^{18}O$ i $H_2{}^{16}O$ a la capa límit de la fulla (Pande et al., 1995), juntament amb la regulació d'intercanvi de gasos foliars (Farquhar 1989). Tanmateix, tot i que els mecanismes d'enriquiment de la fulla deguts a la evaporació estan plenament establerts i la consegüent empremta de la senyal isotòpica en els assimilats més recents (Barbour et al. 2004), hi ha diferents processos que poden complicar la resposta de les plantes a les condicions ambientals (Figura 6), els quals s'expliquen en detall a continuació.

La discriminació isotòpica de l'oxigen entre la font d'aigua i els espais intercel·lulars de la fulla s'expressa de la següent manera (Farquhar i Lloyd, 1993):

$$\Delta^{18}O_e = \frac{R_e}{R_s} - 1 \tag{7}$$

on $\Delta^{18}O_e$ és el grau de enriquiment en ^{18}O de l'aigua de la fulla a les zones d'evaporació i R_e i R_s, són les relacions $^{18}O/^{16}O$ de l'aigua foliar (en zones d'evaporació) i de la font d'aigua, respectivament. Però, en condicions estables, l'enriquiment de la fulla en zones d'evaporació està relacionat amb el fraccionament dels factors de la pressió de vapor i cinètic:

$$\Delta^{18}O_e = \varepsilon^+ + \varepsilon_k + (\Delta^{18}O_v - \varepsilon_k)\frac{e_a}{e_i} \tag{8}$$

on ε^+ és el fraccionament de l'equilibri que es produeix durant la fase del canvi d'aigua líquida a vapor, ε_k és el fraccionament cinètic que es produeix durant la difusió a través dels porus dels estomes en la capa límit de la fulla, $\Delta^{18}O_v$ és l'enriquiment en

^{18}O de l'aigua de vapor atmosfèrica en relació amb l'aigua font, i e_a/e_i és el quocient de la pressió de vapor entre l'atmosfera i l'espai intercel·lular. L'equació 8 suggereix doncs que a una e_a constant, l'increment de la conductància estomàtica resultarà en un menor enriquiment a les zones d'evaporació de les fulles donant lloc a una reducció de la temperatura de la fulla i de e_i causat per una major transpiració. Així, el factor de fraccionament ε^+ depèn de la temperatura (Bottinga i Craig 1968):

$$\varepsilon^+(‰) = 2{,}644 - 3{,}206\,(\frac{10^6}{T_1}) + 1{,}534\,(\frac{10^6}{T_1^2}) \quad (9)$$

on T_1 és la temperatura de la fulla. A més el factor de fraccionament cinètic ε_k es pot calcular a partir de la conductància de l'estoma (g_s) i de la capa límit (g_b) referent al vapor d'aigua (Farquhar et al., 1998)

$$\varepsilon^+ = \frac{32g_s^{-1}+21g_b^{-1}}{g_s^{-1}+g_b^{-1}} \quad (10)$$

on, g_s i g_b són la resistència a la difusió del vapor d'aigua de l'estoma i de la capa límit, respectivament. Sota condicions d'humitat al camp és molt freqüent que la $\Delta^{18}O_v$ sigui equivalent a ε^+ (Farquhar et al., 2007a), així l'equació 10 por quedar ajustada de la següent manera:

$$\Delta^{18}O_e \approx (\varepsilon^+ + \varepsilon_k)(1 - \frac{e_a}{e_i}) \quad (11)$$

Deixant a part els models desenvolupats, en alguns casos s'observà que la $\delta^{18}O$ de l'aigua de la fulla mesurada era menys enriquida de l'esperat (Yakir et al., 1989) i en altres casos és al revés (Helliker i Ehleringer, 2000). Així, es va elaborar un nou model coherent d'enriquiment que englobava la fulla i les venes per explicar el fet que la convecció de l'aigua no enriquida s'oposa a la retrodifusió de la $H_2{}^{18}O$ (aigua enriquida)

a les zones d'evaporació. Així, tenint en compte un increment de la transpiració, el nou model descrit és capaç de predir la discrepància entre l'enriquiment descrit a l'equació 8 i el mesurat a la làmina de la fulla ($\Delta^{18}O_L$) (Farquhar i Lloyd, 1993). Aquest procés s'anomena l'Efecte Péclet (Figura 7) i es caracteritza per ser un paràmetre adimensional que representa el radi de convecció i la difusió:

$$\wp = \frac{EL}{CD} \tag{12}$$

on E és la taxa de transpiració de la fulla (mol m^{-2} s^{-1}), L és la longitud (m) del moviment de l'aigua de les venes a la zona d'evaporació on l'efecte és evident, C és la concentració molar de l'aigua (55,5 x 10-3 mol m-3) i D és la autodifusivitat (m2s-1) de l'aigua enriquida ($H_2{}^{18}O$), $D = 119 \times 10^{-9} e^{\frac{637}{T-137}}$, on la T és la temperatura de l'aire en Kelvin (Cuntz et al., 2007).

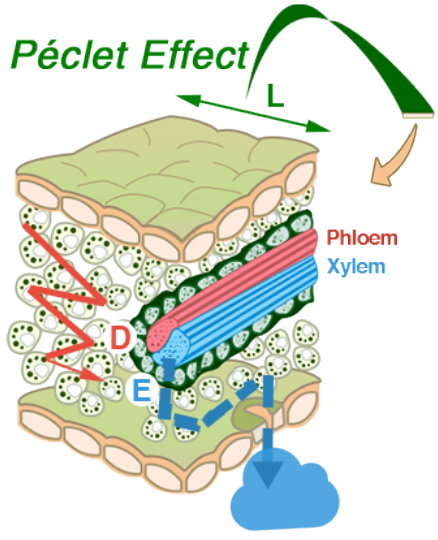

Figura 7*. Efecte Péclet. Il·lustració en una planta C_4 dels factors que intervenen en l'enriquiment de la fulla respecte a l'aigua font, on E és la transpiració, la D és l'autodifusivitat i la L la llargada de la fulla.*

Per altra banda, L no està únicament relacionada amb la distància física entre les venes i els estomes, sinó que també depèn del factor de tortuositat, que pot incrementar la llargària efectiva en almenys dos ordres de magnitud (Barbour i Farquhar, 2004). Així la mitjana de l'enriquiment de l'aigua de la fulla ($\Delta^{18}O_L$) sobre la llargària efectiva pot ser modelat de la següent manera (Farquhar i Lloyd, 1993):

$$\Delta^{18}O_L = \frac{\Delta^{18}O_e\left(1-e^{\wp}\right)}{\wp} \tag{13}$$

En definitiva, a causa de l'efecte de Péclet, l'enriquiment isotòpic mitjà de l'aigua de la fulla és menor a les zones d'evaporació i, com a resultat, hi ha un gradient de la $\delta^{18}O$ de l'aigua des del xilema a la cavitat subestomática, que al mateix temps és proporcional a la transpiració (Farquhar i Lloyd, 1993). A més, les fulles amb venació paral·lela mostren un gradient addicional al llarg del xilema. En un estudi dut a terme per Helliker i Ehleringer (2000) amb plantes C_4 monocotiledònies, la $\delta^{18}O$ de l'aigua transportada per les venes es va enriquir progressivament (a causa de l'evaporació) a les zones distals de la base de la làmina de la fulla. Aquest gradient no només s'observa en l'aigua, sinó que també es pot transmetre a la matèria orgànica, com es va observar a l'estudi dut a terme per Farquhar i Gan (2003) en blat de moro.

La transmissió de la $\delta^{18}O$ des de l'aigua de les fulles fins a la matèria orgànica engloba molts processos, cosa que complica la seva interpretació. Durant la metabolització en el cicle de Calvin, la matèria orgànica integra tres àtoms d'oxigen els quals provenen de dos àtoms d'aigua i un del CO_2 (Sternberg et al., 1986; Schmidt et al., 2001). No obstant això, degut a que el CO_2 dins de la fulla intercanvia ràpidament àtoms d'oxigen amb l'aigua, fa que el CO_2 i l'aigua comparteixin una $\delta^{18}O$ similar (Sternberg et al., 1986). En conseqüència les molècules orgàniques produïdes a les fulles reflectiran per una banda (i) la $\delta^{18}O$ de l'aigua en què es van formar degut a

l'intercanvi isotòpic entre l'aigua i els grups carbonil (Sternberg et al., 1986; Yakir, 1992) i per altra banda (ii) la via metabòlica dels diferents compostos en funció del temps de residència (Gessler et al., 2007). Durant el dia, les molècules trioses fosfat formades a partir de la fotosíntesi, s'exporten des del cloroplast al citosol (Flügge, 1999). En conseqüència, aquestes molècules reflectiran la $\delta^{18}O$ de l'aigua en la qual es van formar, és a dir, reflectiran la $\delta^{18}O$ del cloroplast. Després, les molècules triosa fosfat s'uneixen per formar sacarosa en el citoplasma (Barbour et al., 2000b). Així la $\delta^{18}O$ de la sacarosa reflectirà la $\delta^{18}O$ de l'aigua en què la molècula s'ha format, és a dir en aquest cas haurà de reflectir la $\delta^{18}O$ de l'aigua citoplasmàtica (Barbour et al., 2000b). Posteriorment, la sacarosa s'exporta de la fulla al floema (Barbour i Farquhar 2000).

En definitiva, la $\delta^{18}O$ de la sacarosa englobarà la $\delta^{18}O$ de l'aigua del cloroplast i la del citoplasma (les dues aigües que han participat en la formació de la molècula de sacarosa) més un factor de fraccionament εwc (Yakir i Deniro, 1990) (Figura 8). A l'equilibri, els àtoms d'oxigen dels grups carbonil de la cel·lulosa sintetitzada en teixits fotosintètics (Roden et al., 2000) es troben entre un 25 i 30‰ més enriquits que l'aigua en què es van formar (Sternberg i Deniro, 1983b) tant en espècies llenyoses (Yakir et al., 1990b; Barbour et al., 2000b) com no llenyoses (Helliker i Ehleringer, 2002a; Cernusak et al., 2003), on la sacarosa és de mitja un 27‰ més enriquida que l'aigua de la làmina en condicions isotòpiques estables (Cernusak et al., 2003):

$$\Delta^{18}O_{sacarosa} = 1{,}027\Delta^{18}O_L + 27‰ \quad (14)$$

No obstant això, encara no hi ha evidencies de quin component de l'aigua de la fulla (aigua en el cloroplast i citoplasma) està influint més fortament a la $\delta^{18}O$ de la sacarosa durant la seva síntesi (Barbour et al., 2000b).

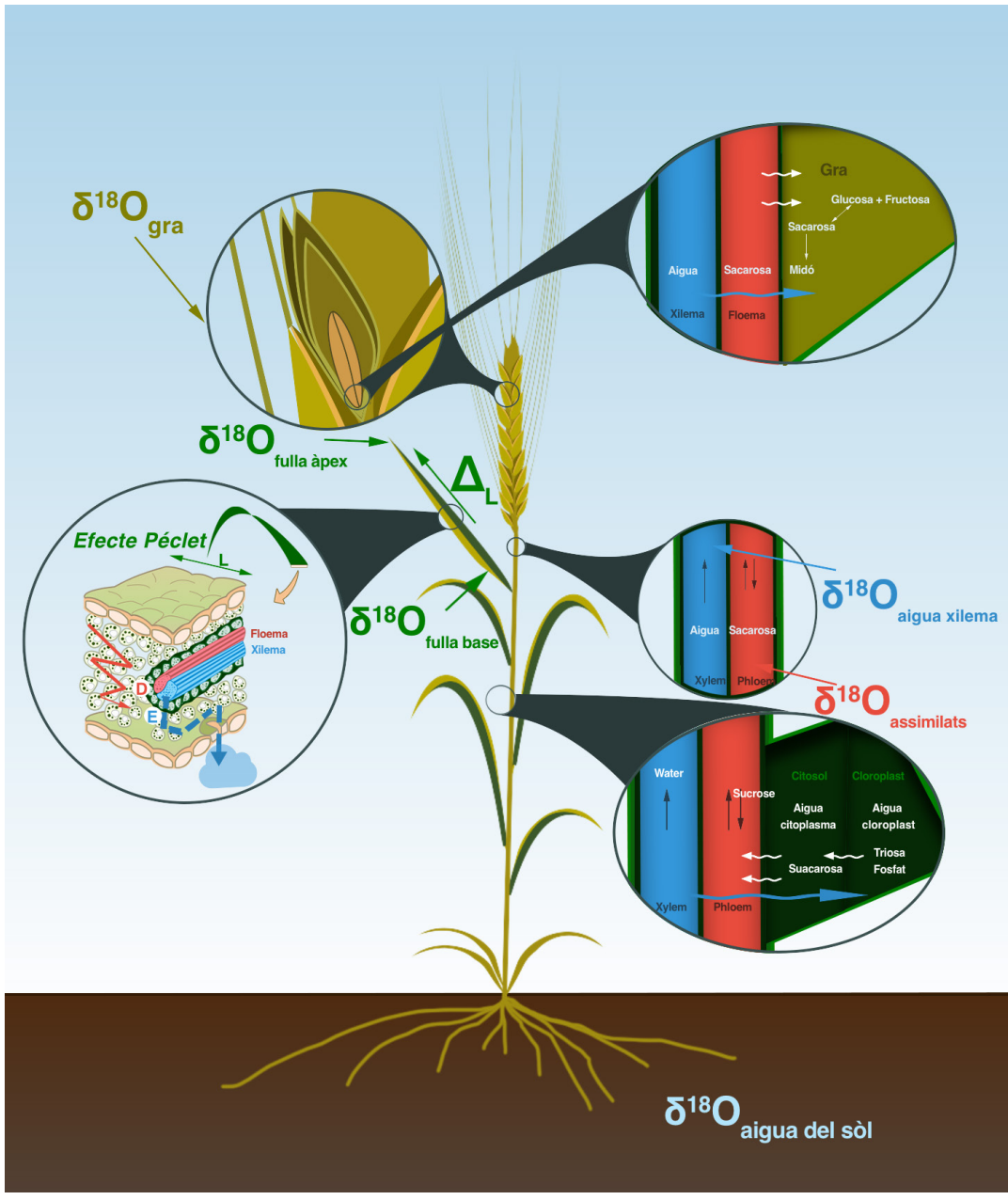

***Figura 8**. Representació gràfica dels diferents factors que poden afectar la $\delta^{18}O$ de la matèria orgànica, en aquest cas d'una planta C_3 com és el blat. Els principals efectes representats engloben les conversions metabòliques de la càrrega, transport i descàrrega dels assimilats des dels òrgans 'font' als òrgans 'destí' durant l'ompliment del gra, la formació de les molècules triosa fosfat i sacarosa a les fulles i la síntesi de midó als grans.*

3.3.1.3 Alteració de la $\delta^{18}O$ associada amb el transport, càrrega i descàrrega d'assimilats

La sacarosa és el component principal de la saba del floema en moltes espècies de plantes (Hayashi i Chino, 1990; Ohshima et al., 1990). La sacarosa és un disacàrid format per monosacàrids de glucosa (hexosa) lligats a la fructosa (pentosa) sense grups carbonil lliures (Preiss, 1982). Durant el seu transport pel floema l'intercanvi àtoms d'oxigen entre l'aigua del floema i la sacarosa és baix a causa de la seva manca de grups carbonil (Gessler, 2011). De fet, en un experiment realitzat per Gessler et al. (2007) en *Ricinus communis*, la $\delta^{18}O$ dels assimilats més recents de la fulla es van conservar en la matèria orgànica del floema (de mitjana durant el cicle diari) i també durant el seu transport a la base de la tija. Així mateix, en un experiment realitzat per Offermann et al. (2011), on els sucres es van incubar durant 5 hores amb aigües de $\delta^{18}O$ diferents, no es van observar diferències significatives entre la $\delta^{18}O$ dels sucres sotmesos a diferents fonts de $\delta^{18}O$. No obstant, el model de pressió-flux que caracteritza el transport del floema (Van Bel, 2003), implica un intercanvi continu de sacarosa entre els tubs cribosos del floema i les cèl·lules de companyia. Per tant, és en aquest moment on apareixen possibilitats per la interconversió metabòlica de la sacarosa a sucres intercanviables dins el citoplasma de les cèl·lules de companyia. En conseqüència, tals conversions dels sucres i el subseqüent intercanvi d'oxigen podrien estar causant una pèrdua parcial del senyal d'enriquiment de la fulla, que en part se substituiria pel valor de la $\delta^{18}O$ de l'aigua en el floema (el qual simultàniament es troba en equilibri amb l'aigua del xilema. En aquest sentit, Offermann et al. (2011a) van observar una disminució significativa de la $\delta^{18}O$ al comparar la fracció soluble de la matèria orgànica de la fulla amb la matèria orgànica del floema, evidenciant les possibles conversions dels sucres i el subseqüent intercanvi d'oxigen. En el mateix estudi, la $\delta^{18}O$ de la matèria orgànica del floema no estava relacionada amb la signatura de la $\delta^{18}O$ de les fulles, però si que hi havia una bona correlació amb les variacions

estacionals d'aigua del sòl i també del xilema. De la mateixa manera, Gessler et al. (2013) van observar una menor $\Delta^{8}O$ dels sucres del floema comparada amb la $\Delta^{18}O$ de la fulla en tres de cada cinc espècies d'arbres estudiats. Malauradament, encara hi ha una manca de coneixement de qualsevol canvi addicional entre les molècules orgàniques i l'aigua durant la càrrega o descàrrega del floema. A les cèl·lules acompanyants, la càrrega o descàrrega de la sacarosa al floema o des del floema pot provocar conversions metabòliques addicionals (Van Bel, 2003). De fet, s'han demostrat en diferents espècies d'arbres, evidències d'intercanvi addicional d'oxigen durant aquests processos de transport (Gessler et al., 2014). Per tant, els canvis en la $\delta^{18}O$ durant el transport del floema són variables i probablement específics de cada espècie, i per això s'han de tenir en compte alhora d'interpretar la $\delta^{18}O$ dels teixits heterotròfics com els grans o els òrgans reproductors.

3.3.1.4 Isòtop estable d'oxigen com a eina de fenotipejat

Com hem vist més amunt, durant les darreres dècades, la $\delta^{18}O$ s'ha proposat com un indicador que integra en el temps les diferències de la conductància estomàtica i de la transpiració del conreu (Barbour i Farquhar, 2000). Com a conseqüència durant la passada dècada la $\delta^{18}O$ s'ha formulat com un caràcter de selecció per la millora genètica, ja que integra les condicions evaporatives de la fulla durant el cicle del cultiu (Barbour et al., 2000a; Barbour, 2007). En aquest sentit, la $\delta^{18}O$ s'ha proposat com un predictiu de rendiment en blat (Barbour et al., 2000a; Ferrio et al., 2007; Cabrera-Bosquet et al., 2009b). Tanmateix, estudis previs han demostrat que la $\delta^{18}O$ és sensible a la temperatura, a la humitat de l'aire (Barbour i Farquhar, 2000; Helliker i Ehleringer, 2002b) a la salinitat (Yousfi et al., 2012) i a la humitat del sòl (Yakir i Deniro, 1990; Ferrio et al., 2007). A més, degut la $\delta^{18}O$ no depèn del metabolisme fotosintètic, el fa un tret molt valuós alhora d'avaluar les condicions evaporatives de la fulla durant el cicle del cultiu (Barbour i Farquhar, 2000; Barbour et al., 2000a)

especialment en plantes amb metabolisme C4. Així, la utilització de la $\delta^{18}O$ en els cultius C_4 pot ser de gran ajuda quan la conservació de la $\Delta^{13}C$ està limitada (Helliker i Ehleringer, 2002b; Araus et al., 2008).

Malauradament i a diferència del que succeeix amb $\delta^{13}C$, en el cas de $\delta^{18}O$ la seva potencialitat com criteri de millora no s'ha assentat i la seva utilització encara és limitada. Tot i que que l'ús de la $\delta^{18}O$ s'ha proposat com a eina de fenotipejat en la millora dels cultius en el blat de moro (Cabrera-Bosquet et al., 2009b) i en altres cultius com el blat (Barbour et al., 2000a; Cabrera-Bosquet et al., 2011), la seva implementació en els programes de millora està menys avançada en comparació a l'isòtop de carboni. Això és degut a que els valors d'isòtops d'oxigen en el material vegetal analitzats no sempre coincideixen amb els pronosticats a partir de models teòrics (Barbour, 2007; Farquhar et al., 2007a). De fet hi ha una manca de coneixement sobre la importància relativa dels diferents processos que poden alterar la $\delta^{18}O$ dins dels diferents òrgans de la planta. Els mecanismes que controlen la transferència de la senyal isotòpica de l'oxigen des dels òrgans autòtrofs (per exemple, fulles) als teixits heterotròfics (per exemple, inflorescències o fruits) encara es desconeixen en bona part. De fet, el coneixement dels mecanismes que controlen la transferència de la senyal isotòpica de l'oxigen entre els diferents teixits dins la planta són bàsics i de particular importància per les interpretacions fisiològiques basades en la composició d'isòtops d'oxigen en els teixits vegetals. Tanmateix, el coneixement d'aquests mecanismes ajudaria a avaluar les diferències genotípiques en el rendiment i l'adaptació a l'estrès hídric en els cultius (Ferrio et al., 2007; Araus et al., 2013b).

3.4.1 Isòtops d'hidrogen

L'estudi de la composició isotòpica de l'hidrogen ha sigut i encara és un repte en el camp de la fisiologia vegetal, ja que hi ha estudis que demostren que la δ^2H de la cel·lulosa de plantes crescudes una al costat de l'altra pot arribar a variar més d'un 160‰. Tot i que l'aigua és l'única font d'hidrogen de la matèria orgànica de la planta, la seva composició isotòpica no es conserva durant el metabolisme de la cel·lulosa (Ziegler, 1989). De fet, s'ha demostrat que la fotosíntesi té un gran impacte en la δ^2H de la matèria orgànica de la planta, però tot i així els mecanismes que afecten a la δ^2H de la planta relacionats amb el metabolisme fotosintètic són encara avui en dia difícils de comprendre (Yakir, 1992). A més, els estudis en el camp de la fisiologia vegetal que fan referència a la composició isotòpica de l'hidrogen són molt escassos, i la literatura que hi ha disponible és de fa més de dues dècades (Epstein et al., 1977; Sternberg i Deniro, 1983a; Yakir i Deniro, 1990; Yakir et al., 1990a; Roden, 1999). No obstant, segon el que s'ha esbrinat fins ara, la informació que aquest isòtop proporciona és bàsicament similar a la que ofereix l'oxigen, excepte per que la δ^2H és molt diferent entre plantes amb diferents vies metabòliques (Ziegler et al., 1976; Stenberg et al., 1984).

3.4.1.1 Composició isotòpica de l'hidrogen de l'aigua font

Al igual que en el cas de l'oxigen, no s'ha demostrat un fraccionament isotòpic de l'hidrogen de la planta durant captació d'aigua del sòl (Wershaw et al., 1966; Dawson i Ehleringer, 1991). De fet la δ^2H s'ha utilitzat per avaluar l'absorció temporal i espaial de l'aigua al sòl a partir de diferents espècies de plantes per obtenir informació sobre la complementarietat de l'ús de l'aigua (Dawson et al., 2002).

Així, la importància d'estudiar la δ^2H de l'aigua font rau en el fet de que pot influir notablement, com ja s'ha comentat en el cas del $\delta^{18}O$, en la δ^2H de la matèria orgànica

de la planta (Yakir, 1992), ja que l'aigua font és el medi on tenen lloc les reaccions fotosintètiques i post-fotosintètiques.

3.4.1.2 Efecte fotosintètic i post-fotosintètic en la δ^2H

L'hidrogen incorporat en els productes fotosintètics durant els primers passos del procés de reducció resulta altament empobrit en ^2H. No obstant això, una gran proporció d'aquests hidrògens són posteriorment substituïts per l'intercanvi amb aigua, donant pas a un enriquiment en ^2H durant el metabolisme heterotròfic (Yakir, 1992). D'aquesta manera els efectes isotòpics associats al metabolisme dels carbohidrats poden proporcionar informació sobre l'estat autotròfic del teixit d'una planta. Això és degut a que la relació isotòpica de l'hidrogen dels carbohidrats reflexa els efectes nets de dos efectes isotòpics antagònics, els associats amb la fotosíntesi i el metabolisme heterotròfic. Mentre que l'efecte de la fotosíntesi produeix carbohidrats amb l'hidrogen unit al carboni empobrit en δ^2H (amb un factor de fraccionament al voltant de -170‰), el metabolisme post-fotosintètic mostra un efecte oposat d'enriquiment en δ^2H (amb un factor de fraccionament al voltant de +150‰) comparat amb la δ^2H de l'aigua del medi. Per tant, petits canvis d'activitat metabòlica (ex. inici del cicle de la glicòlisi-gluconeogènesi) poden produir grans canvis en el quocient $^2H/^1H$ dels hidrògens no intercanviables en els carbohidrats per qualsevol quocient $^2H/^1H$ d'aigua. Aquesta hipòtesi va ser marcadament recolzada pels experiments duts a terme per Luo i Sternberg (1991). En aquest estudi la cel·lulosa formada en el citoplasma (activitat heterotròfica) va mostrar una δ^2H més alta que el midó produït en el cloroplast (activitat autotròfica). Aquest fet, va suggerir que el valor més elevat de la δ^2H de la cel·lulosa formada en el citoplasma és degut a un increment de l'activitat heterotròfica (reaccions post-fotosintètiques produïdes al citoplasma).

Efecte fotosintètic en la δ^2H

Un possible mecanisme associat al fort efecte d'empobriment de la δ^2H degut a la fotosíntesi, es va argumentar en un estudi dut a terme per Luo et al. (1991) de la següent manera: els protons que estan en equilibri amb l'aigua disponibles per la reducció del $NADP^+$ (l'hidrogen donant en la fotosíntesi primària) tenen una δ^2H altament empobrida comparat amb l'aigua del medi. Els protons que s'utilitzen per la reducció del $NADP^+$ venen de la molècula de l'aigua (Hoganson i Babcock, 1997):

$$2\ H_2O \rightarrow O_2 + 4\ H^+ + 4\ e^- \quad (15)$$

Tanmateix, la reducció de l'àcid fosfoglicèric per el NADPH durant la fotosíntesi és una reacció irreversible que consumeix pràcticament tot el potencial reductor i és probable que aquest empobriment de la signatura isotòpica dels protons es transmeti als carbohidrats (Figura 9):

$$NADP^+ + 2e^- + 2H \rightarrow NADPH + H^+ \quad (16)$$

Així doncs les reaccions durant les quals el NADP es redueix a través dels protons de l'aigua i en conseqüència l'hidrogen és donat per el NADPH durant la fotosíntesi són per tant les úniques candidates que podrien provocar un empobriment en la δ^2H dels carbohidrats. Aquesta hipòtesi la va recolzar un estudi realitzat per Yakir i Deniro (1990) amb l'espècie Lemna gibba L. En aquest estudi les plantes es van sotmetre a tres condicions de creixement: heterotròfiques (amb una font de sacarosa i foscor), foto-heterotròfiques (amb una font de sacarosa i llum) i autotròfiques (sense sacarosa i amb llum). Les plantes crescudes en condicions autotròfiques (tram actiu sota condicions de llum) van mostrar un factor de fraccionament de la δ^2H entre l'aigua i la cel·lulosa molt empobrit (-171‰). Aquest factor de fraccionament tant

baix podria estar indicant que el NADPH i la subsegüent reducció del NADP amb els protons de l'aigua (reaccions que ocorren en condicions de llum) podrien ser els responsables de tal empobriment. Així l'anàlisi de δ^2H en els òrgans autotròfics podria ser un indicador de l'efecte isotòpic net associat de la formació del NADPH. Per altra banda en tal experiment les plantes crescudes en condicions heterotròfiques (sota foscor) mostraren un factor de fraccionament de l'hidrogen entre l'aigua i la cel·lulosa molt elevat (+158‰) cosa que indicaria un efecte post-fotosintètic en la δ^2H.

Ziegler et al. (1976) i Sternberg et al. (1984) van demostrar als seus treballs que les plantes CAM tenen una δ^2H molt major que les plantes que utilitzen altres vies fotosintètiques. En canvi, les plantes amb un metabolisme C_3 presenten valors de la δ^2H molt més baixos mentre que les plantes C_4 solen presentar valors entremitjos o similars a les C_3 (Ziegler, 1989).

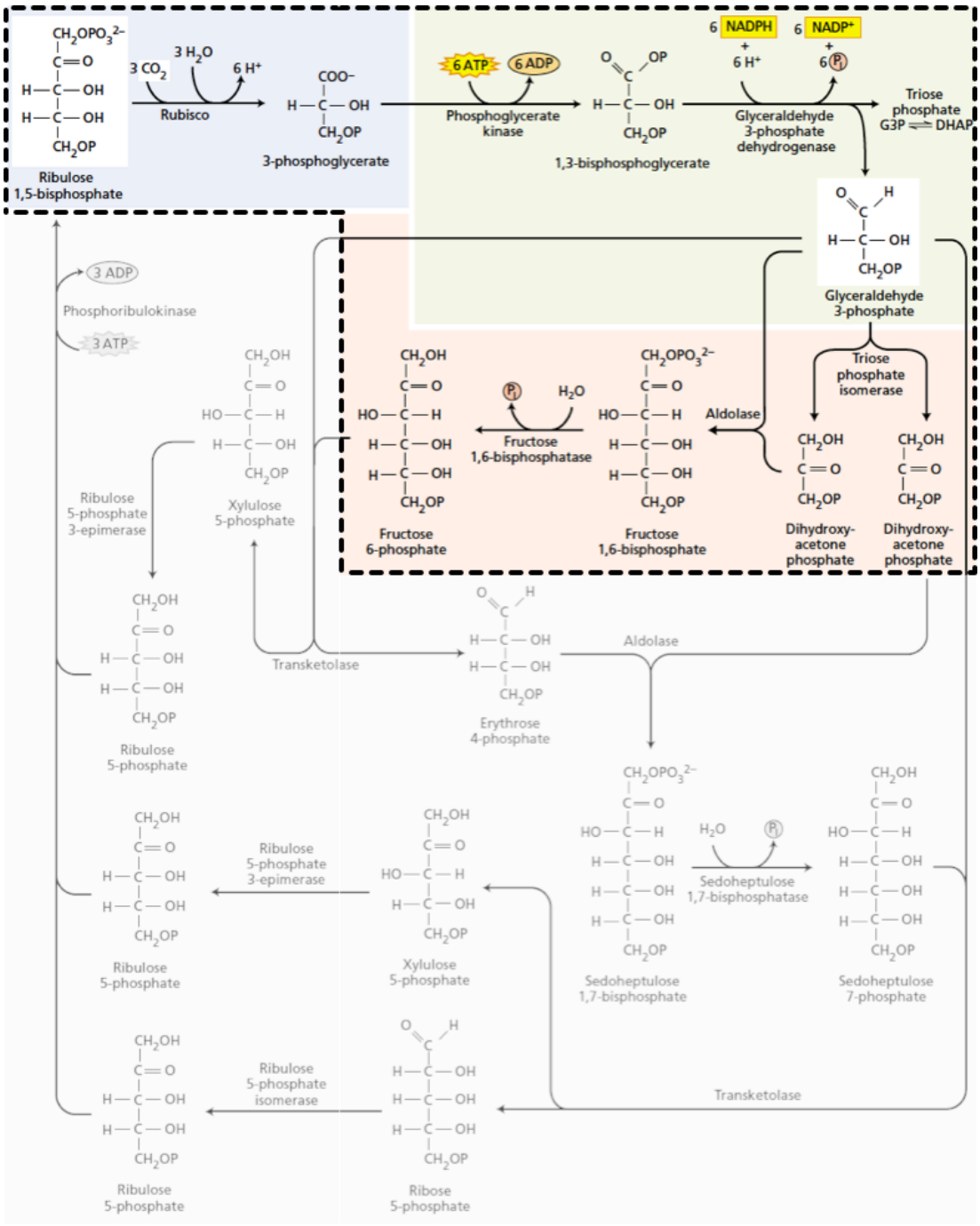

Figura 9*. (Adaptat de Taiz i Zeiger 2002) Enzims i reaccions durant el cicle de Calvin, on es remarca el intercanvi potencial d'hidrògens durant la isomerització de les trioses fosfat, la subseqüent condensació de les trioses fosfats a través de l'aldolasa i interconversió entre els productes fructosa 6-fosfat i glucosa.*

Efecte post-fotosintètic en la δ^2H

Per altra banda, Yakir i Deniro (1990) també van suggerir una possible explicació dels mecanismes que provoquen l'enriquiment del δ^2H observat durant el metabolisme heterotròfic dels carbohidrats en les reaccions subsegüents a la reducció del $NADP^+$. Encara que s'ha demostrat que l'hidrogen unit al carboni no és intercanviable (Epstein et al., 1976), existeixen nombroses possibilitats per intercanviar-lo durant les reaccions de la via metabòlica del complex enzim-catalitzador, ja que el protó abstret del substrat s'uneix temporalment a l'enzim. Tanmateix, el major impacte és aparentment degut a (i) la intervenció de l'enzim que actua en el mecanisme d'intercanvi que es produeix durant la isomerització de les trioses fosfat, (ii) la subseqüent condensació a través de l'aldolasa de les trioses fosfats i (ii) la interconversió al producte de fructosa 6-fosfat (Yakir i Deniro, 1990) (Figura 10). En aquest sentit la meitat dels hidròdens units al carboni de cada unitat de glucosa podrien intercanviar-se amb l'aigua. A més a més, les trioses fosfat són un producte de regeneració a través del cicle de Calvin, amb el qual la majoria dels hidròdens units al carboni poden resultar en un intercanvi en reaccions similars d'isomerització entre els diferents intermediaris tant en la formació del midó com de la cel·lulosa.

3.4.1.3 Isòtop estable d'hidrogen com a eina de fenotipejat

Com s'ha explicat abans la composició isotòpica de l'oxigen, presenta unes certes limitacions com a predictor del rendiment i de la transpiració. Tanmateix, el seu ús en millora és controvertit ja que els valors d'isòtops d'oxigen en el material vegetal no sempre són capaços de captar i reflectir les condicions ambientals de la planta (Barbour, 2007; Farquhar et al., 2007a). Així, l'estudi del comportament potencial d'altres isòtops que també estan directament relacionats amb l'aigua com és l'hidrogen (Figura 10), potser ens podria donar informació addicional sobre les condicions hídriques de la planta. També, tenint en compte la dependència de la

δ^2H al metabolisme fotosintètic, el seu estudi podria ajudar a resoldre l'ambigüitat plantejada en teixits d'una planta amb un metabolisme CAM, el qual permetria obtenir una idea més clara de la via metabòlica utilitzada (Ziegler et al., 1976; Sternberg et al., 1984).

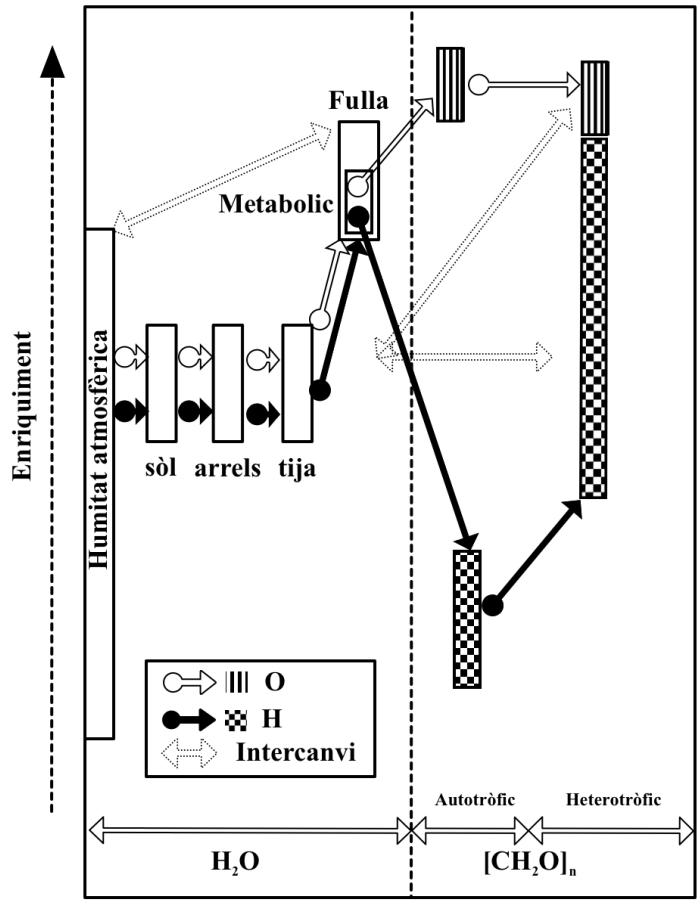

***Figura 10**. (Adaptat de Yakir 1992) Representació esquemàtica dels principals passos del desenvolupament dels quocients isotòpics de l'oxigen i l'hidrogen als carbohidrats de les plantes. Es pot observar la naturalesa conservadora de la captació d'aigua i del transport a la tija, així com el gran enriquiment dels isòtops d'oxigen i d'hidrogen de la fulla degut a l'evaporació i l'efecte diferencial de l'isòtop de l'oxigen i de l'hidrogen durant la fotosíntesi (autotròfic) i durant els processos post-fotosintètics (heterotròfic). Les fletxes discontínues de color blanc indiquen l'intercanvi isotòpic significatiu entre els carbohidrats i l'aigua.*

4. Aplicacions novedoses dels isòtops estables en fenotipatge: Fotosíntesi de l'espiga

En el context del canvi climàtic, una de les tècniques de millora de cultius proposada per augmentar el rendiment potencial i millorar l'adaptació a la creixent incidència del blat a l'estrès abiòtic (com la sequera o la calor) és seleccionar per a una major fotosíntesi de l'espiga (Tambussi et al., 2005, 2007). De fet, en el blat i altres cereals de gra petit l'espiga pot jugar un paper important com a font de foto-assimilats durant l'ompliment del gra, no només en condicions de sequera o d'altres tipus d'estrès abiòtic, sinó també en bones condicions agronòmiques (Araus et al., 1993; Bort et al., 1994; Tambussi et al., 2005, 2007; Maydup et al., 2010). Tot i que en bones condicions agronòmiques la fotosíntesi real dels òrgans 'font' és troba sovint en excés comparat amb la dels òrgans 'destí' (Slafer i Savin, 1994; Borrás et al., 2004), hi ha evidències recents que indiquen que la limitació de la 'font' (Álvaro et al., 2008) comparat amb el 'destí' (Slafer et al., 1999) està sorgint en les varietats modernes de blat. A més, l'espiga presenta unes característiques que poden ser avantatjoses en condicions de sequera: (i) la proximitat als òrgans 'destí' li facilita el transport d'assimilats en comparació a altres òrgans 'font'; (ii) la posició que ocupa a la planta li facilita la captació del CO_2 i de la radiació; (iii) té la possibilitat d'evitar l'absorció de la llum en excés (reflexant-la) gràcies a la presència de ceres epicuticulars i (iv); és l'òrgan que està exposat a més radiació degut a la seva posició en la planta, i en conseqüència és el que més s'escalfa, de manera que la re-fixació del CO_2 reciclat pot suposar un avantatge alhora de disminuir les pèrdues per fotorespiració (Araus et al., 1993). A més, s'ha demostrat que certes malalties fúngiques que poden afectar a les fulles (Robert et al., 2005) no afecten a les espigues (Tiedemann i Firsching, 2000). Per tant, pot ser de gran interès avaluar la contribució fotosintètica de l'espiga durant l'ompliment del gra en condicions on la fotosíntesi de la fulla és limitada.

Per altra banda, les espigues són una font d'assimilats important durant l'ompliment del gra, i es diferencien de les altres fonts de la planta perquè tenen unes característiques singulars: (i) tenen la capacitat de re-fixar el CO_2 respirat pels grans; (ii) una alta eficiència de l'ús de l'aigua (iii) una capacitat d'ajust osmòtic; (iv) característiques xeromòrfiques i (v) un retard de la senescència comparat amb la fulla bandera. Totes aquestes característiques, sobretot la de la presència d'arestes i dels seus trets xeromòrfics junt amb la capacitat d'ajust osmòtic, aporten a l'espiga una major tolerància a la sequera comparat amb les fulles (Morgan, 1980). A més a més, per les seves estructures pròpiament reproductives, les espigues estan composades per varis òrgans fotosintètics: les bràctees (glumes, lemnes i pàlees), (ii) les arestes i (iii) el pericarpi en els grans. Tots aquests òrgans fotosintètics tenen una gran importància durant l'etapa de l'ompliment del gra, (Blum, 1985; Araus et al., 1993; Bort et al., 1994), però la seva contribució (fotosintètica) relativa encara és incerta.

Encara que ja s'ha estudiat àmpliament la fotosíntesi de l'espiga (Araus et al., 1993; Bort et al., 1994; Tambussi et al., 2007; Maydup et al., 2010), la seva contribució durant l'ompliment del gra segueix sent poc clara. Els percentatges trobats a la literatura sobre les contribucions de la fotosíntesi de l'espiga a l'ompliment de gra són molt variables. Així, poden variar des d'aproximadament un 10 a un 76% dels assimilats dipositats en els grans (Gebbing i Schnyder, 2001; Tambussi et al., 2007; Aranjuelo et al., 2011). El motiu d'aquesta variabilitat, podria ser un reflex de la diversitat genètica combinada amb les diferents condicions de creixement. Tot i que s'ha documentat l'existència de variabilitat genotípica en la fotosíntesi de l'espiga (Abbab et al., 2004), aquestes diferències no es poden explicar només en base a la fotosíntesi neta de l'espiga. Les diferències en la taxa de re-fixació de l'espiga també podrien estar involucrades (Tambussi et al., 2007) en l'origen d'aquesta variabilitat.

De fet, s'ha demostrat l'existència d'una re-fixació substancial del CO_2 respirat dins de l'espiga (Bort et al., 1996), arribant a contribuir en un 70% del carboni de la sacarosa acumulada a les bràctees (Gebbing i Schnyder, 2001). No obstant, aquesta variabilitat en la contribució de la fotosíntesi de l'espiga a l'ompliment del gra, és probable que sigui també conseqüència dels inconvenients que sorgeixen en els mètodes utilitzats alhora de mesurar la fotosíntesi de l'espiga. De fet, en comparació amb les fulles, la contribució fotosintètica de l'espiga ha estat menys estudiada especialment en condicions de camp (Maydup et al., 2014) degut a les limitacions metodològiques.

Per altra banda, els assimilats transportats al gra en el blat i altres cereals C_3 durant l'ompliment de gra provenen principalment de tres fonts: i) la fotosíntesi de la fulla bandera (fulla i beina) (Evans et al., 1975); ii) les reserves pre-antesi (Gebbing i Schnyder, 1999); i iii) la fotosíntesi de l'espiga (Tambussi et al., 2007). Ara bé, la proporció de cadascuna de les tres fonts pel que fa a la contribució dels assimilats durant l'ompliment del gra encara roman desconeguda a causa de les esmentades limitacions metodològiques (Evans et al., 1975; Nicolas i Turner, 1993; Tambussi et al., 2007). Aquestes limitacions es deuen en part a la dificultat de quantificar i separar els assimilats que venen dels diferents òrgans fotosintètics i que es re-transloquen durant l'ompliment del gra.

4.1 Eines intrusives versus no intusives per estimar la fotosíntesi de l'espiga

Degut a les limitacions metodològiques (explicades a l'apartat anterior) que suposa l'avaluació a gran escala de la contribució de l'espiga a l'ompliment del gra s'han utilitzat una gran varietat de d'aproximacions alternatives (Borrás et al., 2004; Maydup et al., 2010). Entre les més comunes tenim l'eliminació d'algunes parts (òrgans específics) de la planta, com ara la defoliació de la tija durant l'etapa d'antesi

(Ahmadi et al., 2009), la inhibició de la fotosíntesi a través de l'ombrejat (Aggarwal et al., 1990; Araus et al., 1993; Peralta et al., 2011), l'aplicació d'herbicides (Maydup et al., 2010), o de tractaments dessecants (Blum et al., 1983; Nicolas i Turner, 1993; Saeidi et al., 2012). Tanmateix, aquestes aproximacions no eviten que altres processos fisiològics diferents de la fotosíntesi puguin afectar els òrgans tractats (Tambussi et al., 2007), com per exemple la respiració o la maduració prematura dels fruits (Kriedemann, 1966). Així doncs, s'ha de tenir especial cura alhora d'interpretar els resultats obtinguts a partir d'aquests mètodes aproximatius ja que també els possibles efectes compensatoris provocats per la naturalesa intrusiva de les mateixes en els òrgans no tractats, també podrien esbiaixar el pes final dels grans. En aquest context, és de vital importància la recerca de tècniques no intrusives que siguin capaces de quantificar i separar els assimilats que venen dels diferents òrgans fotosintètics i que es re-transloquen durant l'ompliment del gra.

4.2. Eines no intusives per estimar la fotosíntesi de l'espiga

L'ús de l'abundància natural de l'isòtop estable del carboni pot ajudar a dilucidar la contribució relativa dels diferents òrgans fotosintètics durant l'ompliment del gra. La composició isotòpica de carboni ($\delta^{13}C$) de la matèria seca de la planta reflecteix el seu rendiment fotosintètic, i és un dels trets fisiològics integrats en el temps més utilitzats pels fito-milloradors (Farquhar, 1989; Araus et al., 2002). Com s'ha explicat més amunt, a banda de l'efecte de les condicions hídriques i altres factors ambientals sobre la $\delta^{13}C$, també hi han diferències constitutives de la $\delta^{13}C$ associades amb la part específica de la planta considerada (Araus et al., 1993) i relacionades amb la seva anatomia i possiblement també al metabolisme (Figura 11). Per tant, independentment de les condicions de creixement, la $\delta^{13}C$ dels foto-assimilats produïts a la fulla bandera tindran una senyal més negativa comparat amb els que s'han produït a l'espiga (Araus et al. 1992; Araus et al. 1993). Aquesta variació

en la δ^{13C} entre les diferents parts de la planta pot ser causada per les diferències en la relació Ci/Ca impulsada per una permeabilitat molt inferior a la difusió de gas de l'espiga en comparació amb les fulles.

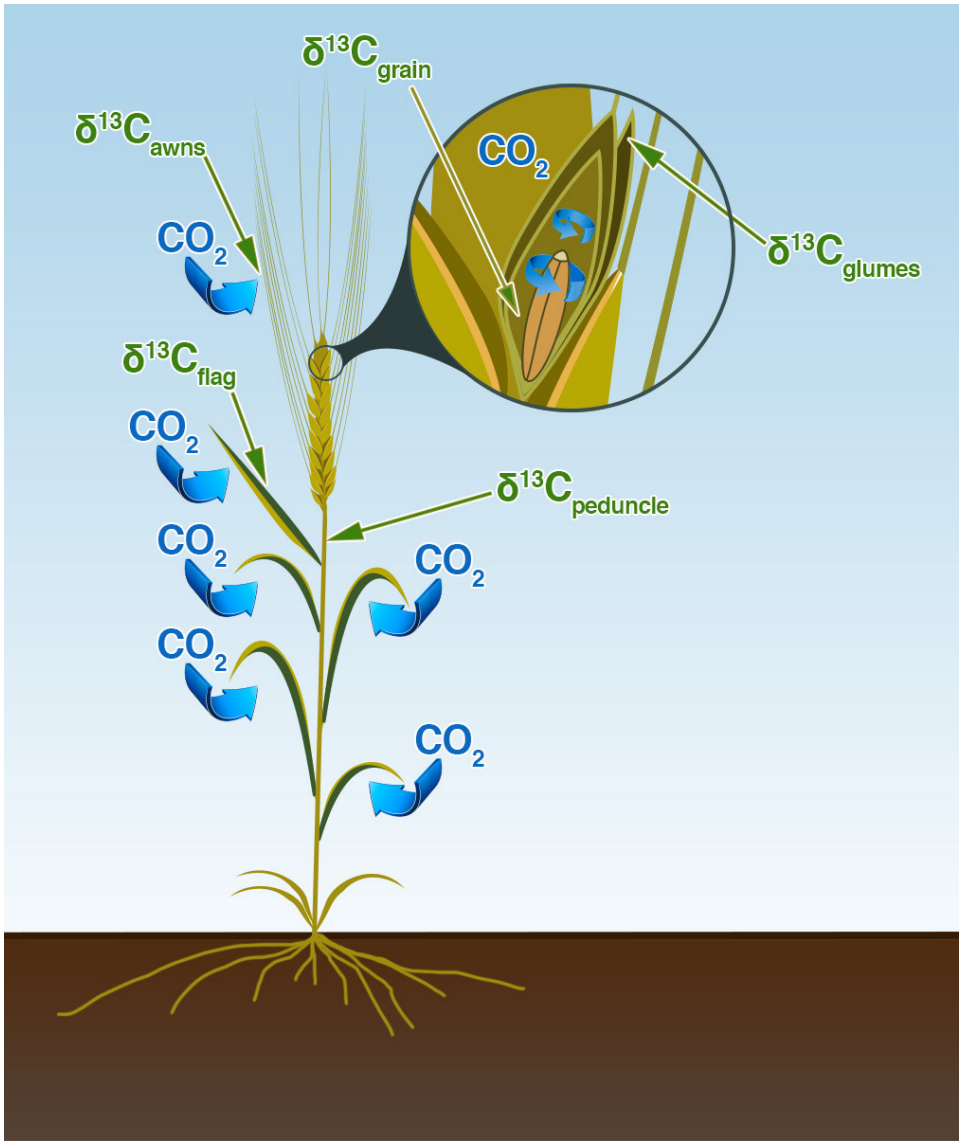

***Figura 11**. Il·lustració d'una planta de blat on es mostren les principals fonts d'assimilats (i de carboni) dels diferents òrgans fotosintètics de les plantes. On, la $\delta^{13}C_{awns}$, $\delta^{13}C_{flag}$, $\delta^{13}C_{glumes}$ $\delta^{13}C_{peduncle}$, i $\delta^{13}C_{grain}$ representen la composició isotòpica de les arestes, la fulla bandera, les glumes, el peduncle i el gra madur. També es mostra la possible re-fixació del CO_2 respirat pel gra.*

Objectius

OBJECTIUS

Aquesta tesis té per objectiu estudiar noves aplicacions de les abundàncies naturals de diferents isòtops estables com a eines de fenotipejat en millora de conreus.

Per tal de dur a terme l'objectiu principal es van plantejar els següents objectius específics:

- L'ús de la composició isotòpica de carboni per avaluar de manera no intrusiva la contribució fotosintètica de l'espiga a l'ompliment del gra en blat dur sota diferents condicions hídriques i de disponibilitat nitrogenada (Capítol 1), de blat fariner en condicions agronòmiques propers a l'òptim (Capítol 2) i la seva comparativa amb les tècniques convencionals d'avaluació de la contribució de l'espiga (Capítol 3).
- Esbrinar els factors que limiten l'aplicació de $\delta^{18}O$ com a criteri de selecció. Reduir les incerteses de la naturalesa dels mecanismes d'alteració de la $\delta^{18}O$ en els teixits dels conreus C_3 (blat dur) i C_4 (blat de moro) (Capítols 4 i 5).
- Estudi comparatiu de les signatures isotòpiques de C, H i O en diferents teixits com a eines de selecció en blat dur sota diferents condicions hídriques i de fertilització nitrogenada (Capítol 5)

Informe dels Directors de Tesi

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Els Drs. Josep Lluís Araus Ortega i Maria Dolors Serret Molins com a Directors de la Tesi que porta per títol: **Ús d'isòtops estables d'O, H, C com eines de selecció de rendiment potencial i adaptació a la sequera i deficiència de nitrogen en cereals C_3 i C_4** que ha dut a terme la doctoranda Rut Sanchez Bragado,

Informen sobre l'índex d'impacte i la participació de la doctoranda en cadascun dels articles inclosos en la memòria de la Tesi. En tots els articles la doctoranda és la primera autora dels treballs.

Capítol 1. Article: "*Contribution of the ear and the flag leaf to grain filling in durum wheat inferred from the carbon isotope signature: Genotypic and growing conditions effects*", publicat a la revista Journal of Integrative Plant Biology, amb un índex d'impacte de 3,448 el 2014, any de la seva publicació, cosa que emplaça aquesta revista en el primer quartil dins l'àmbit de Plant Sciences. En aquest estudi s'avalua l'efecte (combinat de la limitació hídrica i del N) de la contribució relativa de l'espiga i la fulla bandera a l'ompliment del gra en genotips de blat dur anteriors i posteriors a la revolució verda crescuts sota diferents condicions hídriques i de N. És per això, que aquest estudi desenvolupa una nova i original aproximació basada en l'anàlisi de la composició isotòpica de carboni en diferents òrgans de la planta que permet avaluar la contribució relativa de l'espiga i de la fulla bandera a l'ompliment del gra. Tant en els genotips anteriors com posteriors a la Revolució Verda s'observa una contribució relativa de l'espiga major en comparació a la de la fulla bandera.

A més, una menor contribució de l'espiga s'associa a un major índex de collita, possiblement per l'aparició d'una certa limitació de la capacitat fotosintètica de la "font" en els genotips moderns. La doctoranda ha realitzat els mostreigs, les anàlisis de les mostres, el tractament estadístic i l'elaboració de resultats i a més ha participat en la discussió de resultats i en la redacció de l'esborrany de l'article.

Capítol 2. Article "*Relative contribution of shoot and ear photosynthesis to grain filling in wheat under good agronomical conditions assessed by differential organ* $\delta^{13}C$", publicat a la revista Journal of Experimental Botany, amb un índex d'impacte de 5,526 el 2014, any de la seva publicació, el que emplaça aquesta revista en el primer quartil dins l'àmbit de Plant Sciences. En aquest estudi s'avalua l'efecte en condicions agronòmiques òptimes de la contribució relativa de les arestes de l'espiga i de la tija (entès com el conjunt d'òrgans fotosintètics ubicats sota l'espiga) a l'ompliment del gra en línies elit altament productives de blat fariner. Utilitzant una metodologia que presenta un avanç respecte a la desenvolupada en el Capítol 1, la contribució relativa de l'espiga a l'ompliment del gra ha sigut també més alta que la de la tija. A més, l'estudi aporta altres aproximacions metodològiques, com la intercepció de la llum o la capacitat fotosintètica, que també donen suport al paper principal en l'ompliment del gra de l'espiga, comparat amb la fulla bandera. La doctoranda ha realitzat els mostreigs, les anàlisis de les mostres, el tractament estadístic, l'elaboració i discussió de resultats redacció de l'esborrany.

Capítol 3. Article: "*Photosynthetic contribution of the ear to grain filling in wheat: a comparison of different methodologies for evaluation*". Es pretén tornar a enviar (re-submission) a la revista Journal of Experimental Botany, amb un índex d'impacte de 5,526 el 2015. Aquest estudi aprofundeix en l'aproximació metodològica d'avaluació de l'isòtop de carboni realitzada als Capítols 1 i 2 com a eina de millora

de cultius, i es compara amb altres aproximacions convencionals, alternatives als isòtops estables, que avaluen la contribució relativa de l'espiga i la tija a l'ompliment del gra. Un dels mètodes, el d'ombrejat, assigna una contribució de l'espiga i la tija comparable. L'altre mètode, que es basa en l'aplicació de l'herbicida DCMU, assigna un paper més important a la tija, però s'observa que l'aplicació localitzada de l'herbicida a la fulla també afecta a la fotosíntesis de l'espiga, cosa que està esbiaixant els resultats. Finalment, l'aproximació del $\delta^{13}C$, assigna a l'espiga una contribució fotosintètica superior que la de la tija. Aquest estudi podria ajudar a desenvolupar eines més precises de fenotipejat per identificar trets fisiològics, com ara la fotosíntesi de l'espiga que puguin contribuir a augmentar el rendiment del gra. La doctoranda ha realitzat els mostreigs, les anàlisis de les mostres, el tractament estadístic i l'elaboració de resultats i a més ha participat en la discussió de resultats i en la redacció de l'esborrany.

Capítol 4. Article: "*Factors preventing the performance of the oxygen isotope ratios as indicator of grain yield in maize*". Es pretén tornar a enviar (re-submission) a la revista Planta amb un índex d'impacte de 3,263 l'any 2015. En aquest estudi s'avalua (en condicions de sequera semi-controlades i de camp) la importància relativa dels factors transpiratius i els relacionats amb la translocació que afecten la $\delta^{18}O$ en els teixits de les plantes de blat de moro. Tot i que el rendiment del gra i les variables fisiològiques associades a l'estat hídric no mostren correlacions genotípiques clares amb el $\delta^{18}O$ dins de cada assaig, si que es mostren bones correlacions quan es combinen diferents assajos. Aquests resultats indiquen que la resposta de la $\delta^{18}O$ a factors ambientals és prou gran per superar l'efecte de les conversions metabòliques dins dels teixits "destí" encara que poden amagar la variabilitat genotípica. La doctoranda ha participat en les anàlisis de les mostres, tractament estadístic, elaboració i discussió de resultats i escriptura de l'esborrany del manuscrit.

Capítol 5. Article: "*Comparative performance of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ for phenotyping durum wheat adaptation to different water and nitrogen conditions*". Preparat per enviar a la revista New Phytologist, amb un índex d'impacte 7,672 l'any 2014. En aquest treball s'estudia una nova eina de fenotipejat per seleccionar genotips amb un major rendiment potencial i/o més adaptats al dèficit hídric i de nitrogen. L'estudi s'ha dut a terme en un conjunt de genotips de blat dur previs i posteriors a la Revolució Verda crescuts en condicions de camp sota diferents règims hídrics i d'adobat nitrogenat. S'avalua la composició isotòpica de l'hidrogen tant en la matèria seca, como en la fracció soluble de diferents òrgans i en l'aigua extreta dels mateixos teixits i es compara amb la composició isotòpica de l'oxigen i del carboni. La composició isotòpica de l'hidrogen mostra millors resultats que els altres dos isòtops preveient el rendiment del gra i el contingut de N sota estrès hídric. La doctoranda ha realitzat els mostreigs, les anàlisis de les mostres, el tractament estadístic, l'elaboració i discussió dels resultats i la redacció de l'esborrany.

Cal destacar que la Rut Sánchez des de que es va integrar en el nostre grup de treball ha col·laborat de forma autònoma en tots els experiments que s'han plantejat com ja s'ha comentat en els diferents apartats. Té una gran capacitat de feina, ja sigui a les cambres de creixement, al camp i al laboratori. Ha assolit un gran coneixement en l'àmbit dels diferents isòtops i ha demostrat una gran iniciativa i perseverança. És una persona capaç d'ajudar als altres membres de l'equip quan és necessari. Es mostra sempre amable i conciliadora.

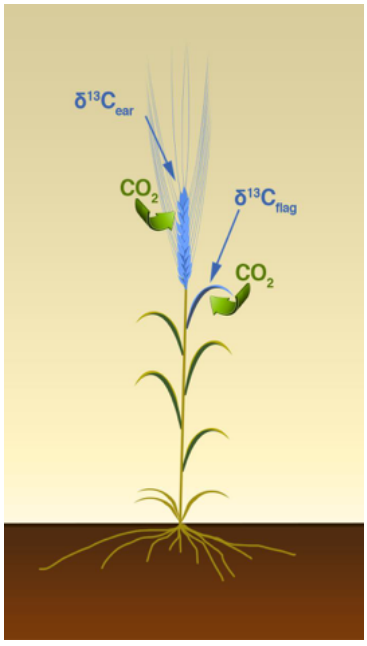
I perquè consti a efectes oportuns,

Dr. Josep Lluís Araus Ortega

Dra. Maria Dolors Serret Molins

Barcelona, 17 de juliol de 2015

Resultats



CAPÍTOL 1

Contribució de l'espiga i la fulla bandera a l'ompliment del gra en blat dur inferit per la signatura isotòpica del carboni: efectes genotípics i de condicions de creixement

CHAPTER 1

Contribution of the ear and the flag leaf to grain filling in durum wheat inferred from the carbon isotope signature: Genotypic and growing conditions effects

Rut Sanchez- Bragado[1] , Abdelhalim Elazab[1] , Bangwei Zhou[1] , Maria Dolors Serret[1], Jordi Bort[1], Maria Teresa Nieto- Taladriz[2] and José Luis Araus[1]

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RESUM CAPÍTOL 1

Als cereals C_3, es creu que l'espiga, juntament amb la fulla bandera, tenen un paper molt important com a font d'assimilats durant l'ompliment del gra. Ara bé, el tipus de metodologies disponibles fins ara per provar-ho fa que els resultats no siguin del tot fiables. En aquest estudi es comparen la composició isotòpica de carboni ($\delta^{13}C$) en la seva abundància natural de les fraccions solubles en aigua de la làmina de la fulla bandera i de l'espiga amb el $\delta^{13}C$ dels grans madurs amb la finalitat de poder avaluar la contribució relativa dels dos òrgans a l'ompliment dels grans en plantes de blat dur (Triticum turgidum L. var. durum). La contribució relativa de l'espiga va ser més elevada als ecotips en comparació amb els cultivars moderns, així com en resposta a l'aport de fertilitzant nitrogenat i a l'estrès per manca d'aigua. Aquestes diferències genotípiques i ambientals es van associar amb variacions en l'índex de collita (HI), mentre que la contribució relativa de l'espiga es va correlacionar negativament amb el HI. En el cas de les diferències genotípiques, la contribució relativament inferior de l'espiga a l'ompliment del gra en els cultivars moderns comparada amb la de les varietats locals possiblement està associada amb l'aparició als primers d'una limitació de la font degut a un HI més elevat. De fet, la contribució relativa de l'espiga va ser més sensible als canvis en HI en els cultivars moderns que en les varietats locals.

JIPB Journal of Integrative Plant Biology

Contribution of the ear and the flag leaf to grain filling in durum wheat inferred from the carbon isotope signature: Genotypic and growing conditions effects

Rut Sanchez-Bragado[1], Abdelhalim Elazab[1], Bangwei Zhou[1], Maria Dolors Serret[1], Jordi Bort[1], Maria Teresa Nieto-Taladriz[2] and José Luis Araus[1]*

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Abstract The ear, together with the flag leaf, is believed to play a major role as a source of assimilates during grain filling in C_3 cereals. However, the intrusive nature of most of the available methodologies prevents reaching conclusive results in this regard. This study compares the carbon isotope composition ($\delta^{13}C$) in its natural abundance in the water-soluble fractions of the flag leaf blade and the ear with the $\delta^{13}C$ of mature kernels to assess the relative contribution of both organs to grain filling in durum wheat (*Triticum turgidum* L. var. *durum*). The relative contribution of the ear was higher in landraces compared to modern cultivars, as well as in response to nitrogen fertilization and water stress. Such genotypic and environmentally driven differences were associated with changes in harvest index (HI), with the relative contribution of the ear being negatively associated with HI. In the case of the genotypic differences, the lower relative contribution of the ear in modern cultivars compared with landraces is probably associated with the appearance in the former of a certain amount of source limitation driven by a higher HI. In fact, the relative contribution of the ear was far more responsive to changes in HI in modern cultivars compared with landraces.

Keywords: Carbon isotope discrimination; ear; flag leaf; grain filling; harvest index; photosynthesis
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INTRODUCTION

Durum wheat is one of the most important crops cultivated within the south and east Mediterranean basin (www://fao.org/). In Mediterranean environments, water limitations followed by low nitrogen availability are the major constraints on wheat yield (Oweis et al. 1998; Araus et al. 2002). Thus, improving adaptation by selecting genotypes of durum wheat better suited to drought (Condon et al. 2004) and low fertility would be a sound way to mitigate such limitations (Araus 2004) and also address the climate and social challenges to come in the next few decades. High-throughput phenotyping will be pivotal to both increase production per unit area and simultaneously improve the resource use efficiency of crops (Araus et al. 2002, 2008; Cabrera-Bosquet et al. 2012; Parry and Hawkesford 2012).

Mediterranean environments are characterized by drought increasing during the last part of the crop cycle; namely, the grain filling period. Although the role of the main photosynthetic organ contributing to grain filling has been assigned traditionally to the flag leaf blade (Evans et al. 1980; Araus and Tapia 1987), photosynthesis of the ear is also believed to play an important role as a source of assimilates during grain filling in wheat and other cereals (Araus et al. 1993; Bort et al. 1994; Tambussi et al. 2007; Maydup et al. 2010). However, reports about the relative contribution of ear photosynthesis to grain filling vary widely, with between 10% and 76% of assimilates deposited in grains deriving from the ear (Gebbing and Schnyder 2001; Tambussi et al. 2007; Aranjuelo et al. 2011). Different studies suggest that the contribution of the ear to growing grains may be affected by environmental and genotypic factors (Araus et al. 2003; Maydup et al. 2010). In particular it has been reported that ears have a higher contribution under stress conditions, particularly drought (Tambussi et al. 2005). Furthermore, for other stresses such as low N fertility, there are currently no reports to be found in the literature. This is not trivial because lack of N may disturb the longevity and activity of photosynthetic organs during grain filling, particularly in the case of the flag leaf (Araus and Tapia 1987), and therefore this potentially affects the relative contribution of each organ. Concerning genotypic differences in the relative contribution to filling grains of the ear compared with the flag leaf, the comparison between old genotypes (pre Green Revolution) and new cultivars is most relevant, since the new cultivars have increased the ear to shoot ratio and

the harvest index (i.e., ear size, and frequently the number of kernels per spike, at the expense of shoot length).

Most of the methodologies designed to infer the photosynthetic contribution of different plant parts have involved very intrusive approaches that have assessed the effect of these organs on grain filling by preventing organ photosynthesis. Such approaches include, for example, shading the ears, the flag leaf blade or the entire shoot (Aggarwal et al. 1990; Araus et al. 1993), application of herbicides that prevent photosynthesis (Mayrup et al. 2010), or simply defoliating leaf blades (Ahmadi et al. 2009). Besides their intrusive nature, manipulation treatments aimed at decreasing photosynthesis in the whole plant may cause an increase in the photosynthetic rate of the remaining organs (Aggarwal et al. 1990; Eyles et al. 2013). In fact, it has been reported for some time that such manipulation treatments may trigger unwanted compensatory mechanisms (Chanishvili et al. 2005) in the remaining organs of the plant or increase the contribution of preanthesis reserves to grain filling (Schnyder 1993; Plaut et al. 2004). Hence, the interpretation of the contribution of different plant organs to grain filling should be taken with caution (Maydup et al. 2010). Alternative approaches involve labeling with radioactive (Austin et al. 1976; Wardlaw and Willenbrink 1994) and stable carbon isotopes (Gebbing et al. 1998; Aranjuelo et al. 2011) during photosynthetic CO_2 uptake by the flag leaf or the ear. However, such approaches, even if not intrusive are not necessarily informative of the whole grain filling process (e.g., pulse chasing experiments) and in general such methods are very time and resource consuming, making them unsuitable as phenotyping tools. In such a way the use of the stable carbon isotope signature in its natural abundance may help to elucidate the relative contribution of the different photosynthetic tissues. The approach we present here is based in the reported differences of the natural abundance of carbon isotope composition of assimilates produced by the different photosynthetic tissues of the plant that are active during grain filling (Brandes et al. 2006), particularly between the ear and the leaf blades (Araus et al. 1992, 1993).

The natural abundance of carbon isotope composition ($\delta^{13}C$) in plant matter reflects photosynthetic performance in the plant (Farquhar et al. 1989; Brugnoli and Farquhar 2000) and is one of the physiological time-integrated successful traits used by plant breeders (Araus et al. 2002). The stable isotope ^{13}C is discriminated against the lighter ^{12}C during photosynthetic carbon fixation (Craig 1954; Bender et al. 1973; Farquhar and Richards 1984). Thus, discrimination of ^{13}C in a photosynthetic organ depends on the ratio of the assimilation rate to CO_2-diffusive conductance (Farquhar et al. 1989). Whereas environmental factors such as water availability may affect $\delta^{13}C$, mostly through an effect on stomatal conductance, there are also constitutive differences in $\delta^{13}C$ associated with the specific plant part considered (Hubick and Farquhar 1989; Sternberg 1989; Araus et al. 1993). This is the case, for example, for $\delta^{13}C$ from photoassimilates produced by different photosynthetic plant parts, such as the leaf blades and the ear (Hubick and Farquhar 1989; Araus et al. 1992, 1993). Thus, regardless of the growing conditions the $\delta^{13}C$ of photoassimilates from the flag leaf blade is lower (more negative) than from the ear (Araus et al. 1992, 1993). Such variation in $\delta^{13}C$ among plant parts may be caused by differences in the ratio of the assimilation rate to the CO_2-diffusive conductance driven by a far lower permeability to gas diffusion in the ear compared to the blades. This is in spite of the fact that metabolic differences, such as the existence of a certain degree of CAM metabolism in inflorescences, have been proposed (Blanke and Lenz 1989; Araus et al. 1992, 1993; Bort et al. 1995; Tambussi et al. 2005, 2007).

The objective of this study is to determine the relative contribution of flag leaf and ear photosynthesis to grain filling under different combinations of water and N regimes under Mediterranean conditions using a non-intrusive approach based on the constitutively different $\delta^{13}C$ of both organs. The effect of genotypic differences associated with changes in harvest index was also investigated. The method we propose here is based on the comparison between the $\delta^{13}C$ of total dry matter (DM) in mature kernels and that of the water-soluble fractions (WSF) of the flag leaf and the ear during grain filling. In fact, the $\delta^{13}C$ in the WSF of a photosynthetic organ mainly reflects the recent assimilates produced by the organ (Brandes et al. 2006) that may be eventually exported through the phloem to active sinks like growing kernels.

RESULTS

Effect of growing conditions on grain yield

Genotype (G), nitrogen (N) and water regime (W) exhibited a significant effect on grain yield (GY) (Table 1). The irrigated, nitrogen-fertilized treatment exhibited the highest GY, whereas the rainfed trials, regardless of the N fertilization showed the lowest GY. Therefore, a significant interaction of water and nitrogen supply ($W \times N$) for GY was observed. Concerning agronomical components, genotype and nitrogen fertilization had a significant effect on harvest index (HI), thousand kernel weight (TKW), and kernel weight per spike (KW SP^{-1}), whereas water regime only affected KW SP^{-1}. The means of modern cultivars compared to old landraces exhibited significantly higher GY and agronomic components. Chlorophyll content on an area basis (SPAD) did not show differences either due to genotype or to nitrogen fertilization. Only water regime had a significant effect.

Carbon isotope signature

Water regime, nitrogen fertilization and genotype had a significant effect on the carbon isotope composition ($\delta^{13}C$) of the different organs (flag leaf, ear, and grains) and fractions (total DM and the WSF) analyzed (Table 2). Water stress increased $\delta^{13}C$ regardless of the nitrogen treatment, organ or fraction analyzed. In general, nitrogen also increased $\delta^{13}C$, whereas landraces exhibited a higher $\delta^{13}C$ in DM than the modern cultivars. In addition, variations in $\delta^{13}C$ values between DM and WSF were observed. The $\delta^{13}C$ of flag leaf ($\delta^{13}C_{flag}$) was higher (less negative) for DM compared with the WSF, whereas the opposite trend occurred in the ear ($\delta^{13}C_{ear}$). Moreover, in the WSF the $\delta^{13}C$ of the ear and the flag leaf blade exhibited values slightly higher (less negative) and lower (more negative) than the $\delta^{13}C$ in the mature kernels.

Table 1. Mean values of grain yield (GY), harvest index (HI), thousand kernel weight (TKW), kernel weight per spike (KW × SP^{-1}) and chlorophyll content in SPAD units (SPAD) of nine durum wheat genotypes (G) grown under a combination of two different water (W; support irrigation, I, vs. rainfed, R) and nitrogen (N; high nitrogen or fertilized, HN, and low nitrogen or non fertilized, LN). Analysis of variance (ANOVA) of the set of durum wheat genotypes grown under different conditions of nitrogen supply, water regime and their respective interaction is also included

	GY (Mg/ha)	HI	TKW (g)	KW × SP^{-1} (g/sp)	SPAD
Treatment					
I + HN	2.3^{c}	0.34^{ab}	42.9^{a}	1.3^{b}	50.7^{a}
I − LN	1.9^{b}	0.36^{ab}	43.6^{a}	1.6^{c}	52.1^{a}
R + HN	1.2^{a}	0.31^{a}	42.4^{a}	1.0^{a}	53.4^{a}
R − LN	1.4^{a}	0.39^{b}	44.6^{a}	1.2^{b}	53.5^{a}
Modern C	1.9^{a}	0.41^{a}	46.45^{a}	1.5^{a}	52.4^{a}
Old L	1.5^{b}	0.28^{b}	39.56^{b}	1.0^{b}	52.4^{a}
Level of significance					
G	0.000***	0.000***	0.000***	0.000***	ns
W	0.000***	ns	ns	0.000***	0.008**
N	0.019*	0.000***	0.000***	0.000***	ns
W X N	0.000***	0.000***	0.028*	ns	ns
W X G	0.000***	0.044*	0.000***	ns	0.000***
W X G	0.004**	0.000***	ns	ns	ns

The set of genotypes included four landraces and five modern cultivars. Values with different superscripted letters are significantly different according to Tukey's HSD test ($P < 0.05$). Treatment values are the means of 27 values (nine genotypes and three replicates per treatment). ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Relationship between $\delta^{13}C$ and grain yield

The relationships of $\delta^{13}C$ against GY across landraces and modern cultivars for each of the different plant parts and fractions were studied (Figure S1). Correlations were in general highly significant, but in the case of the flag leaf and the ear they were stronger for the $\delta^{13}C$ of the WSF than for the $\delta^{13}C$ of the DM. In addition, all plant parts and fractions studied exhibited stronger correlations against GY in old landraces compared to modern cultivars.

Organ contribution to filling grains

The relative contributions of $\delta^{13}C_{ear}$ and $\delta^{13}C_{flag}$ towards the $\delta^{13}C_{grains}$ were assessed via linear fitting using a combination of the $\delta^{13}C$ in the WSF of the ear and flag leaf as the independent variables, and assigning a different weight for the ear and flag $\delta^{13}C$ depending on the growing conditions and genotypes contributing towards the $\delta^{13}C_{grains}$ (Figure 1). This approach is based on the fact that the values of the $\delta^{13}C_{grains}$ were between the range of the $\delta^{13}C_{ear}$ and the $\delta^{13}C_{flag}$ in the WSF

Table 2. Mean values of the stable carbon isotope composition ($\delta^{13}C$) of dry matter (DM) and the soluble fraction (WSF) of the flag leaf ($\delta^{13}C_{flag}$) and the ear ($\delta^{13}C_{ear}$) sampled at mid grain filling plus mature kernels (grain) of nine durum wheat genotypes (G) grown under a combination of two different water (W; support irrigation, I, vs. rainfed, R) and nitrogen (N; fertilized, HN, and non fertilized, LN) conditions). Analysis of variance (ANOVA) of the set of durum wheat genotypes grown under different conditions of nitrogen supply, water regime and their respective interaction is also included

	$\delta^{13}C_{flag\ DM}$ (‰)	$\delta^{13}C_{flag\ WSF}$ (‰)	$\delta^{13}C_{ear\ DM}$ (‰)	$\delta^{13}C_{ear\ WSF}$ (‰)	$\delta^{13}C_{grain}$ (‰)
Treatment					
I + HN	−25.8^{b} (a)	−28.4^{a} (b)	−25.2^{b} (c)	−24.3^{a} (d)	−24.9^{a} (c)
I − LN	−26.7^{a} (b)	−27.4^{b} (a)	−25.7^{a} (c)	−24.6^{a} (e)	−25.2^{a} (d)
R + HN	−24.8^{c} (b)	−26.1^{c} (a)	−23.8^{d} (c)	−22.8^{b} (d)	−23.1^{c} (d)
R − LN	−25.6^{b} (b)	−26.4^{c} (a)	−24.4^{c} (c)	−23.3^{b} (d)	−24.0^{b} (d)
Modern cultivars	−25.9^{a} (b)	−27.0^{a} (a)	−24.5^{a} (c)	−23.3^{a} (d)	−24.6^{a} (bc)
Old landraces	−25.5^{b} (b)	−27.2^{a} (a)	−25.0^{b} (c)	−24.4^{b} (d)	−23.9^{b} (c)
Level of significance					
G	0.000***	0.000***	0.000***	0.000***	0.000***
W	0.000***	0.000***	0.000***	0.000***	0.000***
N	0.000***	0.005**	0.000***	0.000***	0.000***
W × N	ns	0.000***	ns	ns	0.000***
W × G	ns	ns	ns	ns	ns
N × G	ns	ns	ns	ns	ns

Values with different superscripted letters are significantly different within treatments according to Tukey's HSD test ($P < 0.05$). Values with different superscripted letters between brackets are significantly different between organs and factions analyzed within a given treatment according to Tukey's HSD test ($P < 0.05$). Treatment values are the means of 27 values (nine genotypes and three replicates per treatment). ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$.

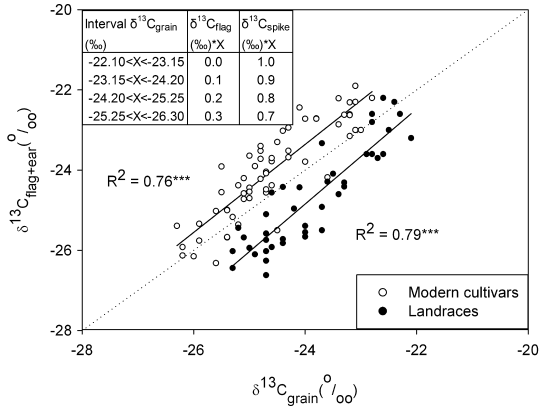

Figure 1. Lineal regression of the relationship between the stable carbon isotope composition in mature grains ($\delta^{13}C_{grain}$) and the combination of $\delta^{13}C$ from the ear and the flag leaf ($\delta^{13}C_{ear} + \delta^{13}C_{flag}$) in the water-soluble fraction (WSF)
The nine genotypes and three replications per genotype and growing condition were considered. For each plot the relative weight assigned to the $\delta^{13}C$ of each of the two organs depended on the water status of the plot assessed by its $\delta^{13}C_{grain}$ (see figure inset). Levels of significance: $^{***}P < 0.001$.

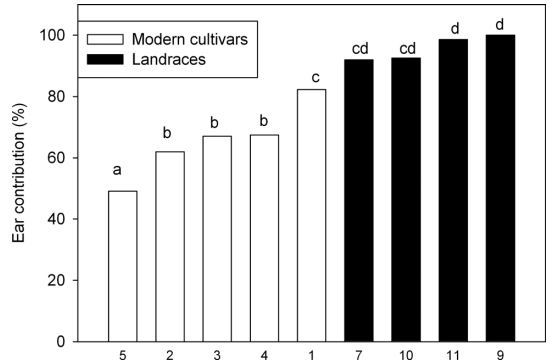

Figure 2. Relative contribution of the ear (%) to grain filling
Values were derived from the relative contribution of the carbon isotope abundances of the ear ($a \times \delta^{13}C_{ear}$) and the flag leaf ($1 - a \times \delta^{13}C_{flag}$) to mature kernels ($\delta^{13}C_{grain}$). Values with different superscripted letters are significantly different according to Tukey's HSD test ($P < 0.05$). Genotype values (*x*-axis), representing five modern cultivars (1, *Anton*; 2, *Bolo*; 3, *Don Pedro*; 4, *Regallo*; and 5, *Sula*) and four old landraces (7, *Blanqueta*; 9, *Griego de Baleares*; 10, *Negro*; and 11, *Jerez* 37), are the means of 12 values (four treatments and three replicates per treatment).

during mid-grain filling (Table 2). The wide range of variation in $\delta^{13}C$ values within organs and fraction analyzed is associated negatively with productivity caused by differences across genotypes (old and modern cultivars) and growing conditions (Figure S1). Thus, a change in the relative contribution of $\delta^{13}C_{ear}$ and $\delta^{13}C_{flag}$ was assumed to be based on the $\delta^{13}C_{grain}$. To simplify, four different levels of relative contribution were considered to contribute to the range of variation in $\delta^{13}C_{grain}$. In that way a good fit was achieved when the $\delta^{13}C_{ear}$ was assigned a relative contribution of 100% to the $\delta^{13}C_{grain}$ ($\delta^{13}C_{ear} \times 1$) for a range of $\delta^{13}C_{grain}$ values between -22.10‰ and -23.15‰. Conversely, the relative contribution of the ear was 70% ($\delta^{13}C_{ear} \times 0.7$) and the flag leaf 30% ($\delta^{13}C_{flag} \times 0.3$) when $\delta^{13}C_{grain}$ values were between -25.25‰ and -26.30‰. In such a way a linear fit with a slope of one and an origin at zero was achieved for the whole set of cultivars ($R^2 = 0.49$, $P < 0.001$; data not shown). However, the fit for the modern cultivars ($R^2 = 0.76$, $P < 0.001$) and old landraces ($R^2 = 0.79$, $P < 0.001$) was different. Thus, even though the fitted line showed a slope close to one for both cases, modern cultivars were placed above the origin at zero and landraces were placed below the line (Figure 1).

In addition, the specific contribution of the ear (a) and the flag leaf ($1 - a$) to growing kernels within each of the nine genotypes and all growing conditions assayed was studied (Figure 2). The relative contributions of $\delta^{13}C_{ear}$ and $\delta^{13}C_{flag}$ accounting for the $\delta^{13}C_{grains}$ were assessed through a linear model, using the ear ($a \times \delta^{13}C_{ear}$ WSF) and the flag leaf ($1 - a \times \delta^{13}C_{flag}$ WSF) as independent variables, and the grain ($\delta^{13}C_{grains}$) as the dependent variable. Thus, old landraces showed higher ear contribution rates compared to modern cultivars. Therefore, ear contribution ranged from 91% to 100% and from 49% to 82% for old landraces and modern cultivars, respectively. Even within modern cultivars there were significant differences across genotypes (Figure 2). In addition, N fertilization also had a significant effect on ear contribution (83.5% and 75.1% for high and low N fertilization, respectively; Table 3). Moreover, water regime also significantly affected the relative contribution of the ear, but only under high fertilization conditions. Thus, the ear contribution under high nitrogen conditions decreased in response to irrigation (86.6% and 81.1%, for rainfed and irrigation, respectively; Table 3). Furthermore, the absolute contribution of the ear to grain filling was calculated as the product of the relative contribution by the total kernel weight per spike (Figure S2). There were significant differences across genotypes but not a clear differentiation between landraces and modern cultivars. Nevertheless, the genotype exhibiting the highest absolute ear contribution was a modern cultivar (*Anton*; Figure S2) despite the fact that in general the relative contribution of the ear in modern cultivars was lower than in landraces (Figure 2).

The relationships of ear contribution against HI within the nine genotypes (five modern cultivars and four old landraces) and all growing conditions assayed were studied (Figure 3). A negative correlation between ear contribution and HI was observed ($R^2 = 0.64$, $P < 0.001$), with landraces exhibiting, in general, higher ear contributions and lower HIs than the modern cultivars. Moreover, the negative relationship across growing conditions of the ear contribution against harvest index (HI) (Figure 4) was clearly different between landraces ($R^2 = 0.69$, $P < 0.001$) and modern cultivars ($R^2 = 0.99$, $P < 0.001$).

Table 3. Mean values of the relative contribution of the ear to grain filling (%) of nine durum wheat genotypes (G) grown a combination of two different water (W; support irrigation, I, vs. rainfed, R) and nitrogen (N; fertilized, HN, and non fertilized, LN) conditions

	Ear contribution (%)
Treatment	
I + HN	81.1^{b}
I − LN	77.1^{ab}
R + HN	86.6^{c}
R − LN	73.0^{a}
Nitrogen supply	
HN	83.5^{a}
LN	75.1^{b}
Genotype	
Old landraces	97.2^{a}
Modern cultivars	65.2^{b}
Level of significance	
Old vs Modern genotypes	0.000^{***}
G	0.000^{***}
W	ns
N	0.000^{***}
W × N	0.002^{**}
W × G	ns
N × G	0.001^{**}

The relative contribution of the ear to grain filling was calculated in Figure 2. Values with different superscripted letters are significantly different within treatments according to Tukey's HSD test ($P < 0.05$). Treatment values are the means of 27 values (nine genotypes and three replicates per treatment). Analysis of variance (ANOVA) of the set of durum wheat genotypes grown under different conditions of nitrogen supply, water regime and their respective interaction is also included. ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$.

DISCUSSION

The study of the natural abundance of carbon isotopic composition in different organs presented here proposes a non-intrusive, comparatively low-cost method that may help to elucidate the relative contribution to grain filling of the ears in relation to the flag leaf. The methodological approach we present is based on the differences in carbon isotope composition that exist among the different photosynthetic organs themselves and between them and the mature kernels. Similar patterns of lower $\delta^{13}C$ in the DM and the WSF of the flag leaf than in ear tissues, and mature kernels exhibiting values between them but closer to the ear, have been reported in the past in durum wheat (Araus et al. 1993) and other cereals (Hubick and Farquhar 1989; Araus et al. 1992). Whereas the effect of growing conditions such as water stress on increasing the $\delta^{13}C$ in all plant parts examined (Yoneyama et al. 1997) fully agrees with the well-established mechanism of carbon isotope fractionation in plants (Farquhar et al. 1989), the differences between photosynthetic organs have a constitutive nature, probably associated with differences in organ permeability to atmospheric CO_2 (Farquhar et al. 1989; Araus et al. 1993). However, the $\delta^{13}C$ of carbohydrates exported from photosynthetic to storage organs could be subjected to fractionation

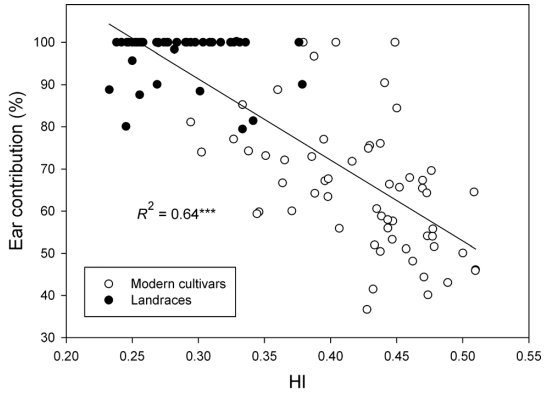

Figure 3. Linear regression of the relationship between ear contribution (%) to grain filling and harvest index (HI)
The relative contribution of the ear to grain filling was calculated in Figure 2. The nine genotypes (five modern cultivars and four old landraces) and three replications per genotype and growing condition were considered. Level of significance: $^{***}P < 0.001$.

during phloem loading and unloading or transport processes. In general, phloem sap sugars tend to be more enriched than bulk leaf tissue and leaf sugars by a few ‰ (Bowling et al. 2008), possibly as a result of fractionation during phloem loading, unloading, or transport (Damesin and Lelarge 2003; Scartazza

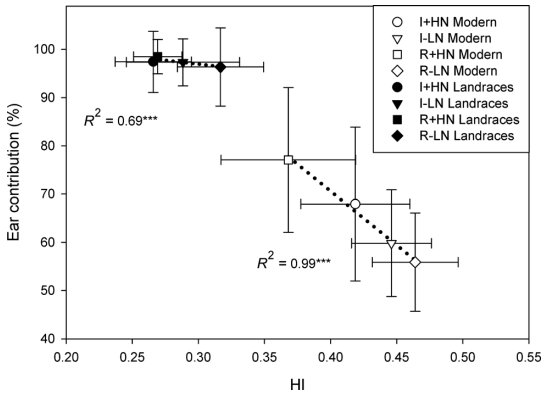

Figure 4. Relationship between ear contribution (%) to grain filling and harvest index (HI) for the landraces and modern genotypes across the four different growing conditions: the combination of two different water (W, support irrigation, I, vs. rainfed, R) and nitrogen (N, fertilized, HN, and non fertilized, LN) conditions
For each category of genotype values are means ± *SE* of the four landraces or five modern cultivars within each of the growing conditions. Level of significance: $^{***}P < 0.001$.

et al. 2004). In that sense, Brugnoli and Farquhar (2000) found differences in discrimination between carbohydrates stored in rice internodes (as sucrose) compared to primary leaves. These differences could be attributed to fractionation during enzymatic reactions or transport processes. Conversely, Yoneyama et al. (1997) studied post-photosynthetic fractionation of stable carbon isotopes between plant organs in different species. In their study, sugars in flag leaf blades, petioles, major veins, and phloem (which transport assimilates from source to sink tissues) were compared. In *Triticum aestivum*, non-significant differences were found between phloem exudates (−29.5‰), sugars and organic acids (−29.4‰) in leaf blades during grain filling. Thus, no evidence was found during transport in wheat for a large discrimination in assimilates after photosynthesis. This supports the conclusion that the $\delta^{13}C$ of grains is the result of the combined $\delta^{13}C$ values of assimilates produced by different photosynthetic organs contributing to grain filling. In the case that some degree of fractionation (enrichment) exists that is associated with phloem metabolism, the actual contribution of the ear to growing kernels may be lower than the value estimated here. However, the effect of growing conditions and genotype on the relative importance of each photosynthetic organ may still be captured by this approach.

Our study found that genotypic differences existed in the relative contribution of ear photosynthesis during grain filling. Such differences appear, at least in part, associated with sink strength. The relative contribution of the ear was negatively correlated with HI, with the ear of modern cultivars providing a lower percentage of assimilates during grain filling than the landraces. Whereas studies in old germplasm in bread wheat have shown no changes in grain weight due to defoliation treatments (Kruk et al. 1997), recent studies involving defoliation and degraining treatments indicate some evidence for the emergence of source- and sink-co-limitation in modern cultivars (Álvaro et al. 2008; Acreche and Slafer 2011; Pedro et al. 2011; Serrago et al. 2013). The increase in sink strength and HI, and the consequent decrease in preanthesis reserves due to *Dwarfism alleles* (Maydup et al. 2012) support the concept that in modern genotypes grain filling cannot be sustained by ear photosynthesis alone. In the case of landraces, our results suggest that besides the preanthesis reserves (Maydup et al. 2012), grain filling is basically sustained through the photosynthetic contribution of the ear. This may represent an evolutionary strategy aimed at minimizing the effect of herbivory or competition for light (Reynolds et al. 2009). In fact, as pointed out above, grain weight in landraces and old genotypes is less affected by defoliation than modern genotypes (see also Maydup et al. 2012). The present results do not contradict a basic role for the flag and the lower leaves in providing assimilates to the growth and development of the reproductive sink; specifically, the leaves determine the number of fertile florets and even further, the number of kernels and their potential size (Slafer and Savin 1994).

However, our results showed that among the set of genotypes studied, the modern cultivar *Anton* had the highest total contribution of the ear to filling grains, suggesting that the ear contribution is not only dependent on sink strength but also on genotypic variation.

On the other hand, we also studied the relative contribution of the ear and flag leaf to grain filling within different growing conditions. The relative contribution of the different organs seems to be subjected not only to source-sink limitations, but also to the growing conditions. Nitrogen fertilization increased the relative contribution of the ear, whereas irrigation decreased its contribution but only under highly fertilized conditions. It has been reported that water stress increases the role of the ear as a photosynthetic organ (Tambussi et al. 2005). In our study the effect of growing conditions on the photosynthetic contribution of the ear seems associated with changes in the HI. Thus, although high N fertilization and water stress caused a decrease in HI, they also increased the relative contribution of the ear during grain filling. However, the relationship between the contribution of the ear and the variation in HI induced by growing conditions (Figure 4) is far stronger for the modern cultivars than for the landraces, which again provides indirect support for some degree of source limitation in the former compared with the latter genotypes. By contrast, differences in the active durations of flag leaves associated with the growing conditions do not appear to have influenced the relative contribution of the flag leaf and ear to filling grains because there were no significant differences in leaf chlorophyll content associated with the treatments at the time that leaf and ear samples were collected. Besides the effect of growing conditions changing the HI, a difference in the response of the ear and the flag leaf to the growing conditions may be also involved. It has been reported that the ear is a photosynthetic organ better adapted to water stress than the flag leaf (Tambussi et al. 2005, 2007).

Regardless of the genotypic or growing conditions, proximity to the sink (i.e., the growing kernels) may also play in favor of a higher photosynthetic contribution during grain filling of the ear compared with the flag leaf. In an experiment conducted by Aranjuelo et al. (2011) in durum wheat using ^{13}C labeling, it was shown that during the beginning of post-anthesis the C fixed by the flag leaf was stored as structural C compounds, starch, and soluble sugars and then respired. Only a small amount of these soluble sugars arrived at the ear. On the other hand, the C synthesized in the ear was used for grain filling.

Our methodological approach does not take into account the potential contribution to grain filling of preanthesis assimilates. This may explain, for example, the different conclusion we reached compared to previous literature (Maydup et al. 2012) regarding the greater role of the ear during grain filling in landraces compared to modern genotypes. Due to their production in earlier stages of the crop cycle when water conditions are better than the later grain filling period, preanthesis assimilates should exhibit a very negative $\delta^{13}C$ compared with assimilates produced during grain filling. Nevertheless, the $\delta^{13}C$ of the kernels is far higher (less negative), which suggests that the contribution of preanthesis assimilates to grain filling is probably minor. In fact, in the methodological approaches of previous studies (Álvaro et al. 2008; Maydup et al. 2012) it has been assumed that the difference in stem DM from anthesis to maturity represented the whole contribution of preanthesis assimilates going to kernels. Besides not taking into consideration respiratory losses (Badeck et al. 2005; Aranjuelo et al. 2011), such an approach underestimates the contribution of the photosynthetic organs during grain filling. In fact during grain-filling, the current photosynthesis usually provides more

than enough carbohydrates for the developing grains (Slafer and Andrade 1991). Only when current photosynthesis drops below the maximum fill rate of the kernel does mobilization from the stem begin (Slewinski 2012).

Our approach also has the inherent limitation of not taking into account the potential contribution of other organs during grain filling such as the rest of the stem (peduncle, flag leaf sheath, lower leaves). However, the available literature has classically considered the flag leaf blade (Evans et al. 1980; Araus and Tapia 1987) and the ear (Tambussi et al. 2007) as the main photosynthetic contributors during grain filling (Evans and Rawson 1970). Even so, future studies should be addressed to assess the relative contribution during grain filling of the ear compared with the photosynthetic contribution of the whole stem. Indeed, the contribution to grain filling of the whole stem rather than the flag leaf blade may be an important parameter to assess.

Summarizing, this study delivers new evidence on the important role of the ear in providing assimilates during grain filling. The results show, however, that the contribution of the ear has decreased in modern cultivars compared to landraces, and this is probably associated with the appearance, to some extent, of source limitation driven by the increase in HI. Moreover, it is not only the genotypic variability but also the growing conditions that may affect the relative contribution of the ear and the flag leaf to grain filling.

MATERIALS AND METHODS

Germplasm used and experimental conditions

Nine durum wheat (*Triticum turgidum* L. var. *durum*) cultivars were used: four historical Spanish landraces (*Blanqueta*, *Griego de Baleares*, *Negro*, and *Jerez 37*) and five modern Spanish commercial varieties delivered after 1990 (*Anton*, *Bolo*, *Don Pedro*, *Regallo*, and *Sula*). The selection criteria for the landraces were based on their phenology, selecting for greatest similarity to the phenology of modern cultivars. The experiment was carried out during the 2010–2011 season at the experimental field of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) in Aranjuez, central Spain (40°03′N, 3°31′E, 500 m above sea level). In the experimental field, soil is Entisol Fluvent Xerofluvent, with the upper 0.4 m having an organic matter content of 4.9 g/kg, total nitrogen content of 0.37 g/kg, carbonate content of 233 g/kg, pH of 8.1 and electric conductivity of 0.164 dS/m. Two water treatments (support irrigation, I, and rain-fed, R) combined with two nitrogen regimes (high nitrogen, HN, and low nitrogen, LN) were assayed. The trials were planted on 30 December 2010 in plots with six rows 0.20 m apart, accounting for a total area of 7.1 m^2 (5 m in length and 1.2 m in width) per plot. The total accumulated precipitation during this season was 275.4 mm. Sprinkler irrigation was applied to irrigation plots around initiation of booting (11 April) and grain filling (15 and 25 May) with an approximated amount of 60 mm for each date. Environmental conditions during the growing season are detailed in Figure 5. Prior to sowing, all trials received 60 kg/ha of phosphorous as superphosphate (18%) and 60 kg/ha potassium as potassium chloride (60%). Further, the HN plants were dressed with nitrogen applied at the beginning of tillering (January 27) and jointing (March 20) using a dose of 45 and 105 kg/ha of urea (46%), respectively. The LN plants were not fertilized, relying exclusively on the N availability in the soil before sowing. The arrangement of water and nitrogen treatments was carried out according to a split–split plot design with three replicates. Experiment plots were kept free of weeds, insect pests, and disease by recommended chemical measures.

Days to anthesis were recorded as the number days from sowing to 50% of ears showing extruded anthers along their head lengths (Simmons et al. 1995). Around 7 days after anthesis (7 May), five representative flag leaves and ears were collected (Figure 6) per plot, oven dried at 70 °C for 48 h, weighed and finely ground for carbon isotope analysis. Flag

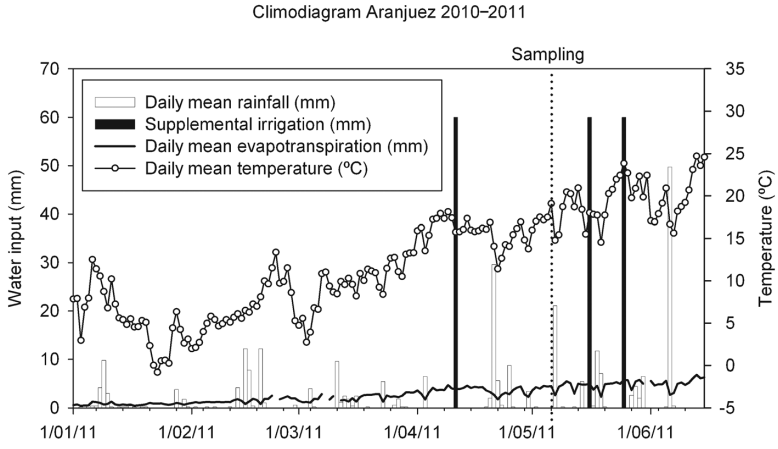

Figure 5. Daily precipitation, irrigation, evapotranspiration and mean air temperature during the growing season at the Aranjuez Experimental Station (Madrid Province, Spain).

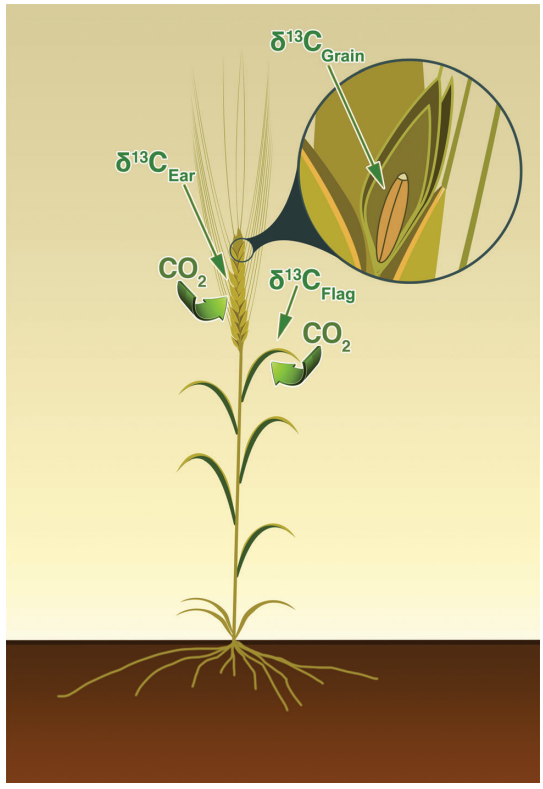

Figure 6. Durum wheat plant illustration showing the organs sampled in this study
The flag leaf and the ear from the main culm of five representative plants were collected for carbon isotope analysis. $\delta^{13}C_{ear}$ and $\delta^{13}C_{flag}$ represent the carbon isotopic composition of the ear and the flag leaf blade, respectively. $\delta^{13}C_{grain}$ represents the carbon isotopic composition of mature kernels.

leaf chlorophyll content was also measured with a SPAD-502 Minolta chlorophyll meter (Spectrum Technologies, Plainfield, IL, USA) in five flag leaves per plot. At maturity, the central four rows of each plot were harvested and grain yield (GY) recorded and grain samples processed as above for isotope analysis. Further, the main agronomical components, harvest index (HI), thousand kernel weight (TKW) and kernel weight per spike ($KW \times SP^{-1}$) were determined.

Carbon isotope analyses
Carbon isotope analyses of mature grains as well as the total DM and WSF of the flag leaf blades and ears were performed using an Elemental Analyzer (Flash 1112 EA; ThermoFinnigan, Bremen, Germany) coupled with an Isotope Ratio Mass Spectrometer (Delta C IRMS, ThermoFinnigan) operating in continuous flow mode in order to determine the stable carbon ($^{13}C/^{12}C$) isotope ratios of the same samples. Samples of approximately 1 mg and reference materials were weighed into tin capsules, sealed, and then loaded into an automatic sampler (ThermoFinnigan) before EA-IRMS analysis. The $^{13}C/^{12}C$ ratios of plant material were expressed in δ notation (Coplen 2008): $\delta^{13}C = (^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1$, where "sample" refers to plant material and "standard" to international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose, and USGS 40 L-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB) with an analytical precision (*SD*) of 0.10‰. Measurements were carried out at the Scientific Facilities of the University of Barcelona.

Water-soluble fraction
The protein-free WSF of the flag leaves and ears were extracted from the same dry samples tested for carbon isotopes, as described previously (Cabrera-Bosquet et al. 2011; Yousfi et al. 2013). Summarizing, 50 mg of either fine leaf or ear powder were suspended with 1 mL of Milli-Q water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany) for 20 min at about 5 °C. After centrifugation (12,000 g for 5 min at 5 °C), the pellet was discarded and the supernatant containing the WSF was heated at 100 °C for 3 min, where the heat denatured proteins precipitated. Subsequently, samples were centrifuged again (12,000 g for 5 min at 5 °C) to separate previously denatured proteins from the soluble fraction. An aliquot of 70 μL of supernatant containing protein-free WSF was transferred to tin capsules for carbon analysis. The capsules containing the aliquots were oven dried at 60 °C.

Relative photosynthetic contribution to grain filling
The relative contribution to grain filling of the different photosynthetic organs of the plants was assessed through a comparison of the $\delta^{13}C$ of the WSF of the different organs and $\delta^{13}C$ of mature kernels. The approach takes into consideration several assumptions. Thus, in this particular study only the whole ear (including awns and bracts) together with the flag leaf blade were considered as potential contributors to the filling of grains. Therefore, other parts of the culm (such as lower leaves as well as the sheath of the flag leaf and the peduncle) were ignored. This was based on two considerations. First, the available literature where the flag leaf blade and the ear are considered as the photosynthetic organs contributing to grain filling (Evans et al. 1980; Araus et al. 1993; Tambussi et al. 2005). Second, the need to economize analysis while developing a feasible technique for breeding, in terms of time and number of analyses, restricting these analyses to particular organs. Moreover, the approach proposed here considers that the relative contribution of each photosynthetic organ to grain filling varies as a result of water status. Thus, the relative contribution of the ear in relation to the rest of the plant increases as water stress develops (Tambussi et al. 2007). Relative water status may be assessed through the $\delta^{13}C$ of grains (Farquhar and Richards 1984; Ferrio et al. 2007; Araus et al. 2013). This approach also assumes that the $\delta^{13}C$ fractionation due to translocation of assimilates from either the culm or the spike to the kernels is negligible (Badeck et al. 2005). Therefore, it is expected that the $\delta^{13}C$ isotopic signal resulting from the combination of the flag leaf blade and the ear will be the same as that of the kernel. This implies that the same slope and origin to zero need to be found between the combination of the $\delta^{13}C$ of the culm and the ear

and that of the kernels. It has been reported that plant organs are typically enriched in ^{13}C relative to leaves and that phloem sap sugars tend to be more enriched than bulk leaf tissue and leaf sugars by a few ‰ (Bowling et al. 2008), possibly as a result of fractionation during phloem loading, unloading, or transport (Damesin & Lelarge 2003; Scartazza et al. 2004). However, such fractionation in wheat appears minimal (Yoneyama et al. 1997). Moreover, it has been reported that respiration may have a minor effect on discrimination (Bort et al. 1996; Badeck et al. 2005).

The ear contribution was calculated as follows:

$$\delta^{13}C_{grain} = a \times \delta^{13}C_{ear} + (1 - a) \times \delta^{13}C_{flag}$$

where "*a*" is the ear contribution to grain filling, $\delta^{13}C_{grain}$ the carbon isotopic composition of mature kernels, $\delta^{13}C_{ear}$ the carbon isotopic composition in the WSF of the ear, and $\delta^{13}C_{flag}$ the carbon isotopic composition in the WSF of the flag leaf blade.

Statistical analysis

Grain yield, agronomic components and isotopic data were subjected to one way analyses of variance (ANOVA) using the general lineal model to calculate the effects of water regime, nitrogen supply, genotype, and their interaction on the studied parameters. Means were compared by Tukey's HSD test and were performed on a combination of water treatments and nitrogen supply. A bivariate correlation procedure was constructed to analyze the relationships between the measured traits. Statistical analyses were performed using the SPSS 18.0 statistical package (SPSS, Chicago, IL, USA). Figures were created using the Sigma-Plot 10.0 program (SPSS).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

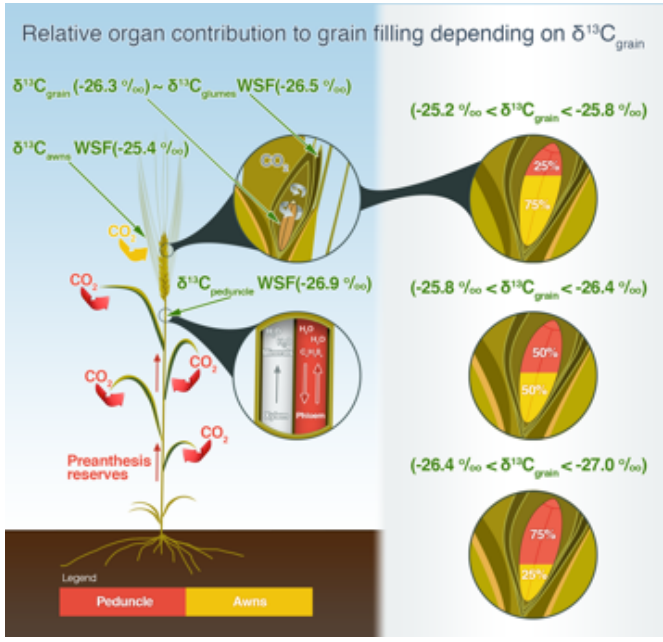
Figure S1. Pearson correlation coefficients of the relationships between stable isotope composition of carbon ($\delta^{13}C$) in the grains as well as in the dry matter (DM) and water soluble

fraction (WSF) of the flag leaf and the ear against the grain yield (GY)

For each relationship the nine genotypes and three replications per genotype and growing condition across the four growing conditions were considered. Levels of significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Figure S2. Total contribution of the ear to grain filling calculated as the product of the relative contribution by the total kernel weight per spike

The relative contribution of the ear to grain filling was calculated in Figure 4. Genotype values (*x*-axis) represent five modern cultivars (1, *Anton*; 2, *Bolo*; 3, *Don Pedro*; 4, *Regallo*; and 5, *Sula*) and four old landraces (7; *Blanqueta*; 9, *Griego de Baleares*; 10, *Negro*; and 11, *Jerez* 37). Values with different superscripted letters are significantly different according to Tukey's HSD test ($P < 0.05$). Genotype values (five modern cultivars and four old landraces) are the means of 12 values (four treatments and three replicates per treatment)



CAPITOL 2

Contribució relativa de la fotosíntesi de la tija i l'espiga a l'ompliment del gra en blat sota bones condicions agronòmiques avaluada per diferencils $\delta^{13}C$ dels òrgans

CHAPTER 2

Relative contribution of shoot and ear photosynthesis to grain filling in wheat under good agronomical conditions assessed by differential organ $\delta^{13}C$

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RESUM CAPÍTOL 2

Als cereals C_3, durant l'ompliment del gra, la tija (sobretot la fulla bandera) a més de l'espiga es creu que tenen un paper molt important com a "fonts" d'assimilats pels grans. El fet que les metodologies utilitzades a l'actualitat siguin intrusives i sovint cares ha fet que actualment no hi hagi resultats concloents. En aquest estudi es comparen la composició isotòpica del carboni ($\delta^{13}C$) en la seva abundància natural dels grans madurs amb la composició isotòpica de la fracció soluble del peduncle i de les glumes i arestes de l'espiga amb l'objectiu d'avaluar les contribucions totals dels òrgans per sota el peduncle a l'ompliment del gra. Es van utilitzar unes línies altament productives procedents del CIMMYT (Centro Internacional para la Mejora del Maíz y el Trigo, Mèxic). En general, la contribució de l'espiga va ser més gran que la de la tija. La contribució específica de la fulla bandera a l'ompliment del gra es va avaluar comparant la $\delta^{13}C$ dels grans amb la $\delta^{13}C$ de la fracció soluble de la fulla bandera i de les arestes. En aquest cas, la contribució de la fulla bandera a l'ompliment del gra va ser només del 3 al 18%. Es van fer anàlisis per complementar aquests resultats com ara intercanvi de gasos i la quantitat de carbohidrats acumulats de la fracció soluble en els dos òrgans, a més de la llum interceptada per la capçada a diferents alçades. Aquest resultats suggereixen que la espiga té una capacitat fotosintètica comparable a la de la fulla bandera. En aquest sentit, els programes de millora podrien utilitzar els isòtops estables per seleccionar plantes de blat en les que hi hagi una elevada contribució de l'espiga al rendiment.

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RESEARCH PAPER

Relative contribution of shoot and ear photosynthesis to grain filling in wheat under good agronomical conditions assessed by differential organ $\delta^{13}C$

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Abstract

During grain filling in C3 cereals, the shoot (particularly the flag leaf) and the ear are believed to play major roles as sources of assimilates. However, both the cost and the intrusive nature of most of the methodologies available to investigate this have prevented conclusive results being obtained. This study compared the carbon isotope composition ($\delta^{13}C$) in its natural abundance in mature kernels with the $\delta^{13}C$ of the water-soluble fraction of the peduncle, glumes, and awns to assess the relative contribution of the shoot (understood as the whole set of photosynthetic organs below the peduncle) and ear to grain filling in a set of highly productive wheat lines from the International Maize and Wheat Improvement Center, Mexico, under good agronomic conditions. In overall terms, the contribution of the ear was greater in comparison with that of the shoot. The specific contribution of the flag leaf blade to grain filling was also assessed by comparing the $\delta^{13}C$ of grains with the $\delta^{13}C$ of the water-soluble fraction of the flag leaf and the awns. The contribution of the flag leaf was minor, ranging between 3 and 18%. Complementary analyses performed such as gas-exchange rates and the accumulated water-soluble carbohydrates in both organs and light intercepted by the canopy at different strata suggested that the ear has a photosynthetic capacity at least comparable to that of the flag leaf. In this sense, selection for a higher contribution of ear photosynthesis to grain yield in breeding programmes could be addressed with the use of stable isotopes.

Key words: Carbon isotope composition, ear, flag leaf, grain filling, photosynthesis, shoot.

Introduction

The United Nations prediction for 2050 is that the world's human population will reach 9.3 billion (Food and Agriculture Organization of the United Nations, 2013). The challenge to accommodate this world population growth in a context of global (i.e. social and climate) change implies adaptations to secure future feed demand and food supply (Foulkes *et al.*, 2011). Hence, the most direct solution to meet this challenge would be to increase productivity through the use of new cultivars with enhanced genetic yield potential. Wheat (*Triticum aestivum* L.) is one of the main staple crops. One of the avenues proposed to increase yield potential and improve adaptation to abiotic stresses, such as drought, is to select for higher ear photosynthesis (Tambussi *et al.*, 2005, 2007; Parry *et al.*, 2011). Indeed, the ear in wheat and other small-grain cereals is believed to play a significant role as a source of photoassimilates during grain filling, not only under drought or other abiotic stresses but also under good agronomical conditions (Araus *et al.*, 1993; Bort *et al.*, 1994; Tambussi *et al.*,

Abbreviations: ANOVA, analysis of variance; BM, biomass; CIMMYT, International Maize and Wheat Improvement Center; DAA, days after anthesis; DM, dry matter; GY, grain yield; HI, harvest index; KW SP^{-1}, kernel weight per spike;PAR, photosynthetically active radiation; PPFD, photosynthetic photon flux density; SE, standard error; TKW, thousand kernel weight; WSC, water-soluble carbohydrates; WSF, water-soluble fraction.

2005, 2007; Maydup *et al.*, 2010) or as response to different fungal diseases that may affect leaves (Robert *et al.*, 2005) more than ears (Tiedemann and Firsching, 2000).

Although several studies have analysed ear photosynthesis (Araus *et al.*, 1993; Bort *et al.*, 1994; Tambussi *et al.*, 2007; Maydup *et al.*, 2010), its contribution to grain filling remains unclear. The reported contributions to grain filling vary widely, with estimates ranging from about 10 to 76% of the assimilate being deposited in grains (Gebbing and Schnyder, 2001; Tambussi *et al.*, 2007; Aranjuelo *et al.*, 2011). The variability of these estimates may reflect genetic diversity in the contribution of ear photosynthesis to grain yield combined with different growing conditions, but this is also likely to be the consequence of drawbacks in the methods used. In fact, compared with the leaves, the photosynthetic contribution of the ear has been less studied, in part due to methodological limitations. In addition, the genotypic differences in the contribution of ear photosynthesis to grain filling cannot be accounted for solely on the basis of the net photosynthesis of the ears. Differences in the refixation rate of the ears could also be involved (Tambussi *et al.*, 2007). Indeed, a substantial refixation of respiratory CO_2 within the ear has been reported (Bort *et al.*, 1996), which can contribute to 70% of the sucrose accumulated in bracts (Gebbing and Schnyder, 2001). While gas-exchange measurements are time-consuming, and even more so if respiration also needs to be monitored, there is no proven link between whole-ear photosynthesis and the relative contribution of this organ during grain filling. This is why an array of alternative approaches has been deployed for a large-scale evaluation of the ear contribution to grain filling.

Most of the methods for inferring the photosynthetic contribution of different plant parts to filling grains have involved intrusive approaches based on a differential (i.e. organ-specific) prevention of photosynthesis of some parts of the plant. Such approaches include, for example, shading the ears, the flag leaf blade, or the entire shoot (Aggarwal *et al.*, 1990; Araus *et al.*, 1993; Peralta *et al.*, 2011), application of herbicides that prevent photosynthesis (Maydup *et al.*, 2010), or simply defoliating leaf blades (Ahmadi *et al.*, 2009). Besides the intrusive nature of such treatments, it should be kept in mind that compensation effects triggered by these treatments may eventually increase the contribution of unaffected photosynthetic organs or of pre-anthesis reserves to grain filling (Aggarwal *et al.*, 1990; Eyles *et al.*, 2013).

The use of the stable carbon isotope signature in its natural abundance may help to elucidate the relative contribution of the different photosynthetic organs (Sanchez-Bragado *et al.*, 2014). The carbon isotope composition ($\delta^{13}C$; frequently expressed as carbon isotope discrimination, $\Delta^{13}C$) in plant matter reflects the photosynthetic performance of the plant (Farquhar *et al.*, 1989) and is one of the most successful time-integrated physiological traits used by plant breeders (Araus *et al.*, 2002). The stable isotope, ^{13}C, is discriminated against the lighter ^{12}C during photosynthetic carbon fixation (Farquhar and Richards, 1984). Thus, discrimination of ^{13}C in a photosynthetic organ depends on the ratio of the intercellular versus the external (atmospheric) CO_2 concentration of CO_2 (c_i/c_a) (Farquhar *et al.*, 1989). Whereas environmental factors such as water availability may affect $\delta^{13}C$ (and thus $\Delta^{13}C$), mostly through an effect on stomatal conductance, there are also constitutive differences in $\delta^{13}C$ associated with the specific plant part considered (Hubick and Farquhar, 1989; Araus *et al.*, 1993). This is the case, for example, for $\delta^{13}C$ from photoassimilates produced by different photosynthetic plant parts, such as the leaf blades and the ear (Hubick and Farquhar, 1989; Araus *et al.*, 1992, 1993). Thus, regardless of the growing conditions the $\delta^{13}C$ of photoassimilates from the flag leaf blade is lower (more negative) than from the ear (Araus *et al.*, 1992, 1993). Such variation in $\delta^{13}C$ among plant parts may be caused by differences in the c_i/c_a ratio driven by a far lower permeability to gas diffusion in the ear compared with the blades. Thus, the higher constitutive $\delta^{13}C$ of the assimilates from the ear compared with the leaves may be associated with a lower c_i/c_a of the former organ.

The main photosynthetic organs of the ear are the glumes and the awns (Gebbing and Schnyder, 2001; Tambussi *et al.*, 2007). While in awned cereals this tissue seems to be the main photosynthetic organ of the ear in terms of fixing atmospheric CO_2 (Li *et al.*, 2006; Tambussi *et al.*, 2007), as pointed out above, the glumes may also have a crucial photosynthetic role mainly in refixing CO_2 respired by the forming grains (Gebbing and Schnyder, 2001).

This study proposed the use of the $\delta^{13}C$ of assimilates from different plant parts as a criterion to assess in a non-disturbing manner the relative contribution of ear and shoot photosynthesis to grain filling. In such a way, the $\delta^{13}C$ of assimilates from the awns and the glumes were analysed at about the mid-stage of grain filling. In order to integrate the $\delta^{13}C$ of the assimilates produced by the different photosynthetic organs of the shoot and then transferred to the ear along with stem reserves, the $\delta^{13}C$ of assimilates from the peduncle was also analysed. In addition, the $\delta^{13}C$ of the assimilates of the flag leaf blade was also analysed, because traditionally this organ has been considered as the main photosynthetic contributor to growing grains, particularly in the absence of water stress (Evans *et al.*, 1975; Araus and Tapia, 1987). The present study was carried out on a set of high-yielding advanced lines of bread wheat from the International Maize and Wheat Improvement Center (CIMMYT), Mexico, growing under well-managed agronomic conditions.

Material and methods

Germplasm used and experimental conditions

Six advanced bread wheat (*T. aestivum* L.) lines were selected on the basis of their similar phenology, high grain yield, and biomass, from the CIMCOG (CIMMYT Mexico Core Germplasm) population, which is composed of 60 elite lines generated from CIMMYT breeding programmes (Table 1). The field experiments were conducted during the spring growing season of 2012 at MEXPLAT (Mexican Phenotyping Platform) situated at CIMMYT's Experimental Station, Norman E. Borlaug (CENEB) in the Yaqui Valley, near Ciudad Obregón, Sonora, México (27°24′N, 109°56′W, 38 m above sea level), under fully irrigated conditions. The soil was a typic calciorthid with low organic matter composition (0.76%) and a slightly alkaline (7.7) pH (Sayre *et al.*, 1997) with a plant-available water-holding capacity of about 200 mm (Lopes and Reynolds, 2012). The experimental design was a randomized lattice with three replications in 8.5 m long plots

Table 1. *Mean values of grain yield (GY), agronomical components and phenology measured in the six selected genotypes*

Each value is the mean±SE of three replications. Thousand kernel weight (TKW), harvest index (HI), biomass at anthesis (BM), number of grains per spike (GSP), kernel weight per spike (KW SP^{-1}), plant height (Height), and number of days from sowing to anthesis (DTA) and maturity (DTM) were determined. ANOVA for the effect of genotype is shown. Pedigrees of the genotypes detailed in the Genotype column are as follows: 1, CNO79//PF70354/MUS/3/PASTOR/4/BAV92*2/5/FH6-1-71; 2, PBW343*2/KUKUNA*2//FRTL/PIFED2; 3, SOKOLL//PBW343*2/KUKUNA/3/ATTILA/PASTOR3; 4, TACUPETO F2001/BRAMBLING*2//KACHU4; 5, UP2338*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ/5/MILAN/KAUZ//CHIL/CHUM18/6/UP2338*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ5; 6, WBLL1*2/KURUKU*2/5/REH/HARE//2*BCN/3/CROC_1/AE.SQUARROSA(213)//PGO/4/HUITES6. Mean values with different superscript letters were significantly different according to Tukey's HSD test ($P<0.05$). NS, not significant.

Genotype	GY (Mg ha^{-1})	BM (Mg ha^{-1})	HI	TKW (g)	KW SP^{-1} (g)	Height (cm)	GSP	DTA (d)	DTM (d)
1	7.2±0.2^{a}	16.0±0.2^{b}	0.45±0.01^{a}	45.5±0.1^{b}	2.8±0.0^{bc}	110.8±0.9^{a}	61.7±0.8^{a}	87^{bc}	132^{a}
2	6.9±0.3^{a}	14.4±0.3^{a}	0.48±0.03^{a}	42.2±0.9^{a}	2.0±0.1^{a}	99.8±0.6^{b}	46.9±2.9^{b}	84^{a}	126^{a}
3	6.6±0.1^{a}	15.2±0.2^{ab}	0.43±0.01^{a}	43.1±0.3^{ab}	2.1±0.1^{a}	107.4±0.9^{b}	48.0±1.2^{b}	86^{b}	127^{a}
4	6.6±0.0^{a}	14.7±0.4^{ab}	0.45±0.02^{a}	43.0±0.8^{ab}	2.7±0.1^{b}	101.3±2.7^{a}	61.8±2.1^{a}	89^{c}	131^{a}
5	6.9±0.2^{a}	15.7±0.6^{a}	0.44±0.02^{a}	40.3±0.7^{a}	2.1±0.0^{a}	105.1±0.7^{ab}	51.3±1.1^{a}	88b^{c}	131^{a}
6	6.6±0.3^{a}	13.7±0.4^{a}	0.48±0.02^{a}	49.0±0.7^{c}	3.1±0.1^{c}	108.1±1.1^{b}	63.2±2.3^{b}	87^{bc}	132^{a}
Level of significance									
Genotype	NS	0.010*	NS	0.000***	0.000***	0.001**	0.000***	0.000***	NS

consisting of two raised beds (0.8 m wide per bed) with two rows per bed (0.24 m between rows) with an additional shared bed in each plot side. The seeding rates were 108 kg ha^{-1}. The experiments were sown on 9 December 2011 and 23 November 2012, and immediately irrigated to promote germination. The respective emergence dates were on 16 December 2011 and 2 December 2012. Harvesting was performed by machine on 15 May 2012 and manually on 6–7 May 2013, respectively, about 15–20 d after reaching physiological maturity. The mean rainfall was 14.2 and 15.4 mm and evapotranspiration was 4.6 and 3.8 mm, respectively, during the 2012 and 2013 crop cycles. The maximum average temperatures were 28.0 and 25.9 °C and the minimums were 8.4 and 8.3 °C (in 2012 and 2013, respectively). The relative moisture ranged from 27.5 to 88.5% in 2012 and from 34.4 to 90.9% in 2013. A total of five auxiliary irrigations were provided totalling more than 500 mm of water applied in 2012 and 2013. In 2012, the auxiliary irrigation dates during grain filling were on 16 March and 31 March, about 8 and 17 d after anthesis (DAA), respectively. For the crop cycle in 2013, auxiliary irrigation dates were on 15 March and 4 April, about 8 and 28 DAA. Appropriate fertilization and weed, disease, and pest control were implemented to avoid yield limitations. Plots were fertilized with 50 kg ha^{-1} of N and 50 kg ha^{-1} of P at soil preparation and another 150 kg ha^{-1} of N with the first irrigation.

Agronomic traits

For each plot, grain yield, biomass, yield components, and plant height were determined in approximately 5.7 m^2 using standard protocols (Pask *et al.*, 2012). In addition, phenology was recorded throughout the cycle using the Zadocks scale (Zadocks *et al.*, 1974).

Leaf and ear photosynthesis and respiration

Photosynthetic and respiration rates of the flag leaf blade and the ear were measured as carbon uptake using a LI-6400XT portable gas-exchange photosynthesis system (LI-COR, Lincoln, NE, USA). Photosynthesis measurements were performed approximately 2 weeks after anthesis. The flag leaf photosynthetic assimilation rate was estimated at a saturating photosynthetic photon flux density (PPFD) of 1500 µmol m^{-2} s^{-1} and 30 °C. Ear photosynthesis was measured using a hand-made chamber connected to the Li-6400XT as described previously for other purposes (Aranjuelo *et al.*, 2009). Ears were enclosed inside the chamber and ingoing air was passed through the chamber at a rate of 1 l min^{-1}. The molar fractions of CO_2 and humidity were measured with the infrared gas analyser of the LI-6400XT. The CO_2 partial pressure was maintained as constant with the infrared gas analyser-controlled CO_2 injection system. To ensure steady-state conditions inside the chamber, the system was left to stabilize for a few minutes. An external light source composed of LED lights was placed around the chamber during the measurement achieving a saturating PPFD of approximately 1200 µmol m^{-2} s^{-1} measured inside the chamber. The photosynthetic rates presented here are based on the whole organ area measured with a LI3050A/4 (LI-COR). Dark respiration of the flag leaf and the ear were measured immediately after the photosynthetic measurements at a temperature of 30 °C.

Assimilates produced

The potential contribution of the two organs as a source of assimilates was assessed taking into account the instantaneous net photosynthesis plus the dark respiration (here defined as gross photosynthesis) of the whole organs multiplied by the duration of the daylight period with a saturating PPFD and the number of days from heading to organ senescence. For each organ, the active duration of the flag leaf and ear was assessed periodically from heading to maturity. In the case of the flag leaf, chlorophyll content was measured once a week with a SPAD-502 Minolta chlorophyll meter (Spectrum Technologies, Plainfield, IL, USA) in five flag leaves per plot. The active duration of the flag leaf was considered to end when SPAD values went below 20. For the ear, senescence was assessed visually and the active ear duration was considered to end when the peduncle changed colour. In addition, the total amount of assimilates produced per organ was estimated from the accumulated gross photosynthesis from heading to maturity, assuming all the fixed C was converted into carbohydrates (CH_2O).

Light interception

Incident and transmitted photosynthetically active radiation (PAR) was measured about 1 week after anthesis on clear days as close to solar noon as possible (from 11:00 to 14:00), with a Linear PAR Ceptometer (AccuPAR LP-80; Decagon, Washington, CD, USA). Different strata of the canopy were considered for the measurements of transmitted PAR: the base of the ear, the flag leaf blade (which also included the peduncle), the penultimate leaf (including the sheath of the flag leaf and the first internode), and the third leaf (including the sheath of the penultimate leaf and the second internode). A single measurement was performed at each stratum

in each of the three replicates. The light intercepted by each stratum was estimated from the PAR measured by adapting the equations described by Pask *et al.* (2012) to each stratum.

Total water-soluble carbohydrates (WSCs)

For the 2012 and 2013 crop seasons, WSCs were analysed in plants around mid-grain filling. Sampling was performed twice in 2012 and once in 2013. In 2012, WSCs were sampled 17 and 24 DAA (before and after irrigation, respectively). In 2013, WSCs were sampled 18 DAA. Ten representative ears, flag leaves, and peduncles per plot were harvested, cleaned, and immediately frozen with liquid nitrogen. Additionally, for the 2013 crop season, the entire peduncle was sampled and thereafter separated into two sections, the upper section (peduncle 1) and the lower sections (peduncle 2). The samples were stored at –20 °C and then lyophilized for 48 h in 2012. For the 2013 crop season, samples were oven dried at 70 °C for 48 h. In addition, the glumes, awns, flag leaves, and peduncles were separated, weighed, and finely ground. WSCs were analysed as described by Yem and Willis (1954) using the anthrone method and following the procedures described in Galicia *et al.* (2009). Briefly, the anthrone procedure is based on the reaction of anthrone (9,10-dihydro-9-oxoantraceno) with the furfural conformation of carbohydrates (treatment of carbohydrate in strong sulfuric acid) to give a coloured hemi-acetal, which is determined spectroscopically at 630 nm. Total soluble carbohydrates are expressed on a dry-weight basis. In addition, total soluble carbohydrates per whole organ were calculated.

Carbon isotope analysis

Analyses were only performed in the 2012 experiment. The stable carbon isotope composition ($\delta^{13}C$) in the dry matter (DM) of glumes, awns, flag leaves, and peduncles was analysed in the same samples used for WSCs and taken before irrigation (17 DAA). $\delta^{13}C$ was also analysed in mature kernels. For $\delta^{13}C$ analysis of the DM, approximately 1 mg of each sample was weighed into tin capsules and measured with an elemental analyser coupled with an Isotope Ratio Mass Spectrometer (Delta C IRMS; ThermoFinnigan, Bremen, Germany) operating in continuous flow mode in order to determine the stable carbon ($^{13}C/^{12}C$) isotope ratios of the same samples. The $^{13}C/^{12}C$ ratios of plant material were expressed in δ notation (Coplen, 2008): $\delta^{13}C=(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1$, where 'sample' refers to plant material and 'standard' to international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose and USGS 40 L-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate with an analytical precision (standard deviation) of 0.10‰.

The water-soluble fraction (WSF) of the flag leaf, peduncle, glumes, and awns was further extracted, as described previously (Yousfi *et al.*, 2013), from the same dry-matter samples used for WSCs and taken before and after irrigation. Briefly, 50 mg of fine leaf and ear powder was suspended in 1 ml of Milli-Q water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany) for 20 min at about 5 °C. After centrifugation (12 000*g* for 5 min at 5°C), the pellet was discarded and the supernatant containing the WSF was heated at 100 °C for 3 min where the heat-denatured proteins precipitated. Subsequently, samples were centrifuged again (12 000*g* for 5 min at 5 °C) to separate previously denatured proteins from the soluble fraction. An aliquot of 40 µl of supernatant containing the protein-free WSF was transferred to tin capsules for carbon analysis. The capsules containing the aliquots were oven dried at 60 °C for 1 h. Then, the $\delta^{13}C$ of the WSCs was determined following the same procedure as that used for DM. Isotopic analyses were carried out by the Scientific-Technical Services of the University of Barcelona, Spain.

Isotopic composition of respired CO_2

Analysis of the isotopic composition of respired CO_2 was performed as described previously by Nogués *et al.* (2004). Entire flag leaves and ears were placed separately in the same chamber used to measure ear photosynthesis, and this was connected in parallel to the sample air hose of a LI-6400XT (LI-COR). The measurements were performed in the field in intact plants about 2 weeks after anthesis. Measurements were done twice: during the day (covering the entire plant with a black blanket) and the subsequent night. Ingoing air was passed through the chamber at a rate of 1 l min^{-1}. The CO_2 respired by the plant was monitored by the LI-6400XT in order to determine respiration rates so that the time of accumulation could be defined to obtain a concentration in the chamber of approximately 350 ppm of CO_2. The gas-analysis chamber was first flushed with CO_2-free air to ensure that only the CO_2 respired in the chamber was accumulated. According to the respiration rates, the time required for the plant to respire 350 ppm of CO_2 was calculated and the chamber system was closed until the CO_2 concentration inside the chamber reached the desired concentration. For each analysis, 25 ml of gas sample was collected from inside the chamber with a 50 ml syringe (SGE, Ringwood, Victoria, Australia) and immediately injected into a 10 ml BD vacutainer. The vacutainers were sent for analysis at the Scientific-Technical Services of the University of Barcelona, Spain, and were analysed by gas chromatography combustion isotope ratio mass spectrometry as previously described (Aranjuelo *et al.*, 2009).

Relative photosynthetic contribution to grain filling

The relative contribution to grain filling of the different photosynthetic organs of the plant was assessed by a comparison of the $\delta^{13}C$ of the WSF of the different organs (averaged values before and after irrigation) and the $\delta^{13}C$ of mature kernels. The approach takes into consideration several assumptions. It considers that the photosynthetic organs fixing CO_2 from the atmosphere are the awns and the green culm parts (leaf blades, sheaths, and peduncles) and therefore it excludes the glumes because this organ mainly fixes CO_2 from grain respiration (Gebbing and Schnyder, 2001). It assumes that the WSF in the peduncle reflects the pooled assimilates produced by the different photosynthetic organs (leaf blades, sheaths and the peduncle itself) during grain filling (plus the pre-anthesis reserves) eventually moving to growing kernels (assuming no downstream fractionation). Analysing only the WSF of the peduncle as an indicator of the pooled photosynthetic contribution of the stem is a way to economize analyses while developing a feasible technique for breeding in terms of numbers of analyses required.

The specific contribution to grain filling of the flag leaf blade was also assessed through analysis of the WSF in this organ (averaged values before and after irrigation) compared with the WSF of the awns (also averaged values), in order to estimate the potential maximum contribution of the flag leaf to grain filling. This was based on the fact that the flag leaf blade has traditionally been considered the main photosynthetic organ contributing to grain filling (Evans *et al.*, 1975; Araus and Tapia, 1987).

In addition, the approach proposed here considers that the relative contribution of each photosynthetic organ to grain filling varies as a result of water status and that it is reflected in the stable carbon isotope signature of mature grains (Araus *et al.*, 2003). In our study, relative water status was assessed through the $\delta^{13}C$ of mature kernels (Farquhar and Richards 1984; Ferrio *et al.*, 2007; Araus *et al.*, 2013). Water stress may induce stomatal closure in the different photosynthetic organs and then a decrease in the ratio of intercellular to atmospheric partial pressure of CO_2, therefore increasing the $\delta^{13}C$ of assimilates (Farquhar and Richards, 1984; Condon *et al.*, 2004) and finally the $\delta^{13}C$ of kernels. Thus, we assumed that the relative contribution of the awns in relation to the rest of the organs increased as water stress developed. This agrees with existing reports on the increased role of the ear (compared with the leaves) providing photoassimilates to growing kernels under water stress (Araus *et al.*, 1993; Tambussi *et al.*, 2007). Variability in crop water status may be present even under what are considered good agronomic conditions, with these frequently exposing the plants to mild water stress conditions (Cuenca, 1989).

Another assumption of the method proposed here was to neglect the $\delta^{13}C$ fractionation due to translocation of assimilates from

either the culm or the awns to the kernels (Yoneyama *et al.*, 1997). In fact, it has been reported that respiration associated with translocation may only have a minor discrimination effect (Bort *et al.*, 1996; Badeck *et al.*, 2005). Therefore, it was expected that the $\delta^{13}C$ of the kernels would directly reflect the isotopic signal resulting from the combinations of the $\delta^{13}C$ of assimilates coming from different photosynthetic sources. This implied that the same slope and origin to zero need to be found between the combined $\delta^{13}C$ of the culm and the awns and the $\delta^{13}C$ of the kernels.

Statistical analysis

Data were subjected to one-way analyses of variance (ANOVA) using the general linear model in order to calculate the effects of genotype and organ on the studied parameters. Means were compared by Tukey's honestly significant difference (HSD) test. A bivariate correlation procedure was constructed to analyse the relationships between the measured traits. Statistical analyses were performed using the SPSS 18.0 statistical package (SPSS, Chicago, IL, USA). Figures were created using the Sigma-Plot 10.0 program (SPSS).

Results

Effect of growing conditions on grain yield

The six selected genotypes were advanced lines that in general presented high biomass (BM) and grain yield (GY). Thus, GY across plots ranged between 6.5 and 7.2 Mg ha^{-1} (Table 1), but no significant differences across genotypes were observed. Concerning the agronomical components, thousand kernel weight (TKW) ranged from 40.3 to 49.0 g and kernel weight per spike (KW SP^{-1}) from 2.0 to 3.1 g. All agronomic components exhibited genotypic variation except for GY and harvest index (HI). The phenology range across genotypes according to date of anthesis was 5 d, and no differences were observed for date of maturity. The duration from planting to maturity was about 130 d, whereas grain filling extended for approximately 6 weeks (counted as the number of days from anthesis to maturity).

Photosynthetic contribution of the flag leaf and the spike to grain filling

Instantaneous net and gross carbon fixation were higher in the flag leaf compared with the spike (Fig. 1a). However, total photosynthetic productivities of the flag leaf and the ear (based on the accumulated gross carbon fixation) were calculated as the total carbohydrates produced by each organ from heading to maturity, and while they were comparable to the KW SP^{-1}, they were not significantly different from each other (Fig. 1b).

During the 2012 crop season, the amount of WSCs per whole organ present at mid-grain filling (17 and 24 DAA, before and after irrigation, respectively) in the awns, glumes, and flag leaf blades was similar, whereas in the peduncles the WSCs were significantly higher (Fig. 2, upper panel). In the 2013 crop season, the WSCs (Fig 2, lower panel) were similar in the awns, glumes, flag leaf blades and sheaths and in peduncle 1 (upper section of the peduncles). Conversely, WSCs in peduncle 2 (lower section of the peduncles) were higher than in the rest of the organs studied.

The amount of light intercepted at the different crop strata was different among plant organs (Fig. 3). The ear and the flag leaf blade (including the peduncle) strata showed similar percentages of light intercepted (around 30%). The amount of light intercepted by the penultimate leaf (plus the first internode) was lower in comparison with that in the ear and flag leaf but higher than that of the third leaf plus the second internode.

Carbon isotope signature

The carbon isotope composition ($\delta^{13}C$) was different between DM and the WSF, where DM showed higher values (i.e. ^{13}C enriched, less negative $\delta^{13}C$) compared with the WSF before and after irrigation (Table 2). Moreover, the $\delta^{13}C$ in the WSF

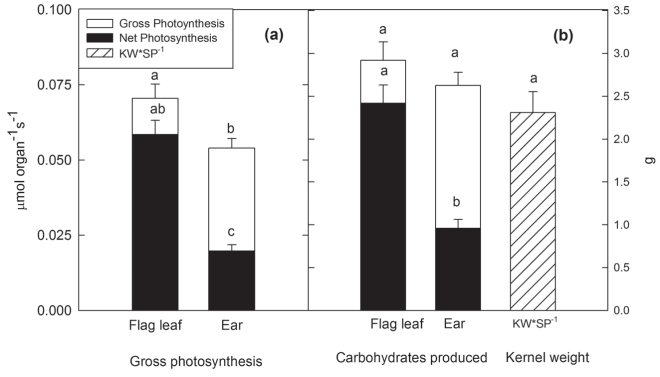

Fig. 1. Comparative photosynthetic contribution of the ear and flag leaf during grain filling, expressed as instantaneous net photosynthetic rate plus dark respiration (gross photosynthesis) (a) and the carbohydrates produced by both organs during the reproductive stage (accounted from heading to maturity) compared with the accumulated kernel weight per spike (KW*SP^{-1}) at maturity (b). Carbohydrates produced were calculated by multiplying gross photosynthesis, duration of the daylight period (at saturating PPFD), active organ duration (as the number of days from heading to maturity), and molecular weight (C_2OH) of the basic carbohydrates produced. For more details, see Materials and methods. Each bar represents the mean values±standard error (SE) of the five genotypes with the three replications per genotype. Mean values with different superscript letters are significantly different according to Tukey's HSD test (P<0.05).

before irrigation showed a tendency to higher values (less negative $\delta^{13}C$) compared with the WSF after irrigation, but only the peduncle showed significant differences. Significant differences in $\delta^{13}C$ values were observed across plant organs, with both the DM and WSF of the awns and flag leaf blades having the highest and the lowest $\delta^{13}C$ values, respectively. The $\delta^{13}C$ values of the different plant organs were significantly different to the $\delta^{13}C$ of mature kernels with the exception of the DM and WSF $\delta^{13}C$ of the glumes. Thus, the $\delta^{13}C$ of awns and peduncles exhibited slightly higher (^{13}C enriched) and lower (^{13}C depleted) values, respectively, than the $\delta^{13}C$ of grains. Moreover, genotypic differences were found in the $\delta^{13}C$ of the peduncle and flag leaf DM, whereas for the WSF only the $\delta^{13}C$ in the peduncle (before and after irrigation) and the flag leaf blade (after irrigation) showed genotypic differences (see Supplementary Table S1 at *JXB* online). In spite of no significant genotypic differences in the $\delta^{13}C_{grain}$, the range of variation across plots in the $\delta^{13}C_{grain}$ was about 2‰. Moreover, a negative correlation across plots was observed between the $\delta^{13}C_{grain}$ and GY (see Supplementary Fig. S1 at *JXB* online), which suggested that the studied trial exhibited some differences in water status across plots.

Assimilate contribution to filling grains

The $\delta^{13}C_{grains}$ values were between the range marked by the WSF $\delta^{13}C$ of the awns ($\delta^{13}C_{awns}$) and the peduncle ($\delta^{13}C_{peduncle}$) at mid-grain filling (Table 2), measured before and after irrigation. The relative contribution of the $\delta^{13}C_{awns}$ and $\delta^{13}C_{peduncle}$ that accounted for the $\delta^{13}C_{grains}$ was assessed through a linear fit. The $\delta^{13}C_{grains}$ was used as a dependent variable and a combination of the $\delta^{13}C$ in the WSF of awns and peduncle were used as the independent variables, with assignment of a different weight for the awn

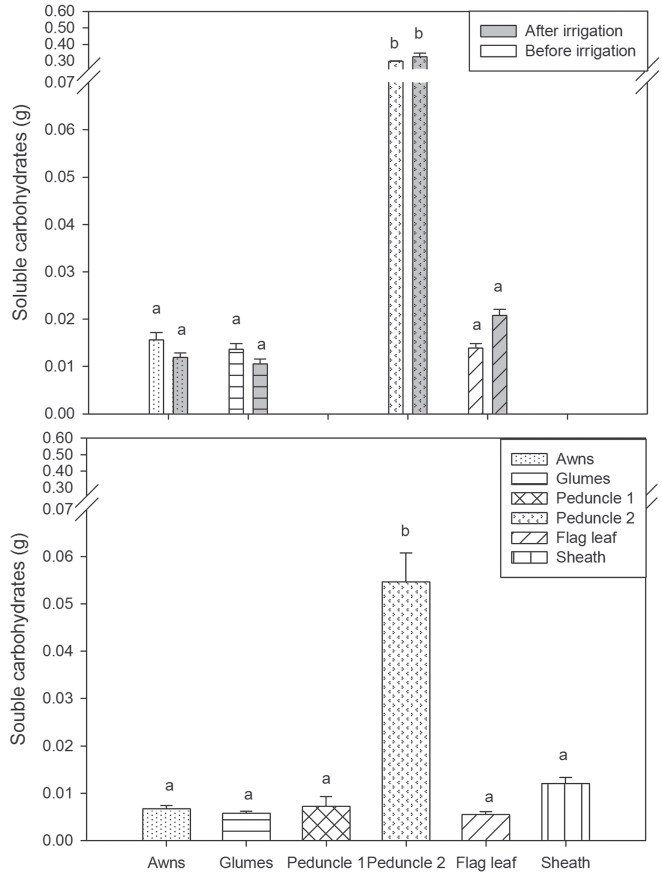

Fig. 2. Total amount of soluble carbohydrates in 2012 (upper panel) and 2013 (lower panel) per whole organ in the awns, glumes, peduncle 1 (upper section of the peduncle), peduncle 2 (lower section of the peduncle), flag leaf, and sheath blades around mid-grain filling. Each bar represents the mean values plus standard error of the five genotypes before irrigation and after irrigation (2012) and six (2013) genotypes with three replications per genotype. The total amount of soluble carbohydrates in peduncle 1 in 2012 was calculated with the full stem weight. Mean values with different superscript letters are significantly different according to Tukey's HSD test (P<0.05).

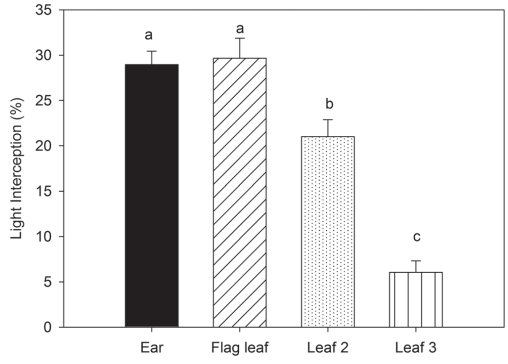

Fig. 3. Light intercepted at different strata of the canopy: the base of the ear, flag leaf, penultimate leaf (leaf 2), and third leaf (leaf 3) around 1 week after flowering. Each bar represents the mean values±SE of the six genotypes and the three replications per genotype. Mean values with different superscript letters are significantly different according to Tukey's HSD test (P<0.05).

Table 2. *Mean values and ANOVA of stable carbon isotope composition ($\delta^{13}C$) of DM and the WSF of different plant parts sampled at mid-grain filling (before and after irrigation) plus mature kernels*

Each value is the mean±SE of three replications. Mean values across plant tissues with different superscripted letters are significantly different according to Tukey's HSD test (P<0.05). Values with different superscript letters between brackets are significantly different between fractions and sampling dates analysed within a given organ according to Tukey's HSD test (P<0.05).

	Before irrigation		After irrigation
	$\delta^{13}C$ DM (‰)	$\delta^{13}C$ WSF (‰)	$\delta^{13}C$ WSF (‰)
Plant tissue			
Flag leaf	$-28.1\pm0.1^{a(b)}$	$-29.7\pm0.1^{a(a)}$	$-30.1\pm0.1^{a(a)}$
Peduncle	$-25.9\pm0.1^{c(c)}$	$-26.9\pm0.1^{b(b)}$	$-28.2\pm0.1^{b(a)}$
Glumes	$-26.2\pm0.1^{b(b)}$	$-26.5\pm0.1^{c(a)}$	
Awns	$-25.5\pm0.1^{d(a)}$	$-25.4\pm0.1^{d(a)}$	$-26.1\pm0.1^{c(a)}$
Grains	-26.3 ± 0.1^{b}		
Level of significance			
Genotype	**	NS	**
Organ	***	***	***
G × O	NS	NS	NS

***P<0.001; **P<0.01; *P<0.05; NS, not significant.

and peduncle $\delta^{13}C$ depending on the water status accounted for by the $\delta^{13}C_{grains}$. Thus, the $\delta^{13}C_{awns}$ had a relative contribution of 90% ($\delta^{13}C_{awns}\times0.90$) and the peduncle 10% ($\delta^{13}C_{peduncle}\times0.10$) towards the $\delta^{13}C_{grain}$, when the $\delta^{13}C_{grain}$ values were between –25.2 and –25.8‰. Conversely, the relative contribution of the awns was 58% ($\delta^{13}C_{awns}\times0.58$) and the peduncle 42% ($\delta^{13}C_{peduncle}\times0.42$) when $\delta^{13}C_{grain}$ values were between –26.4 and –27.0‰. In such a way, a linear fit with a slope of 1 and origin to zero was achieved (R^2=0.73, P<0.001) (Fig. 4).

The same approach was developed to assess the maximum relative contribution of the $\delta^{13}C_{flag}$ to grain filling. In such a way, a combination of the WSF $\delta^{13}C_{flag}$ and $\delta^{13}C_{awns}$ was used as an independent variable and $\delta^{13}C_{grain}$ as a dependent variable. Thus, the estimated contribution of the flag leaf was 18% ($\delta^{13}C_{flag}\times0.18$) and the awns 82% ($\delta^{13}C_{awns}\times0.82$) when the $\delta^{13}C_{grain}$ values were between –26.4 and –27.0‰. By contrast, the relative contribution of the awns was 97% ($\delta^{13}C_{awns}\times0.97$) and the flag leaves 3% ($\delta^{13}C_{flag}\times0.03$) when the $\delta^{13}C_{grain}$ values were between –25.2 and –25.8‰. As before, a linear fit with a slope of 1 and origin to zero was achieved (R^2=0.69, P<0.001) (Fig. 5).

The $\delta^{13}C$ of the CO_2 respired by the flag leaf and the ear was higher (^{13}C enriched) than the $\delta^{13}C$ in the WSF of all the organs studied (Fig. 6). In fact, the $\delta^{13}C_{flag}$ of the WSF exhibited values that were far more depleted than the $\delta^{13}C$ of the CO_2 respired by the flag leaf. On the other hand, the $\delta^{13}C_{glumes}$ and $\delta^{13}C_{awns}$ of the WSF and the $\delta^{13}C_{grains}$ showed values only slightly more depleted than the $\delta^{13}C$ of the CO_2 respired by the ear.

Discussion

Our study proposes a non-intrusive methodology to quantify the relative contribution of different organs to grain filling. The approach was based on the constitutive differences in natural abundance of carbon isotopic composition ($\delta^{13}C$) of assimilates from the different photosynthetic organs active during grain filling. This method aimed to compare the $\delta^{13}C$ of these assimilates with the $\delta^{13}C$ of mature kernels (Fig. 7). Since the method was applied in intact (i.e. non-manipulated) plants, the results were not biased by compensatory mechanisms. Moreover, and compared with pulse-chasing approaches, this is a relatively low-cost method that may help to elucidate the relative photosynthetic contribution to grain filling of different plant organs. Further application of this methodology into breeding programmes could be considered when selection for 'high' spike photosynthesis is desirable (Parry *et al.*, 2011).

In order to develop a feasible technique for breeding and keeping a balance between economy and accuracy, only a few photosynthetic organs were considered. From the available literature, there is evidence that the flag leaf blade and the ear are considered the main photosynthetic organs that contribute to grain filling (Evans *et al.*, 1975; Araus and Tapia, 1987; Araus *et al.*, 1993; Bort *et al.*, 1994; Tambussi *et al.*, 2007; Maydup *et al.*, 2010). Thus, the awns and glumes, which are the two main photosynthetic parts of the ear (Bort *et al.*, 1994) were analysed separately. In addition, the $\delta^{13}C$ of the assimilates was also analysed in the peduncle because this organ represents the pathway through which the current shoot assimilates (i.e. produced by the different shoot organs during grain filling, including the blades and sheaths of the flag and the lower leaves) plus the pre-anthesis reserves (assimilates accumulated before flowering) are mobilized towards growing kernels (Gebbing *et al.*, 1999). Because the use of the pre-anthesis reserves is reported to

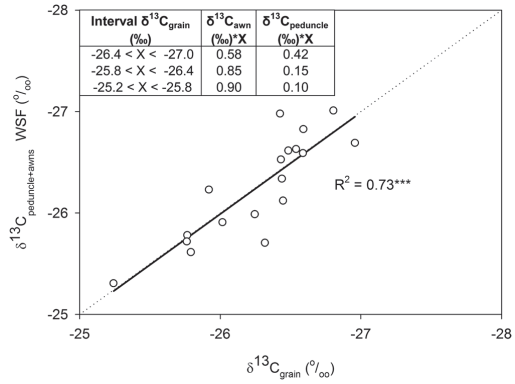

Fig. 4. Linear regression of the relationship between the stable carbon isotope composition in mature grains ($\delta^{13}C_{grains}$) and the combination of $\delta^{13}C$ from awns and the peduncle ($\delta^{13}C_{awns}+\delta^{13}C_{peduncle}$) in the WSF. The individual values of $\delta^{13}C_{awn}$ and $\delta^{13}C_{peduncle}$ used in the linear regression were the average of the $\delta^{13}C$ in the WSF before and after irrigation. The six genotypes with three replications per genotype were considered, accounting for a total of 18 plots. For each plot, the relative weight assigned to the $\delta^{13}C$ of each of the two organs depended on the water status of the plot assessed by its $\delta^{13}C_{grains}$ (see inset). Level of significance: ***P<0.001.

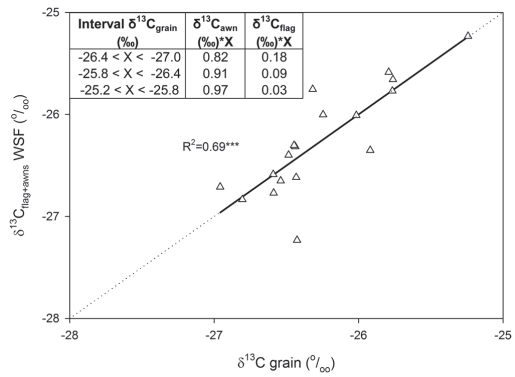

Fig. 5. Linear regression of the relationship between stable carbon isotope composition in mature grains ($\delta^{13}C_{grains}$) and the combination of the $\delta^{13}C$ from the flag leaf blade and the awns ($\delta^{13}C_{flag}+\delta^{13}C_{awns}$) in the WSF. The individual values of $\delta^{13}C_{awn}$ and $\delta^{13}C_{flag}$ used in the linear regression were the average of the $\delta^{13}C$ in the WSF before and after irrigation. The six genotypes with three replications per genotype were considered. For each plot, the relative weight assigned to the $\delta^{13}C$ of each of the two organs depended on the water status of the plot assessed by its $\delta^{13}C_{grains}$ (see inset). Level of significance: ***P<0.001.

take place during the first the half of grain filling (Wardlaw and Willenbrink, 1994; Gebbing *et al.*, 1999; Zhou *et al.*, 2009), assimilates present in the peduncle at the time that samples were collected in our study may integrate the potential contribution of such reserves. Therefore, the $\delta^{13}C$ in the peduncle informs us about the relative contribution of the entire culm to the grains (see Fig. 7). In any case, the potential contribution of pre-anthesis reserves to growing kernels seems at first small because the plants were grown under good agronomical conditions and so the photosynthetic capacity of the plants during grain filling exceeds the sink demand of growing grains (Slafer and Andrade, 1991; Bingham *et al.*, 2007; Dreccer *et al.*, 2009).

A basic point of our approach was that water-soluble organic matter is a proper indicator for newly produced assimilates, which agrees with available literature (Brandes *et al.*, 2006; Gessler *et al.*, 2009*b*). An additional requirement of our approach was that the $\delta^{13}C$ signature in the sink was the direct consequence of the $\delta^{13}C$ of assimilates produced by photosynthetic organs. Post-carboxylation fractionation effects in the $\delta^{13}C$ of the newly assimilated compounds in photosynthetic organs and further fractionation during their remobilization (i.e. phloem loading, unloading, or transport) to heterotrophic tissues have been reported mostly in tree species (Damesin and Lelarge, 2003; Scartazza *et al.*, 2004; Brandes *et al.*, 2006; Bowling *et al.*, 2008; Gessler *et al.*, 2009*a*) and other woody species (Tcherkez *et al.*, 2004; Badeck *et al.*, 2005). Nevertheless, for herbaceous plants, such post-photosynthetic fractionation appears less evident. Indeed, a lack of a clear diel variation in $\delta^{13}C$ in the organic WSF has been reported in sunflower (Ghashghaie *et al.*, 2001) and wheat (Kodama *et al.*, 2011). In the same sense, Yoneyama *et al.* (1997) studied post-photosynthetic fractionation of stable carbon isotopes between plant organs in different species. In their study, sugars in flag leaf blades, petioles, major veins, and phloem (which transport assimilates from source to sink tissues) of wheat were compared. Non-significant differences were found between phloem exudates (–29.5‰), sugars, and organic acids (–29.4‰) in leaf blades during grain filling. Similarly, Badeck *et al.* (2005) did not show consistent isotopic differences in the $\delta^{13}C$ of sugars in the leaf blades, petioles, and major phloem veins of French bean concluding that fractionation during assimilate transport, leading to preferential export of heavy carbon isotopes from photosynthesizing leaves, cannot be proven. For herbaceous plants at least, the existence of fractionation during assimilate transport from leaves to sink tissues could not be confirmed from these results. In addition, Gessler *et al.* (2009*b*) did not find differences in $\delta^{13}C$ composition of the phloem compared with the WSF and the assimilates of the leaf in *Ricinus communis*. Moreover, in our study, the CO_2 respired in the different organs was ^{13}C enriched compared with the corresponding soluble fractions (Fig. 6). This pattern of enrichment of the respired CO_2 has been reported before in different plants (Klumpp *et al.*, 2005; Ocheltree and Marshall, 2004; Gessler *et al.*, 2009*b*), including wheat (Kodama *et al.*, 2011), and does not support an enrichment of the remaining assimilates that may eventually be translocated to the sink tissues. Such differences between the $\delta^{13}C$ of the WSF (more negative) and the respired CO_2 (less negative) were more evident in the leaves compared with other parts of the plants, which agrees with previous studies (Ocheltree and Marshall, 2004; Klumpp *et al.*, 2005).

In our study, the $\delta^{13}C$ of the flag leaf was lower (more negative) than that of the ear parts, whereas the $\delta^{13}C$ of mature kernels exhibited values in between. Previous studies in durum wheat (Araus *et al.*, 1993) and other cereals

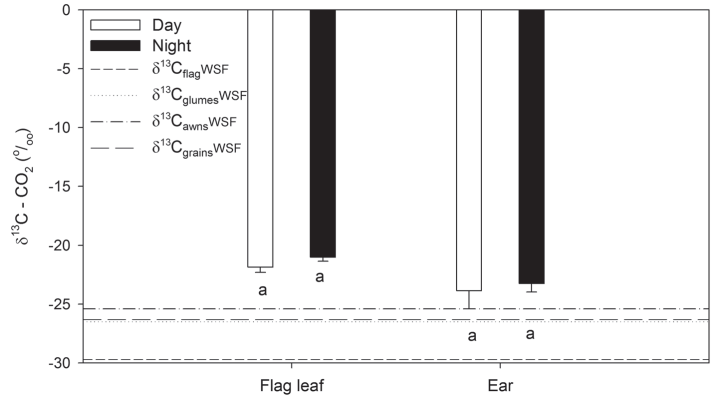

Fig. 6. Carbon isotope composition ($\delta^{13}C$) of the CO_2 respired by the flag leaf and the spike at mid-grain filling during the day and night (filled and open bars) compared with the $\delta^{13}C$ of the WSF in the flag leaf, glumes, awns, and grains (dashed horizontal lines). Each bar represents the mean respiration. The $\delta^{13}C$ values are means±SE of the six genotypes with three replicates per genotype. Mean values with different superscript letters are significantly different between day and night according to Tukey's HSD test (P<0.05).

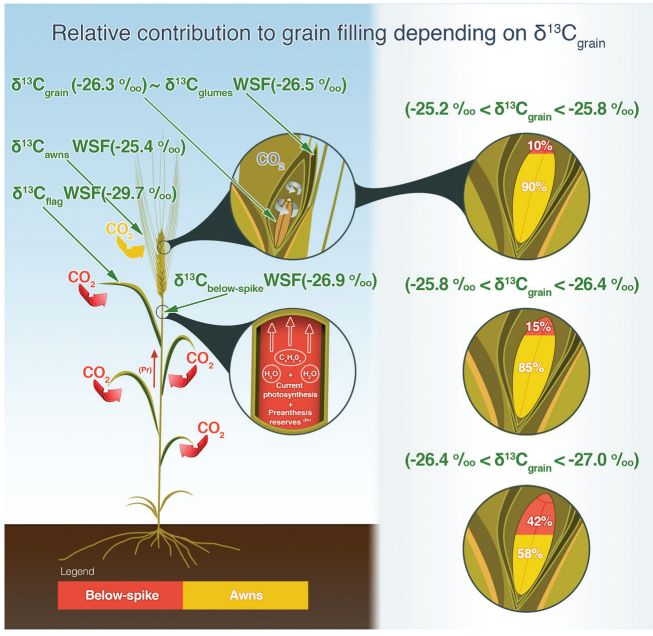

Fig. 7. Illustration of a wheat plant showing the relative photosynthetic contributions of the ear and shoot to grain filling. The below-spike integrates the $\delta^{13}C$ of the assimilates produced by the different photosynthetic organs of the shoot plus the stem reserves. The $\delta^{13}C_{awns}$, $\delta^{13}C_{glumes}$, and $\delta^{13}C_{below\text{-}spike}$ represent the carbon isotopic composition of the WSF in the awns, glumes, and below-spike, respectively. $\delta^{13}C_{grain}$ represents the carbon isotopic composition of mature kernels. (This figure is available in colour at *JXB* online.)

(Hubick and Farquhar, 1989; Araus *et al.*, 1992) have found similar patterns of lower $\delta^{13}C$ in the DM and the WSF of the flag leaf in comparison with the different ear parts, while the mature kernels exhibited values between them but closer to the ear parts. Differences in organ permeability to atmospheric CO_2 between photosynthetic organs probably explain the constitutive differences in $\delta^{13}C$ of the ear compared with the flag leaf (Farquhar *et al.*, 1989; Araus *et al.*, 1993). The fact that the $\delta^{13}C$ values in the WSF of the awns are far closer to the $\delta^{13}C$ of mature kernels than the WSF the $\delta^{13}C$ of the flag leaf supports the idea that in our study the ear has a more important role in providing assimilates during grain filling

than the flag leaf. On the other hand, the values of $\delta^{13}C$ in the peduncle were far higher (less negative) than those in the flag leaf, and closer (even when still more negative) to the $\delta^{13}C$ of the kernel. These results provide empirical evidence that the contribution of the flag leaf to the growing grains is minor compared with the rest of the shoot (including pre-anthesis reserves).

Our results showed that the contribution of the ear represented on average about 70% of the total assimilates contributing to grain filling (Fig. 4), while the role of the flag leaf blade was markedly smaller, with an average contribution of 10% (Fig. 5). Some evidence in the past has shown that only 49% of carbon assimilated by the flag leaf moves to the grain in comparison with 80% of the ear-derived photosynthates (Carr and Wardlaw, 1965). In an experiment carried out by Aranjuelo *et al.* (2011) in durum wheat, the C fixed by the flag leaf during the beginning of post-anthesis was studied using ^{13}C labelling. In this study, only a small amount of the soluble sugars coming from the C fixed by the leaf arrived at the ear, and the rest was stored as structural C compounds and starch and then respired. This study also concluded that the C synthesized in the ear was used for grain filling.

The potential contribution of the ear during grain filling is also supported by other indirect evidence. For example, the calculated total CO_2 fixed by the ear (including the respiratory losses) was comparable to that of the flag leaf blade and of similar magnitude to the total kernel weight per spike attained at maturity (Fig. 1B) from heading to maturity. In addition, the total WSCs per whole organ at mid-grain filling, which represents the potential amount of assimilates available in this organ, also supports this assumption. Thus, the WSC values were similar in the awns, glumes, flag leaf blades (Fig. 2, upper panel) and peduncle 1 and sheaths (Fig. 2, lower panel) despite the fact that these values were approximately one sixth of the level recorded in peduncle 2 (Fig. 2, lower panel). In this sense, grain filling may be limited by the sink rather than the source in wheat (Slafer and Savin, 1994), especially under good agronomical conditions, and therefore only the assimilates from the upper part of the plant are needed to fill the grains.

Moreover, the percentage of incoming light intercepted by the ear was similar to that captured by the flag leaf blade (plus the peduncle), whereas light absorption by the rest of the shoot still accounted for about 40% of the total incoming light (Fig. 3). These results indicate that the ear may have a photosynthetic contribution during grain filling that is at least similar to that of the flag leaf, with the additional advantage that the structures of the ear are physically closer than the flag leaf to the growing kernels (Evans *et al.*, 1975). They also provide indirect evidence supporting the fact that the flag leaf blade is not the only source of assimilates from the shoot. In a study performed in durum wheat under well-watered conditions, the photosynthetic rate of the whole ear correlated much better with GY than the photosynthetic rate of the whole flag leaf blade (Abbad *et al.*, 2004). On the other hand, the relative contribution of the $\delta^{13}C$ in the awns against the $\delta^{13}C$ of the stem (peduncle) varied depending on the water status (see Fig. 7). The results indicate that the awns had a higher contribution to filling grains compared with the stem, especially when the water status was less optimal (i.e. less negative $\delta^{13}C_{grains}$). Indeed, it has been reported that the ear is a photosynthetic organ better adapted than the flag leaf to water stress (Tambussi *et al.*, 2005). In a study carried out by Motzo and Giunta (2002) in durum wheat under Mediterranean conditions, it was concluded that the presence of awns increased the average GY by 10–16%. The positive role of awns may be even higher under drought stress. A study performed by Evans *et al.* (1972) using $^{14}CO_2$ labelling revealed that the presence of awns doubled the net photosynthesis rate in the ear, and the proportion of assimilate contributed by ear photosynthesis to grain filling was greater in awned ears compared with awnless ears under drought conditions.

Our results indicated that total shoot photosynthesis (i.e. combining the contribution of the different leaves plus the peduncle together with the pre-anthesis stem reserves) represents on average 22% of total assimilates going to the grain, and up to 42% of the assimilates during grain filling when water conditions were the most optimal (and thus $\delta^{13}C_{grains}$ the most negative). In addition, in order to assess which contribution of assimilates of the peduncle was actually due to the flag leaf, the maximum relative contribution of the $\delta^{13}C$ in the flag leaf compared with the awns was analysed (Fig. 5). The maximum relative contribution achieved by the flag leaf was 18% (when the water conditions were the most optimal and thus the $\delta^{13}C_{grains}$ the most negative). In addition, from Fig. 5, the relative contribution of the flag leaf blade appeared to be five times lower than that of the awns when water conditions were the most optimal (and thus the $\delta^{13}C_{grains}$ was the most negative). Extrapolating this proportion to Fig. 4, the contribution of the flag leaf with respect to the awns was 13%. If this calculation is applied to all three water conditions, the contribution of the flag leaf with respect to the awns ranged from 3 to 13%, from less optimal to most optimal conditions, respectively. In summary, this indicates that the flag leaf blade contributes on average only 8% of grain C and that the proportion changes with the degree of water stress (experienced in this study). Moreover, the proportion of grain C coming from the below-spike photosynthetic organs other than the flag leaf blade also decreases as water stress increases. Moreover, genotypes showing higher ear contributions do not necessarily reflect higher GY. In fact, water stress (assessed by $\delta^{13}C_{grains}$) may cause a decrease in GY, and thus an increase in the relative contribution of the ear to filling grains in comparison with non-ear organs (Tambussi *et al.*, 2007).

Furthermore, the glumes, which are photosynthetically active, are believed to be a significant source of assimilates for grain filling in wheat and other cereals (Araus *et al.*, 1993; Bort *et al.*, 1994). Hence, the importance of the ear's contribution to grain filling may thus be underestimated because, in the approach presented here, the glumes were not included (Fig. 7). The glumes mainly refix CO_2 (Bort *et al.*, 1996). In a study performed by Gebbing and Schnyder (2001) with labelling of the atmospheric CO_2 surrounding the ear, the view was supported that the CO_2 used for glume photosynthesis was derived mainly from CO_2 respired by the grains.

In addition, this view is reinforced by our results where the $\delta^{13}C$ in the glumes and the grains was not significantly different (Table 2). In fact, no discrimination occurs during reassimilation of CO_2 respired by the grain if the ear parts are completely gas tight (Farquhar *et al.*, 1989). These findings suggest that the ear could potentially contribute more to grain filling as was initially postulated in the approach.

Summarizing, it is not only the flag leaf that plays an important role in grain filling, as has traditionally been considered (Evans *et al.*, 1975; Araus and Tapia, 1987); the ear is also an essential organ during grain development. In accordance with the results obtained in this study, even under good agronomical conditions, the ear may be more important than the flag leaf during grain filling. Such a conclusion is also supported by the results of WSC in the whole organs, with the flag leaf blades and sheaths showing similar values to the awns and glumes as well as the similar photosynthetic contribution per whole organ recorded during the reproductive period by the ears and flag leaf blades. Whereas awns may be the organ of the ear that is pre-eminent in fixing atmospheric CO_2 (Motzo and Giunta, 2002), the glumes may also play a major photosynthetic role in reassimilating CO_2 respired by the ear.

Previous studies using a simplified version of the $\delta^{13}C$ approach also support a very limited role for the flag leaf blade in durum wheat under both moisture stresses and low nitrogen conditions (Sanchez-Bragado *et al.*, 2014). Our results are not in conflict with a basic role of the flag and the lower leaves in providing assimilates to the growth and development of the reproductive sink; specifically, shoot photosynthesis may determine the number of fertile florets and even further, the number of kernels and their potential size (Slafer and Savin, 1994). In addition, the flag leaf also plays an important role as a source of nitrogen that is later remobilized to the grain (Bahrani and Joo, 2010).

By analysing the $\delta^{13}C$ of the WSF at the peduncle, the photosynthetic contribution of the complete shoot to growing kernels has been assessed. Therefore, our methodological approach avoids the inherent limitation of not taking into account the potential contribution of other organs such as the flag leaf sheath and the peduncle, as well as the lower parts of the shoot and the pre-anthesis reserves.

The main purpose of our study was to estimate the relative organ contribution to grain filling using a non-intrusive and relatively low-cost approach (three $\delta^{13}C$ analyses per plot). While such an approach may potentially be deployed as a phenotyping tool, the relative contribution of each organ to grain filling is probably strongly affected by growing conditions. Therefore, for breeding, care should be taken to assess all genotypes under similar growing conditions, avoiding as much as possible spatial (across the trial) variability in the level of soil moisture.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Mean values of the stable carbon isotope composition in the flag leaf ($\delta^{13}C$flag), peduncle ($\delta^{13}C$peduncle), glumes ($\delta^{13}C$glumes), awns ($\delta^{13}C$awns) and mature kernels ($\delta^{13}C$grains) of the six genotypes of bread wheat.

Supplementary Fig. S1. Polynomial quadratic regression of the relationship between the stable carbon isotope compositions ($\delta^{13}C$) of mature grains and grain yield (GY).

Acknowledgements

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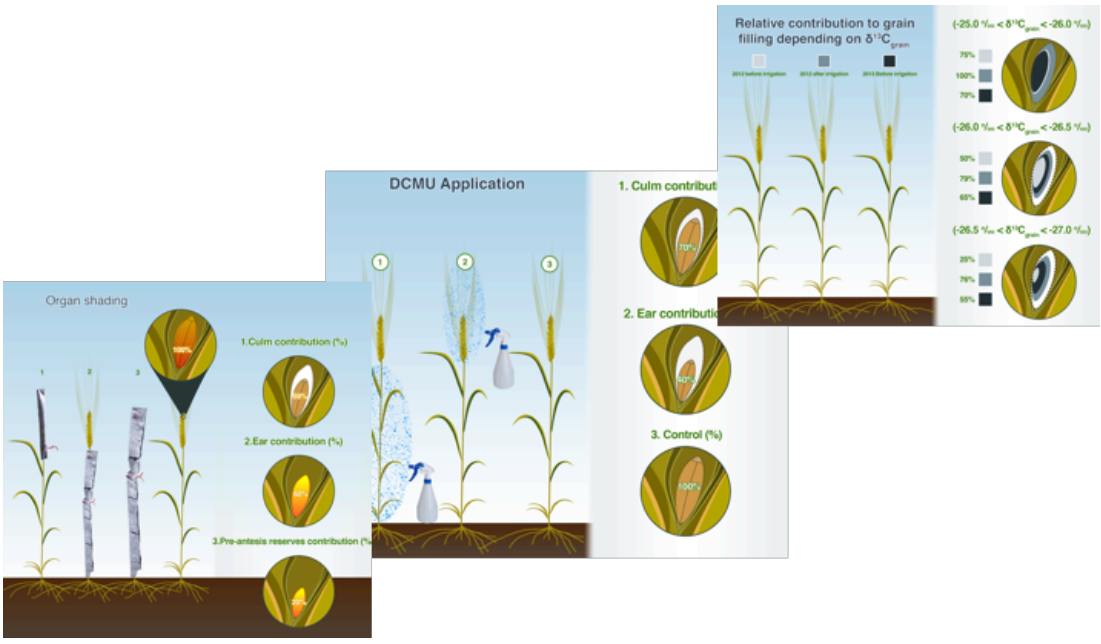

CAPITOL 3

Contribució fotosintètica de l'espiga a l'ompliment del gra: una comparació de diferents metodologies per la seva avaluació

CHAPTER 3

Photosynthetic contribution of the ear to grain filling in wheat: a comparison of different methodologies for evaluation

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RESUM CAPÍTOL 3

La tija, (sobretot la fulla bandera) i l'espiga es creu que juguen un paper molt important alhora d'aportar assimilats durant l'ompliment del gra en el blat. Ara bé, la qualitat dels resultats obtinguts en estudis del passat depèn de la metodologia utilitzada. En el nostre estudi compararem 3 metodologies diferents per tal d'avaluar la contribució relativa de la tija (incloent tots els òrgans fotosintètics ubicats per sota l'espiga) i de l'espiga a l'ompliment del gra. Las dues primeres consisteixen en l'aplicació de tractaments que inhibeixen fotosíntesi, mitjançant la utilització de l'herbicida DCMU (primera) i l'ombrejat dels òrgans (segona). La tercera metodologia és no intrusiva i es basa en comparar la composició isotòpica del carboni ($\delta^{13}C$) dels grans madurs amb la $\delta^{13}C$ de la fracció soluble de l'aigua del peduncle i de les arestes. L'experiment es va fer en línies avançades de blat del CIMMYT cultivades sota bones condicions agronòmiques. Aquesta aproximació metodològica basada en la $\delta^{13}C$ indica una contribució fotosintètica de l'espiga més elevada que la de la tija, mentre que l'aproximació de l'ombrejat assigna una contribució similar dels dos òrgans. L'aproximació del DCMU atribueix un paper més important a la tija tot i que l'aplicació de l'herbicida sembla afectar a l'espiga i com a conseqüència, al rendiment (pes dels grans). Per altra banda el DCMU i l'ombrejat poden causar efectes compensatoris que sobreestimen la contribució dels òrgans no afectats.

Aquest estudi pot ajudar a desenvolupar eines de fenotipejat més precises per identificar la contribució fotosintètica de l'espiga a l'ompliment del gra, i en conseqüència contribuir a l'increment del rendiment. De totes formes, l'estudi suggereix determinades modificacions que poden fer més eficient aquesta la metodologia d'avaluació.

Photosynthetic contribution of the ear to grain filling in wheat: a comparison of different methodologies for evaluation

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Summary

The culm (particularly the flag leaf) and the ear are believed to play a major role in providing assimilates for grain filling in wheat. However, the results obtained in the past varied depending on the methodology applied. Three different methodologies were compared that aimed to assess the relative contribution of the culm (photosynthetic organs below the ear) and the ear to grain filling. The first two consisted of applications of photosynthesis inhibition treatments, including the use of the herbicide DCMU and organ shading. The third was a non-intrusive method that compared the carbon isotope composition ($\delta^{13}C$) of mature kernels with the $\delta^{13}C$ of the water-soluble fraction of the peduncle and the awns. Several advanced CIMMYT lines were tested under good agronomic conditions. The $\delta^{13}C$ approach assigned a higher photosynthetic contribution to the ear than to the culm. The shading approach assigned a comparable contribution to the ear than to the culm. The DCMU approach assigned a greater role to the culm but herbicide application to the culm affected the ear, thus biasing the final grain weight. Moreover DCMU and shading approaches may cause compensatory effects which overestimated the contribution of unaffected organs. This study may help to develop precise phenotyping tools to identify physiological traits such as ear photosynthesis that could contribute towards increasing grain yield. However, some methodological considerations should be taken into account.

Key words: carbon isotope composition, culm, ear, grain filling, photosynthesis, wheat

Introduction

The prediction of future world population growth in the context of global (i.e. social and climate) change entails an urgent need to adapt food crops to ensure the security of future feed and food supply demands (Deryng *et al.*, 2011). In order to meet this challenge, annual cereal production will need to rise. Currently, wheat (*Triticum aestivum* L.) is an important crop worldwide, and it is considered the second main source of carbohydrate and protein in developing countries after rice (Food and Agriculture Organization of the United Nations, 2013).

Whereas breeding efforts in recent decades have been focused on improving crop adaptation to disease and abiotic stresses (Araus *et al.*, 2002; Reynolds and Borlaug, 2006), interest in raising the yield potential has grown only recently (Reynolds *et al.*, 2012). Although wheat breeding programs still achieve steady genetic gains (Manès *et al.*, 2012; Sharma *et al.*, 2012), there is a need to develop more efficient wheat breeding methodologies that complement existing (traditional) breeding techniques (Araus *et al.,* 2008). One of the breeding techniques proposed to increase yield potential and improve the adaptation to the increasing incidence of abiotic stresses (such as drought and heat) due to climate change is to select for higher ear photosynthesis (Reynolds *et al.*, 2005; Tambussi *et al.*, 2005a, 2007b; Araus *et al.*, 2008; Parry *et al.*, 2011). In fact, ear photosynthesis has been suggested as an important trait in the conceptual model for yield potential (Reynolds *et al.*, 2011). Hence, ear photosynthesis is thought to play an important role in terms of the source of photoassimilates during grain filling, not only under drought, but also under good agronomical conditions (Araus *et al.*, 1993; Bort *et al.*, 1994; Abbad *et al.*, 2004; Tambussi *et al.*, 2005a, 2007b; Maydup *et al.*, 2010; Sanchez-Bragado *et al.*, 2014b). Although under good agronomical conditions the actual photosynthetic source is often in excess of the sink (Slafer and Savin, 1994; Borrás *et al.*, 2004), recent evidence indicates that limitations to the source (Álvaro *et al.*, 2008) rather than the sink (Slafer *et al.*, 1999) have been emerging in modern cultivars of wheat. In addition, it is widely reported that different fungal diseases may affect leaves (Robert *et al.*, 2005) more than ears (Tiedemann and Firsching, 2000). Therefore, in conditions where leaf photosynthesis is limited, assessing the photosynthetic contribution of the ear to grain yield may be relevant.

Although the importance of the contribution of ear photosynthesis to final grain weight has been widely studied (Araus *et al.*, 1993; Bort *et al.*, 1994; Tambussi *et al.*, 2005a, 2007b; Maydup *et al.*, 2010; Saeidi *et al.*, 2012), its actual percentage contribution to grain filling is not completely understood (Tambussi *et al.*, 2007a). At present it is well known that assimilates transported to the grain during grain filling in C_3 cereals are mainly provided by three sources: i) flag leaf (blade and sheath) photosynthesis (Evans *et al.*, 1975); ii) pre-anthesis reserves (Gebbing and Schnyder, 1999); and iii) ear photosynthesis (Tambussi *et al.*, 2007b). However, the proportion in terms of the contribution of assimilates to grain filling of each of the three mentioned sources still remains imperfectly known due to methodological constraints (Evans *et al.*, 1975; Nicolas and Turner, 1993; Tambussi *et al.*, 2007a). Such methodological limitations are closely related to the quantification and separation of the ear photosynthesis from assimilates that come from the leaves and are retranslocated during grain filling. In point of fact, compared with the leaves, the photosynthetic contribution of ears has been less studied and still remains unclear, particularly under field conditions (Maydup *et al.*, 2014).

Thus, alternative approaches to solve such methodological constraints have been deployed to evaluate the ear contribution to grain filling (Borrás *et al.*, 2004; Maydup *et al.*, 2010). The most commonly used approaches include detachment (i.e. organ-specific) of some plant parts, such as stem defoliation at the anthesis stage (Ahmadi *et al.*, 2009); inhibition of photosynthesis based on shading (Aggarwal *et al.*, 1990; Araus *et al.*, 1993; Peralta *et al.*, 2011); application of herbicides (Maydup et al. 2010); or desiccant treatments (Blum *et al.*, 1983; Nicolas and Turner, 1993; Saeidi *et al.*, 2012). Nevertheless, these approaches do not exempt organs from being affected by physiological processes other than photosynthesis (Tambussi *et al.*, 2007*a*), such as respiration, ripening, etc. (Kriedemann, 1966) that hypothetically may bias the final grain weight. For example, shading treatments have been criticized because they might increase organ temperature affecting the final grain weight (Tambussi *et al.*, 2007a) or may contribute to the accumulation of ethylene (Cheng and Lur, 2008). However, in a study performed by Maydup *et al.* (2010), no significant differences in temperature were observed between control and shaded organs. Furthermore, an additional source of variation in growing grains

may be related to remobilization of stem reserves due to a decrease in photoassimilate production after anthesis (Chanishvili *et al.*, 2005). Nonetheless, such remobilization has been observed to begin only when the maximum fill rate of the grains cannot be maintained by the current photosynthesis (Bingham *et al.*, 2007; Slewinski, 2012). Likewise, the potential contribution of stem reserves during grain filling under good agronomical conditions seems to be low because the photosynthetic capacity of plants during grain filling exceeds the sink demand of growing grains (Slafer and Andrade, 1991; Dreccer *et al.*, 2009).

Therefore, use of the stable carbon isotope signature in its natural abundance ($\delta^{13}C$) may help to elucidate the relative contribution of the different photosynthetic organs with the added advantage of being a non-intrusive approach (Sanchez-Bragado *et al.*, 2014a,b). Moreover the novel approach using $\delta^{13}C$ in its natural abundance may help to avoid unwanted compensatory effects triggered by intrusive methods (Chanishvili *et al.*, 2005). However, this approach using $\delta^{13}C$ still needs to be investigated in more detail to be confident in it as a methodological tool.

The main objective of this work was to compare different experimental approaches aiming to assess the relative contribution of ear photosynthesis and the rest of the plant to grain filling. The study was performed in a set of high-yielding advanced lines of bread wheat from CIMMYT (International Maize and Wheat Improvement Center) growing under well-managed agronomic conditions. Three different techniques were used: inhibition of ear and culm photosynthesis through i) herbicide DCMU application, or ii) by shading each organ and iii) the analysis of the $\delta^{13}C$ of assimilates from different plant parts (awns and peduncle) as a criterion to assess in a non-disturbing manner the relative contribution of ear and culm photosynthesis to grain filling. In such a way the $\delta^{13}C$ of assimilates from the awns and peduncles were analysed around the mid stage of grain filling.

Materials and Methods

Germplasm used and experimental conditions

Six advanced bread wheat (*Triticum aestivum* L.) lines were selected on the basis of their similar phenology, high grain yield and biomass, from the CIMCOG (CIMMYT Mexico Core Germplasm) panel. The field experiments were conducted during the spring growing seasons of 2012 and 2013 at MEXPLAT (Mexican Phenotyping Platform) situated at CIMMYT's Experimental Station, Norman E. Borlaug (CENEB) in the Yaqui Valley, near Ciudad Obregón, Sonora, México (27°24' N, 109°56' W, 38 m asl), under fully irrigated conditions. The experimental design was a randomized lattice with three replications in 8.5 m long plots as explained elsewhere (Sanchez-Bragado *et al.,* 2014b). The experiment was sown on 9 December 2011 and 23 November 2012, and immediately irrigated to promote germination. The emergence dates were 16 and 2 December of 2011 and 2012, respectively. Environmental conditions during the growing seasons are detailed in Fig. 1. Harvesting was performed by machine on 15 May 2012 and manually on 6-7 May 2013, respectively, about 15-20 days after reaching physiological maturity.

Agronomic traits

For each plot, yield components were determined in approximately 5.7 m^2 using standard protocols (Pask et al., 2012). In addition, phenology was recorded throughout the cycle using the Zadocks scale (Zadoks et al., 1974).

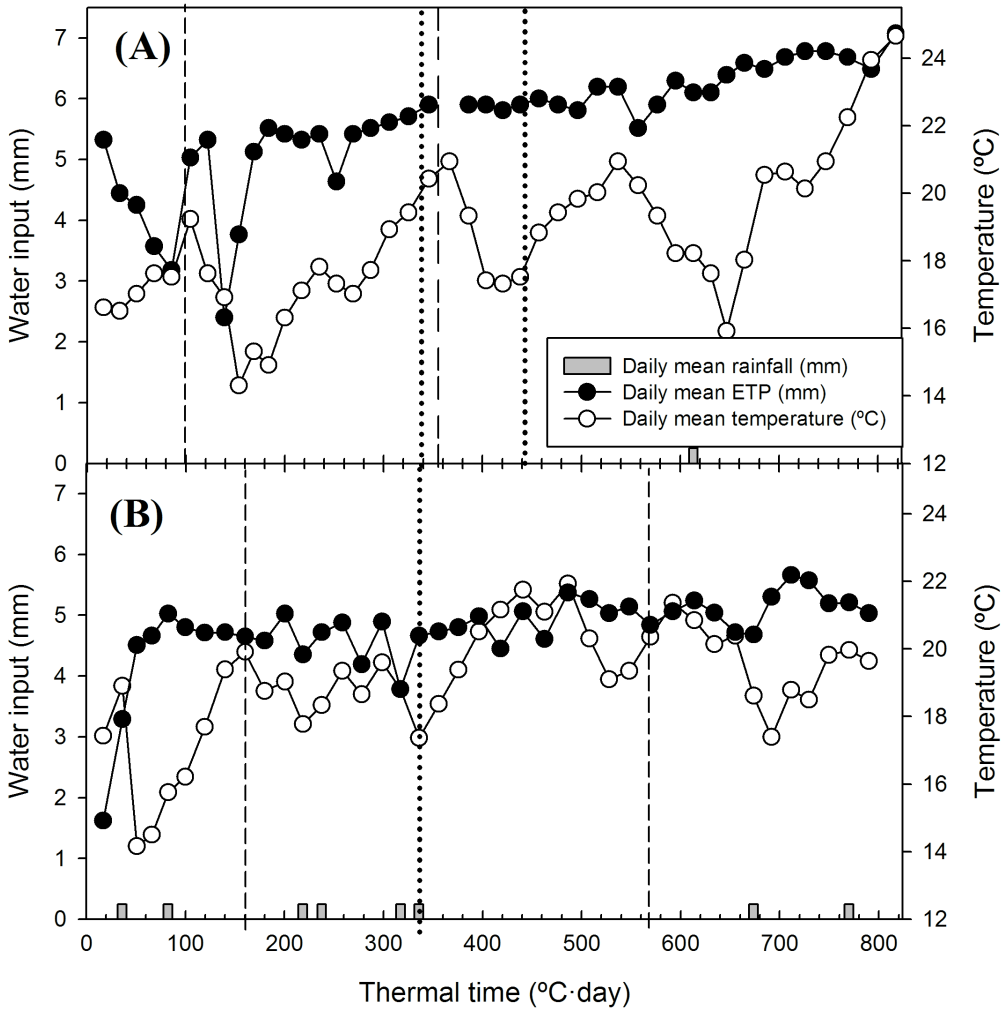

***Fig. 1**. Daily mean precipitation (mm), evapotranspiration (mm) and air temperature (ºC) during the growing season from flowering to physiological maturity expressed as thermal time (ºC·day) during (a) the 2012 crop season and (b) the 2013 crop season. Vertical dotted lines symbolize sampling dates and vertical dashed lines represent dates of irrigation.*

Leaf and ear photosynthesis and respiration

Photosynthetic and respiration rates of the flag leaf blade and the ear were measured during both seasons (2012 and 2013) as carbon uptake using a LI-6400XT portable gas exchange photosynthesis system (Li-COR, Lincoln, Nebraska, USA). Photosynthesis measurements were performed approximately two weeks after anthesis. The flag leaf photosynthetic assimilation rate (*A*) was estimated at a saturating PPFD of 1500 μmol $m^{-2}s^{-1}$ and 30ºC. Ear photosynthesis was measured using a hand-made chamber connected to the Li-6400XT as described elsewhere (Sanchez-Bragado *et al.*, 2014b). Dark respiration of the flag leaf and the ear were measured immediately after the photosynthetic measurements at a temperature of 30ºC.

Assimilates produced

The potential contribution of the two organs as a source of assimilates was assessed taking into account the instantaneous net photosynthesis plus the dark respiration (here defined as gross photosynthesis) of the whole organs multiplied by the duration of the daylight period with a saturating PPFD and the number of days from heading to organ senescence. For both seasons the active duration of the flag leaf and the ear was assessed periodically from heading to maturity. In the case of the flag leaf, chlorophyll content was measured once a week with a SPAD-502 Minolta chlorophyll meter (Spectrum Technologies Inc., Plainfield, IL, USA) in five flag leaves per plot. The active duration of the flag leaf was considered to end when SPAD values went below 20. For the ear, senescence was assessed visually and the active ear duration was considered to end when the peduncle changed colour. Further, the total amount of assimilates produced per organ in 2012 and 2013 was estimated from the accumulated gross photosynthesis from heading to maturity, assuming all the fixed C was converted into carbohydrates (CH_2O).

Incoming radiation and potential production

Incident and transmitted photosynthetically active radiation (PAR) was measured about one week after anthesis on clear days as close to solar noon as possible (from 11:00h to 14:00h), with a Linear PAR Ceptometer (AccuPAR LP-80, Decagon, Washington, USA). Different *strata* of the canopy were considered for the measurements of transmitted PAR: base of the ear and flag leaf blade (which

also included the peduncle). A single measurement was performed at each *stratum* in each of the three replicates. The light intercepted by each *stratum* was estimated from the PAR measured by adapting the equations described by Mullan and Pietragalla (2012). The integrated incoming radiation from heading to maturity (MJ_{H-M}) was calculated and divided by the number of ears per unit ground area. Thereafter, the (MJ_{H-M}) was multiplied by the light interception in the ear and flag leaf *stratums* in order to obtain the integrated incoming radiation in those *stratums*. Furthermore the potential production was calculated from the integrated incoming radiation in the ears and flag leaf assuming a photosynthetic efficiency (solar energy conversion efficiency) of 2.4% (Zhu *et al.*, 2008).

Inhibition of photosynthesis with DCMU

Five main tillers were randomly selected in each plot for the photosynthetic inhibition treatments in 2012. Seven days after anthesis, ears or culms (including leaf blades, sheaths and the peduncle) were sprayed with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) in order to inhibit photosynthesis. DCMU is a specific inhibitor of photosynthetic electron transport through photosystem II. The inhibition of photosynthesis was checked by measuring photosynthetic gas exchange 3-4 days after DCMU application. Subsequently, the carbon isotopic composition ($\delta^{13}C$) in mature grains of different treatments was analysed (see Carbon isotope analysis section).

Shading treatment

A total set of three main tillers was randomly selected in each plot for the shading treatment in 2013. Eight days after anthesis the ear and the culm (leaf blades, sheaths and the peduncle) were shaded. In the shaded culm treatment, the entire vegetative part of the plant was covered, enabling the ears to remain in full sunlight. Shading treatment consisted of wrapping each ear, culm or entire tiller with textile foil, such that light transmitted is below the light compensation point while being gas permeable to avoid ethylene accumulation (Molero *et al.*, 2014).

At maturity, the weight and number of grains per ear in the different treatment (including DCMU and shading) and control groups were measured in order to estimate the photosynthetic contribution of the ear and the culm to grain filling calculated based on the grain weight per ear (GW_{ear}) of the treatments relative to the control (Maydup *et al.*, 2010).

Carbon isotope analysis

Carbon isotope composition was analysed in plants around mid-grain filling. Sampling was performed twice in 2012 and once in 2013. In 2012, samples were collected 17 and 24 days after anthesis (DAA), before irrigation (named as BI) and after irrigation (named as AI), respectively, and in 2013, 18 DAA. In the growth chamber (see below), samples were collected 8 weeks after sowing. For each sampling in the field, ten representative ears, flag leaves, and peduncles per plot were harvested and cleaned in the field trials. In the 2012 crop season, a full set of BI samples were collected and immediately frozen with liquid nitrogen after sampling. For AI samples collected in 2012, only half of the sample set was frozen with liquid nitrogen immediately after sampling (named 'frozen'). The other half of the sample set was stored at room temperature in paper bags for approximately 3 hours after sampling (named 'not-frozen'). All samples from the 2012 field trial (samples frozen with liquid nitrogen and samples stored in paper bags) were finally stored at –20 ºC and then lyophilized for 48 h. For the 2013 crop season, samples were stored at room temperature for approximately 3 hours after sampling and subsequently oven dried at 70 ºC for 48 h. Once dried, the glumes, awns, flag leaves, and peduncles were separated, weighed, and finely ground.

The stable carbon isotope composition ($\delta^{13}C$) in the water-soluble fraction (WSF) of the peduncles, awns and leaves in the field trials (and only leaves in the growing chamber experiment) were analysed as described previously (Yousfi *et al.*, 2013). The $\delta^{13}C$ was also analysed in mature kernels. For $\delta^{13}C$ analysis, approximately 1 mg of each dry sample (100 µl for WSF) was weighed into tin capsules and measured with an elemental analyser coupled with an Isotope Ratio Mass Spectrometer (Delta C IRMS, ThermoFinnigan, Bremen, Germany) operating in continuous flow mode in order to determine the stable carbon ($^{13}C/^{12}C$) isotope ratios of the same samples. The $^{13}C/^{12}C$ ratios of plant material

were expressed in δ notation (Coplen 2008): $\delta^{13}C = (^{13}C/^{12}C)_{sample} / (^{13}C/^{12}C)_{standard} - 1$, where 'sample' refers to plant material and 'standard' to international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose and USGS 40 L-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB) with an analytical precision (SD) of 0.10‰. Isotopic analyses were carried out in the Scientific-Technical Services of the University of Barcelona, Spain.

Relative photosynthetic contribution to grain filling

The approach proposed here considers that the relative contribution of the awns and peduncle to grain filling varies as a result of water status and that it is reflected in the stable carbon isotope signature of mature grains (Araus *et al.*, 2003). Based on the approach developed and explained in detail previously (Sanchez-Bragado *et al.*, 2014b) it is expected that the $\delta^{13}C$ of the kernels will directly reflect the isotopic signal resulting from the combinations of the $\delta^{13}C$ of assimilates coming from different photosynthetic sources. Through the comparison within $\delta^{13}C$ in the WSF of the different organs and the $\delta^{13}C$ of mature kernels, it was possible to assess the relative contribution of the different photosynthetic organs to grain filling. This implies that the same slope and origin at zero needed to be found between the combined $\delta^{13}C$ of the peduncle and the awns and the $\delta^{13}C$ of the kernels. The approach was performed during the 2012 (BI and AI) and 2013 crop seasons.

Effect of sampling conditions on $\delta^{13}C$ of the water-soluble fraction

In order to reduce possible divergences in the $\delta^{13}C$ of the water-soluble fraction triggered by the different sampling and drying methods used during 2012 (BI and AI) and between the 2012 and 2013 seasons, a correction factor was calculated. Differences in the $\delta^{13}C$ of the water-soluble fraction (WSF) of samples collected AI (not-frozen) minus samples collected BI (frozen), compared to samples collected AI (frozen) minus samples collected BI (frozen), were calculated according to equation 1:

$$CF = [(AI\ not\ frozen - BI\ frozen)] - [(AI\ frozen - BI\ frozen)]] \quad (1)$$

Where, CF is the correction factor; AI not frozen, represents the samples collected after irrigation that were not frozen with liquid nitrogen; AI frozen, represents the samples collected after irrigation and frozen with liquid nitrogen immediately after sampling; BI frozen, represents the samples collected before irrigation and frozen with liquid nitrogen immediately after sampling.

The input parameters used for calculating the correction factor were: the $\delta^{13}C$ in the WSF of the peduncle, awns, and flag leaves collected in 2012. The correction factor was estimated to be on average, 0.4 ‰ (Table 1). In 2012 samples collected AI (not frozen) and samples collected in 2013 (which were oven-dried) were corrected with the CF obtained in equation 1. In both cases the $\delta^{13}C$ in the WSF was transformed by adding a constant CF of 0.4‰ to each individual value.

Experimental estimation of the effect of sampling conditions on $\delta^{13}C$ in the WSF

A modern Spanish durum wheat (*Triticum turgidum* var. *durum*) cultivar (Regallo) was grown in 3L pots (3 replicates) filled with sand (1 plant per pot). Plants were watered three times a week with Hoagland nutrient solution and were grown under controlled conditions in a growth chamber (Conviron E15, Controlled Environments Ltd., Winnipeg, Canada). Plants were supplied with a PPFD of about 400 μmol m^{-2} s^{-1} during the light period (14 hours). A constant relative humidity of 50 to 60% and a temperature of 23/17ºC during the light and dark periods, respectively, were also maintained. Three leaves of each plant were collected and divided longitudinally into two sections. One section was frozen with liquid nitrogen immediately after sampling and the other section (of the same leaf) was oven-dried 6 hours after sampling for 48 hours. Further leaf segments were finely ground. Subsequently, $\delta^{13}C$ in the WSF of leaf segments was analysed as previously mentioned (see the carbon isotope analysis section).

In order to confirm the existence of possible divergences in the $\delta^{13}C$ of the water- soluble fraction triggered by different sampling and drying methods, an additional correction factor was experimentally calculated in leaves obtained in the growing chamber experiment. The correction factor was calculated from the difference in $\delta^{13}C$ in the WSF between leaves oven-dried six hours after

***Table 1**. Mean values of $\delta^{13}C$ in the WSF of the peduncle minus mature kernels ($\delta^{13}CWSF_P$-$\delta^{13}C_G$), the awns minus mature kernels ($\delta^{13}CWSF_A$-$\delta^{13}C_G$) and the flag leaf minus mature kernels ($\delta^{13}C\ WSF_F$-$\delta^{13}C_G$) after irrigation during the 2012 crop season. Differences in the $\delta^{13}C$ in the WSF after irrigation (AI) minus before irrigation (BI) in the peduncle ($\delta^{13}C\ WSF_{AI-P}$ - $\delta^{13}C\ WSF_{BI-P}$), awns ($\delta^{13}C\ WSF_{AI-A}$ - $\delta^{13}C\ WSF_{BI-A}$) and flag leaf ($\delta^{13}C\ WSF_{AI-F}$ - $\delta^{13}C\ WSF_{BI-F}$) during the 2012 crop cycle are also shown. Mean values are divided into two categories (see Materials and Methods); samples frozen immediately after sampling (frozen) and samples not frozen immediately after sampling (not frozen). Analysis of variance (ANOVA) for the sampling method effect (P values on the right column). Mean values with different superscripted letters are significantly different according to the Tukey's honestly significant difference test ($P<0.05$). Each value represents eleven plots for not-frozen samples, seven plots for frozen samples.*

	not-frozen	**frozen**	**not-frozen/frozen**	***P***
$\delta^{13}C\ WSF_P$-$\delta^{13}C_G$	-2.10^a	-1.63^b	-0.47	0.02*
$\delta^{13}C\ WSF_A$-$\delta^{13}C_G$	-0.10^a	0.44^b	-0.54	0.049*
$\delta^{13}C\ WSF_F$-$\delta^{13}C_G$	-3.91^a	-3.64^a	-0.27	0.149^{ns}
Average	*-2.03*	*-1.61*	*-0.43*	
$\delta^{13}C\ WSF_{AI-P}$ - $\delta^{13}C\ WSF_{BI-P}$	-1.56^a	-0.91^b	-0.65	0.02*
$\delta^{13}C\ WSF_{AI-A}$ - $\delta^{13}C\ WSF_{BI-A}$	-0.74^a	-0.49^b	-0.25	0.25^{ns}
$\delta^{13}C\ WSF_{AI-F}$ - $\delta^{13}C\ WSF_{BI-F}$	-0.45^a	-0.14^a	-0.31	0.32^{ns}
Average	*-0.91*	*-0.51*	*-0.40*	

sampling and leaves frozen with liquid nitrogen and subsequently lyophilized. The correction factor was estimated to be on average 0.8 ‰ (Table S1).

Statistical analysis

One-way analysis of variance (ANOVA) using the general linear model was calculated in order to quantify the effects of genotype and organ interaction on the studied parameters. Genotype and organ were included as fixed factors including 3 blocks and 3 replicates per block. Means were compared by Tukey's HSD test. A bivariate correlation procedure was constructed to analyse the relationships between the measured traits. Statistical analyses were performed using the SPSS 21.0 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using the Sigma-Plot 10.0 program (SPSS Inc.).

Results

Contribution of the ear and the culm to grain filling: DCMU application

In order to estimate the relative contribution of the ear and the culm to filling grains, the photosynthesis of either ears or culms (which represent all the assimilation organs below the ear) was inhibited with DCMU (Table 2). Mean values of carbon isotope composition in mature grains of control plants ($\delta^{13}C_{grain}$) were higher (less negative $\delta^{13}C$) in comparison to the $\delta^{13}C_{grain}$ in DCMU-ear plants (ear photosynthesis inhibited) but similar to the $\delta^{13}C_{grain}$ in DCMU-culm plants (culm photosynthesis inhibited); however, grain weight per ear (GW_{ear}) and thousand kernel weight (TKW) in the DCMU-culm plants showed the lowest values (19.8 g and 1.29 g, respectively) compared to the control plants (44.3 g and 2.89 g, respectively) and the DCMU-ear treatment (31.6 g and 1.99 g, respectively), whereas the number of grains per ear (NG_{ear}) did not differ within the treatments and control plants. In addition, genotypic differences existed for $\delta^{13}C_{grain}$ and GW_{ear} and TGW, whereas genotype comparisons to treatment interactions were not significant.

Table 2. *Mean values of stable carbon isotope composition in mature grains ($\delta^{13}C_{grain}$), total grain weight per ear (GW_{ear}), the number of grains per ear (NG_{ear}) and thousand kernel weight (TKW) in control, DCMU-culm (inhibition of the whole culm photosynthesis) and DCMU-ear (inhibition of ear photosynthesis) plants. Analysis of variance (ANOVA) for the effect of genotype and treatment is shown. Mean values with different superscripted letters are significantly different according to the Tukey's honestly significant difference test (P<0.05). Each value represents six genotypes and three replications per genotype. Experiment performed in the 2012 crop season.*

Treatment	$\delta^{13}C_{grain}$ (‰)	NG_{ear}	GW_{ear} (g)	TKW (g)
DCMU culm	-26.0^{b}	65.7^{a}	1.29^{a}	19.8^{a}
DCMU ear	-26.7^{a}	62.7^{a}	1.99^{b}	31.6^{b}
control	-26.3^{b}	65.5^{a}	2.89^{c}	44.3^{c}
Level of significance				
Genotype (G)	0.000***	ns	0.000***	0.009***
Treatment (T)	0.008**	0.000***	0.009**	0.000***
GxT	ns	ns	ns	ns

Monitoring effects of DCMU on photosynthesis

In order to monitor the efficiency of the inhibition method with DCMU, the photosynthesis of the ear and the flag leaf blade (Table 3) was measured. As expected, when DCMU was applied to ears, the net ear photosynthesis was significantly inhibited (-11.45 $\mu mol \cdot m^{-2} \cdot s^{-1}$) compared to the control ears (9.95 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Concerning the flag leaf blade, net photosynthesis was not inhibited when DCMU was applied to the ears. Thus, net photosynthetic rates in the flag leaf blade showed similar values to the DCMU ear treatment (17.95 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and control (18.66 $\mu mol \cdot m^{-2} \cdot s^{-1}$); however, when DCMU was applied to the culms, net photosynthesis was not only inhibited in the stem, but also the photosynthesis of the ears was affected (3.82 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Table 3. *Mean values of ear and flag leaf blade photosynthesis expressed as the instantaneous net photosynthetic rate (Net photo.) and instantaneous dark respiration (Dark resp.) for the control and the two DCMU treatments. Analysis of variance (ANOVA) for the effect of genotype and treatment is shown. Mean values with different superscripted letters are significantly different according to the Tukey's honestly significant difference test (P<0.05). Each value represents six genotypes and three replications per genotype. Experiment performed in the 2012 crop season.*

	Flag leaf ($\mu mol \cdot m^{-2} \cdot s^{-1}$)		**Ear ($\mu mol \cdot m^{-2} \cdot s^{-1}$)**	
	Net photo.	**Dark resp.**	**Net photo.**	**Dark resp.**
DCMU culm	-1.81^{a}	-1.95^{b}	3.82^{b}	-16.69^{a}
DCMU ear	18.66^{b}	-3.05^{ab}	-11.45^{a}	-13.89^{a}
Control	17.95^{b}	-3.70^{a}	9.95c	-17.17^{a}
Level of significance				
Genotype (G)	ns	ns	0.028**	ns
Treatment (T)	0.000***	0.018*	0.000***	ns
GxT	ns	ns	ns	ns

Contribution of the ear and the culm to grain filling: shading treatment.

Mean values of NG_{ear} and TKW for shaded-culms and shaded-ears were both lower than control plants (Table 4). Moreover, mean values of GW_{ear} were similarly affected by shading the ears and the culms, and did not show significant differences. Both NG_{ear} and $GW_{ear,}$ exhibited genotypic effects, whereas only GW_{ear} showed significant genotype by environment interactions.

***Table 4**. Mean values in the set of six selected genotypes of total grain weight per ear (GW_{ear}), the number of grains per ear (NG_{ear}) and thousand kernel weight (TKW) in control, shaded-ear and shaded-culm plants. Analysis of variance (ANOVA) for the effect of genotype and treatment is shown. Mean values with different superscripted letters are significantly different according to the Tukey's honestly significant difference test (P<0.05). Experiment performed in the 2013 crop season.*

Treatment	NG_{ear}	GW_{ear} (g)	TKW (g)
Shaded ear	50.8^{a}	1.50^{a}	30.2^{b}
Shaded culm	55.3^{b}	1.51^{a}	27.3^{a}
Control	60.9^{c}	2.53^{b}	41.5^{c}
Level of significance			
Genotype (G)	0.000^{***}	0.000^{***}	0.000^{***}
Treatment (T)	0.000^{***}	0.000^{***}	0.000^{***}
GxT	ns	0.003^{*}	ns

Monitoring effects of shading treatments on photosynthesis

In order to monitor the efficiency of the shading method, the photosynthesis of the ear and the flag leaf blade (Table 5) was measured. Mean values of flag leaf blade photosynthesis under shaded ear treatment (16.15 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were not significantly different compared to control (17.89 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Whereas ear photosynthesis under shaded culm treatment (8.64 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was higher compared to control (6.44 $\mu mol \cdot m^{-2} \cdot s^{-1}$), dark respiration was lower under shaded culm treatment (-9.67 $\mu mol \cdot m^{-2} \cdot s^{-1}$) compared to control (-13.24 $\mu mol \cdot m^{-2} \cdot s^{-1}$). In addition, dark respiration in the ear was higher in control than shaded-culm plants (Table 5).

***Table 5**. Mean values of ear photosynthesis and flag leaf blade expressed as instantaneous net photosynthetic rate (Net Photo) and instantaneous dark respiration (Dark respiration) for control, shaded ear and shaded culm treatments. Analysis of variance (ANOVA) for the effect of genotype and treatment is shown. Mean values with different superscripted letters are significantly different according to the Tukey's honestly significant difference test (P<0.05). Each value represents six genotypes and three replications per genotype. Experiment performed in the 2013 crop season.*

	Flag leaf ($\mu mol \cdot m^{-2} \cdot s^{-1}$)		Ear ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	
	Net photo.	**Dark respiration**	**Net photo.**	**Dark respiration**
Shaded ear	16.15^{a}	-0.99^{a}	-	-
Shaded culm	-	-	8.64^{b}	-9.67^{b}
Control	17.89^{a}	-0.97^{a}	6.44^{a}	-13.24^{a}
Level of significance				
Genotype (G)	ns	ns	0.016^{*}	ns
Treatment (T)	ns	ns	0.012^{*}	0.000^{***}
GxT	ns	ns	ns	ns

Monitoring sampling procedure

In order to monitor the efficiency of the sampling procedure (samples frozen or not frozen), mean values of $\delta^{13}C$ in the WSF of the peduncle and awns minus the mean values of $\delta^{13}C$ in the mature kernels ($\delta^{13}CWSF_P$ - $\delta^{13}C_G$ and $\delta^{13}CWSF_A$ - $\delta^{13}C_G$, respectively) were compared in 2012 (Table 1). Differences within the $\delta^{13}C$ in the WSF of the peduncles and awns when compared to the grains were on average higher in the plots in which plants were not frozen immediately (-2.03‰) relative to frozen plants (-1.61‰). In addition, differences within the peduncles and awns sampled BI and AI in 2012 ($\delta^{13}C$ WSF_{AI-P} - $\delta^{13}C$ WSF_{BI-P}, and $\delta^{13}C$ WSF_{AI-A} - $\delta^{13}C$ WSF_{BI-A}, respectively), were calculated for frozen and not-frozen samples (Table 1). Thus, samples from plots that were not frozen exhibit greater differences within organs sampled BI and AI (-0.91‰) compared to the plots whose plants were frozen (0.51‰).

Photosynthetic contribution of the ear and the culm to grain filling: $\delta^{13}C$ comparison.

The relative contribution of the $\delta^{13}C_{awns}$ and the $\delta^{13}C_{peduncle}$ that accounted for the $\delta^{13}C_{grains}$ was assessed through a linear fit (Table 6 and Fig. 2). The $\delta^{13}C_{grain}$ was used as a dependent variable and the $\delta^{13}C$ in the WSF of awns and peduncles were used as the independent variables, with assignment of a different weight for the $\delta^{13}C$ of the awns and peduncles depending on the $\delta^{13}C_{grain}$. Thus, in 2012 before irrigation (Table 6) the $\delta^{13}C_{awns}$ showed a relative contribution of 75% ($\delta^{13}C_{awns}$*0.75) and the peduncles 25% ($\delta^{13}C_{peduncle}$*0.25), when the $\delta^{13}C_{grain}$ values were between -25.2 ‰ and -25.8‰. Conversely, the relative contribution of the awns was 25% ($\delta^{13}C_{awns}$*0.25) and the peduncle 75% ($\delta^{13}C_{peduncle}$*0.75) when $\delta^{13}C_{grain}$ values were between -26.4‰ and -27.0‰. In this way a linear fit with a slope of one and origin at zero was achieved (R^2 = 0.61, P<0.001). Furthermore, the awns showed a higher relative contribution in the linear regression in 2012 after irrigation (Table 6) compared to linear regression before irrigation. As mentioned in the Materials and Methods (see '*Correction factor on the relative contribution to grain filling'* section), values in the $\delta^{13}C$ in the WSF of awns and peduncle (not frozen samples obtained AI in 2012) were re-calculated using the experimentally calculated correction factor (see Table 1 and Table S1). Hence, from the linear fit after irrigation (R^2 = 0.70 P<0.001), the relative contribution of the $\delta^{13}C_{awns}$ ranged from 66% (when $\delta^{13}C_{grain}$ values were within the most negative interval, -26.4‰ and -27.0‰) to 100% (when the $\delta^{13}C_{grain}$ values were within the most positive interval, -25.2 ‰ and -25.8‰).

***Table 6**. Pearson correlation coefficient of the relationship between stable carbon isotope composition in mature grains ($\delta^{13}C_{grains}$) and the combination of the $\delta^{13}C$ from the peduncle and the awns ($\delta^{13}C_{peduncle}$+$\delta^{13}C_{awns}$) in the water-soluble fraction (WSF). The Peduncle (%) represents the relative contribution of the culm (i.e. the whole plant below the ear) to grain filling as a percentage and Awns (%) represents the relative contribution of the awns to grain filling as a percentage. The individual values of $\delta^{13}C_{awn}$ and $\delta^{13}C_{peduncle}$ used in the linear regression belong to $\delta^{13}C$ in the WSF before and after irrigation during the 2012 crop season. After irrigation for samples that were not frozen with liquid nitrogen a correction factor of 0.4‰ in the values of the $\delta^{13}C$ in the WSF was applied (see Materials and Methods and Table 1). The six genotypes and three replications per genotype were considered, accounting for a total of 18 plots per sampling date. For each plot the relative weight assigned to the $\delta^{13}C$ of each of the two organs depended on the water status of the plot assessed by its $\delta^{13}C_{grains}$ based on Sanchez-Bragado et al., 2014b. Level of significance: ***, P < 0.001*

	Interval $\delta^{13}C_{grain}$(‰)	**Awns (%)**	**Peduncle (%)**	R^2
Before Irrigation				
	[-25.2, -25.8]	75	25	
	[-25.8, -26.4]	50	50	
	[-26.4, -27.0]	25	75	
	$\delta^{13}C_{grain}$	*vs* $[\delta^{13}C_{awns}$*(%) + $\delta^{13}C_{peduncle}$*(%)]		0.61^{***}
After Irrigation				
	[-25.2, -25.8]	100	0	
	[-25.8, -26.4]	80	20	
	[-26.4, -27.0]	66	33	
	$\delta^{13}C_{grain}$	*vs* $[\delta^{13}C_{awns}$*(%) + $\delta^{13}C_{peduncle}$*(%)]		0.70^{***}

Conversely, in 2013 the relative contribution of the $\delta^{13}C_{awns}$ and the $\delta^{13}C_{peduncle}$ that accounted for the $\delta^{13}C_{grain}$ was achieved through a linear fit (R^2 = 0.53; P<0.002) with a slope of one but without an origin at zero (Fig. 2). Thus, the $\delta^{13}C$ in the WSF of awns and peduncles exhibited more negative values in 2013

compared to the 2012 experiment. As mentioned in the Materials and Methods (see '*Correction factor on the relative contribution to grain filling*' section), values of $\delta^{13}C$ in the WSF of awns and peduncles obtained in 2013 were re-calculated with the correction factor (see Table 1 and Table S1) to account for the deviation associated with sampling and further drying conditions, whereby a linear fit with a slope of one and an origin at zero was then possible to achieve. Hence, the relative contribution of the $\delta^{13}C_{awns}$ in the linear fit (Fig. 2) was quite steady around 90% irrespective of the $\delta^{13}C_{grain}$ values.

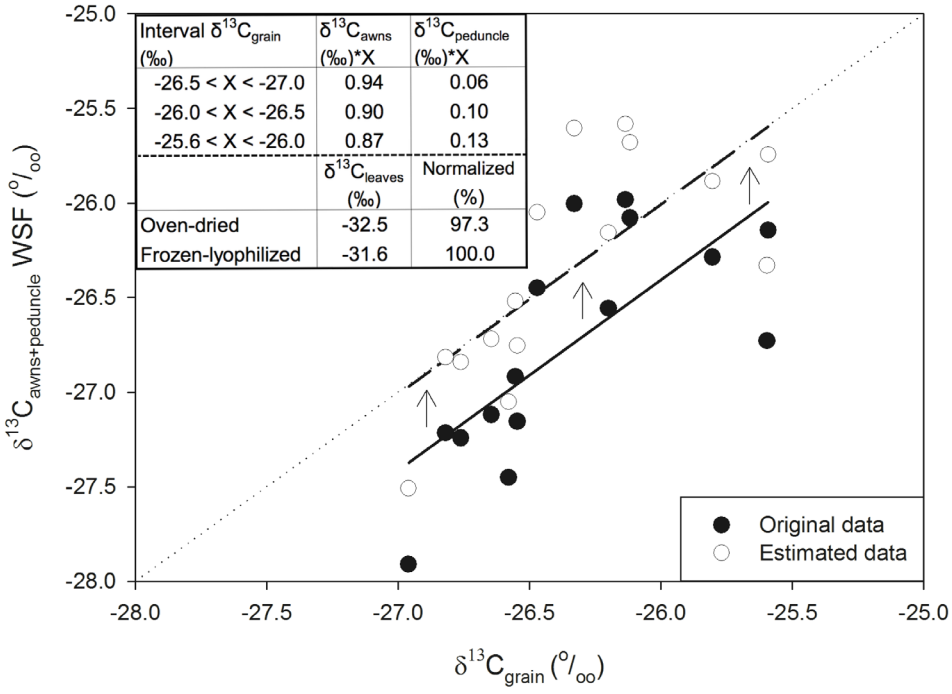

***Fig. 2**. Linear regression of the relationship between the stable carbon isotope composition in mature grains ($\delta^{13}C_{grain}$) and the combination of $\delta^{13}C$ from awns and the peduncle ($\delta^{13}C_{awns}$+$\delta^{13}C_{peduncle}$) in the water-soluble fraction (WSF) during the 2013 crop season (R^2=0.52; P=0.002). Closed symbols (original data) indicate raw data, and open symbols (estimated data) indicate original data with a correction factor of 0.4 ‰ (see Materials and Methods and Table 1). The six genotypes and three replications per genotype were considered, accounting for a total of 18 plots. For each plot the relative weight assigned to the $\delta^{13}C$ of each of the two organs depended on the water status of the plot assessed by its $\delta^{13}C_{grains}$ (see figure inset).*

Summarizing the DCMU approach assigned a lower relative contribution (40%) for the ear compared to the other two approaches (between 60-74%). Besides, compensatory effects were observed in the DCMU and shading treatment. Hence, the relative contribution of ears and culm together (Fig. 3) accounted for more than the expected for the intact (100%) plants, being 110% for DCMU and 119% for shading treatment. Concerning the $\delta^{13}C$ in the WSF, the greatest relative contribution was observed in the ear (74%) compared to DCMU and shading treatments.

Potential biomass production and assimilates produced

The potential contribution of the ear (in terms of photoassimilates produced during the reproductive stage) increased with the proportion of awn tissue relative to the ear (Fig. 4). Furthermore, ears without awns ($Awn \cdot ear^{-1}=0$) exhibited a potential assimilates production level accounting for half the grain weight (Fig. 4).

Besides, total carbohydrates produced by each organ from heading to maturity (calculated as total photosynthetic productivities of the flag leaf and the ear based on the accumulated gross carbon fixation) were comparable to the GWear and did not show significant differences from each other (Fig. 5). Moreover, the potential amount of biomass produced by the flag and the ear (Fig. 5), as inferred from light interception and photosynthetic assimilation accumulated from heading to maturity (see material and methods), surpassed the total grain weight of the ear.

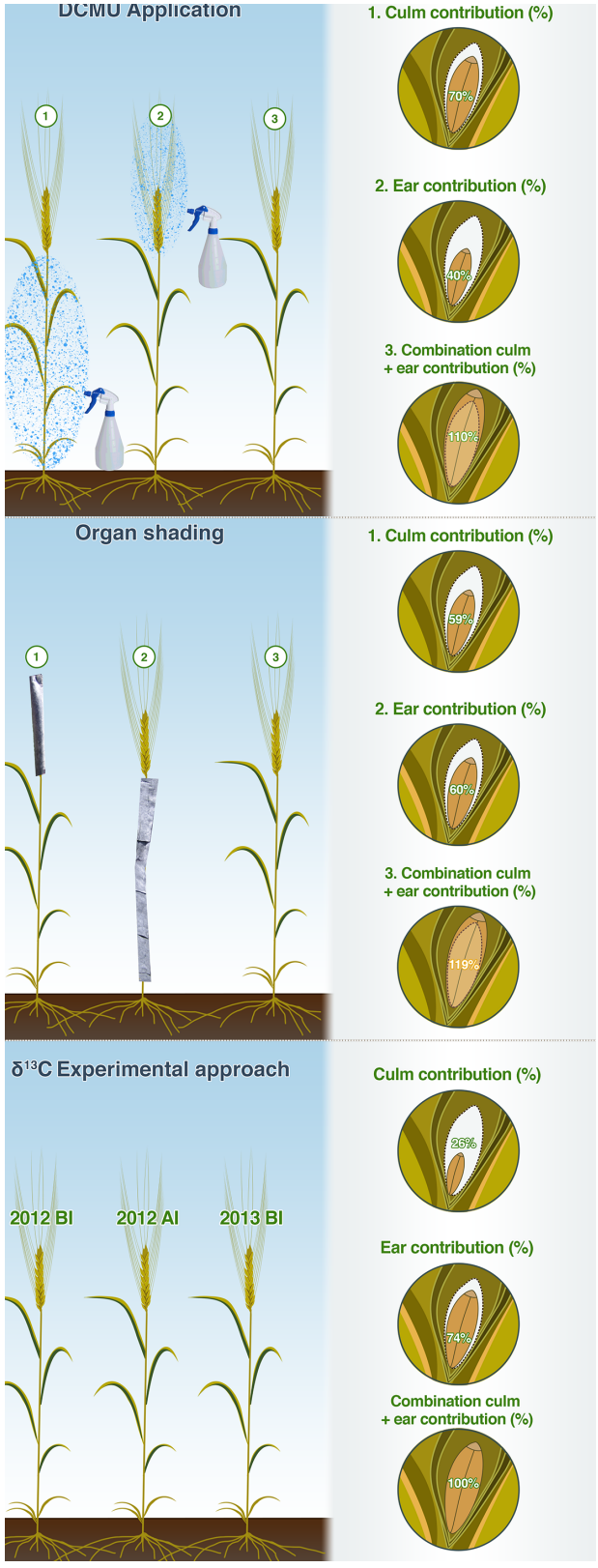

***Fig. 3**. Illustration of a wheat plant showing the relative photosynthetic contributions of the ear and culm to grain filling as estimated with three methodologies. The upper panel shows shading treatment with a shaded ear (1), shaded culm (2) and combination of culm plus ear contribution to grain filling (3). The middle panel shows DCMU treatment with DCMU application to the ear (1), culm (2) and combination of culm plus ear contribution to grain filling (3). The percentage contribution (%) of the culm, ear were calculated relative to control. The lower panel represents the $\delta^{13}C$ of assimilates as a % produced by the awns ($\delta^{13}C$ in the WSF) relative to the $\delta^{13}C$grain during grain filling, during 2012 before irrigation (BI), 2012 after irrigation (AI) and 2013 before irrigation (BI).*

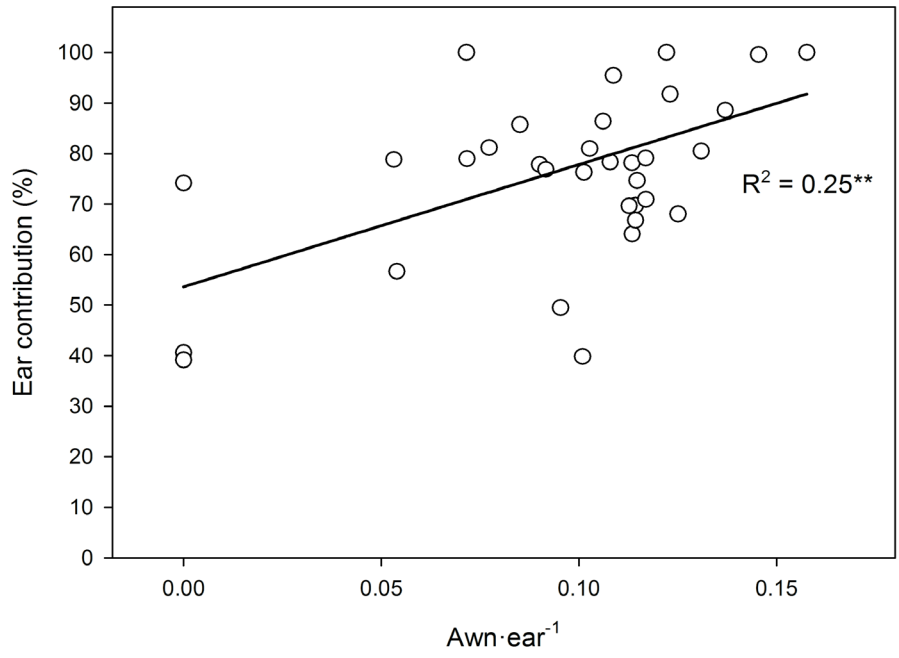

Fig. 4*. Linear regression of the relationship between the potential contribution (%) of the ear to total grain weight per ear (Y-axis) and the proportion of awns relative to the ear dry weight (X-axis). The potential contribution of the ear was calculated from the estimated carbohydrates produced by the ear during the reproductive stage (measured from heading to maturity) divided by the kernel weight per ear (GW_{ear}) at maturity. Carbohydrates produced were calculated by multiplying gross photosynthesis, duration of the daylight period (at saturating PPFD), active organ duration (as the number of days from heading to maturity) and molecular weight (C_2OH) of the basic carbohydrates produced. For more details see the Materials and Methods section. The six genotypes and three replications per genotype were considered, accounting for a total of 18 plots. 2012 and 2013 crop seasons. Level of significance: **, P < 0.01.*

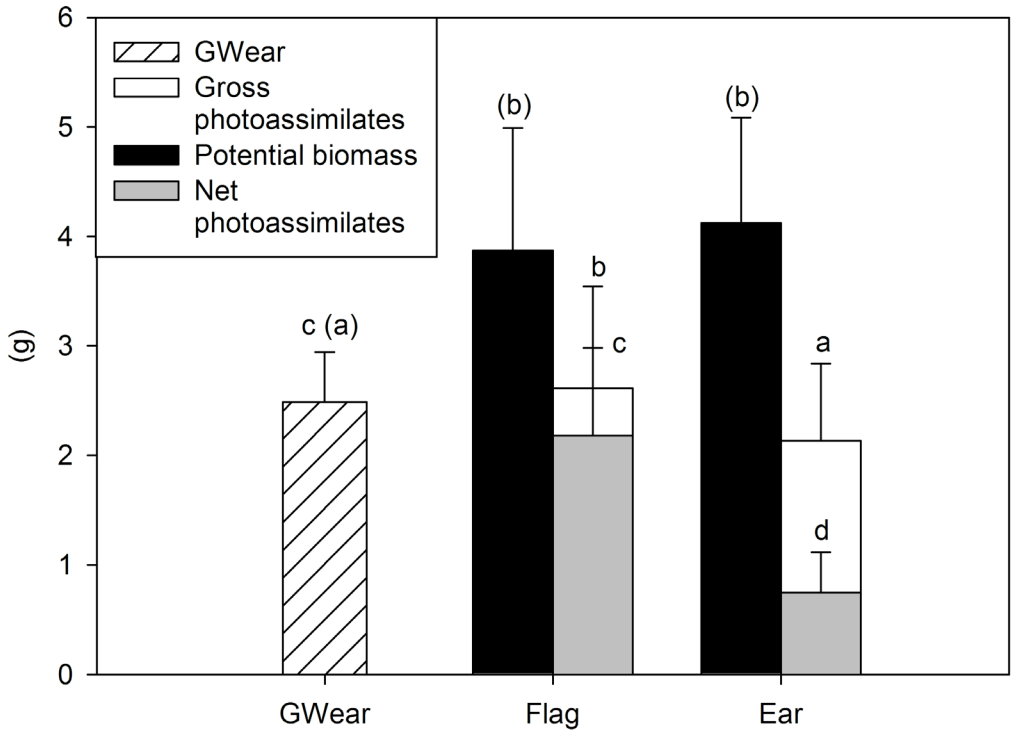

Fig. 5*. Comparison of kernel weight per ear (g) at maturity (GWear) with the photosynthetic contribution of the ear and the flag leaf during grain filling, estimated from the potential biomass (g) produced by each of the two organs from heading to maturity based in the time-integration of the irradiance intercepted by the canopy layers where the ear and the flag leaf are placed (see Material and Methods section) and from the carbohydrates produced (a) during the same interval estimated from the net and gross photosynthesis rates of the whole organ (for more details see Material and Methods). Each bar represents the mean values ± standard deviation (SD) of the 6 genotypes and the three replications per genotype during 2012 and 2013 crop cycle. Mean values with different superscripted letters are significantly different according to the Turkey's honesty significant difference test (P<0.05).*

Discussion

Photosynthetic contribution of the ear and the culm to grain filling: DCMU application.

The GW_{ear} exhibited lower values with DCMU applied to the culm compared to GW_{ear} with DCMU applied to the ear (Table 2). This indicates that the organ that most affected grain filling following photosynthesis inhibition was the culm. Thus, the culm contributed around 70%, whereas the ear contributed 40% (Fig. 3). However, when the DCMU was applied to the culm, not only was the photosynthesis of the stem (measured in the flag leaf) affected (Table 3), but the photosynthesis of the ear was also partly inhibited. These results suggest that DCMU is transported acropetally to the ear, causing a premature yellowing of awns and glumes, but that DCMU is not transported to the culm from the ears. In fact, in a study by Nicolas and Turner (1993), although leaves and stems were sprayed with three different desiccant treatments (paraquat, magnesium chlorate and sodium chlorate), the ears were also affected and consequently grain weight was reduced by more than 70%. Such findings suggest that chemicals were transported from stem and leaves (via phloem) to the ears and subsequently to the growing grains (Blum *et al.*, 1983). In similar studies plants were also sprayed with potassium iodide, which is a desiccant that appears to be transported only by xylem (Herrett *et al.*, 1962) and not by phloem into the grains. Consequently, leaves and stems sprayed with potassium iodide resulted in a lower reduction in grain growth compared to treatments with other desiccants that are transported through the phloem. A recent study by Saeidi *et al.* (2012) using iodide potassium as a desiccant also concluded that the contribution of the ear to growing grains was higher than that of the shoot. Moreover, in our study mean values of the $\delta^{13}C_{grain}$ in control plants were similar to the $\delta^{13}C_{grain}$ in DCMU stems, but different to the $\delta^{13}C_{grain}$ DCMU ears (Table 2). These results also suggest that the DCMU method underestimates the photosynthetic contribution of the ear due to the fact that the $\delta^{13}C_{grain}$ is not maintained when the ear photosynthesis is reduced by 40% as a side effect of spraying the stem with DCMU.

Photosynthetic contribution of the ear and the culm to grain filling: shading treatment.

The importance of ear photosynthesis was also supported by the other two experimental approaches of this study. In the textile-shading approach, mean values of GW_{ear} from shaded ears (Table 4) were similar to shaded culms, indicating that ear photosynthesis was comparable to culm photosynthesis (leaf blades, sheaths and peduncles) in terms of contribution to grain filling; however, the intrusive nature of treatments such as DCMU or shading should be kept in mind. These results should therefore be interpreted with caution because potential compensation effects triggered by these treatments may eventually increase the contribution of unaffected photosynthetic organs or preanthesis reserves to grain filling (Aggarwal *et al.*, 1990; Eyles *et al.*, 2013). Indeed, the total contribution to grain filling attributed to the ear and culm together in DCMU (110%) and shading (119%) treatments was higher than the control (100%), suggesting possible compensation effects by unaffected photosynthetic organs (Fig. 3). In studies presented by Aggarwal *et al.* (1990) and Ahmadi *et al.* (2009), leaf detachment did not result in any significant decreases in final grain weight, and authors stated that grain filling was controlled by sink rather than source strength. However, in those works the photosynthetic role of ears was not considered (Saeidi *et al.*, 2012), and therefore the interpretation of results could go in two directions. On the one hand, the results might have been biased by compensation mechanisms in the remaining unaffected ears of the plant (Chanishvili *et al.*, 2005). On the other hand, the final grain weight was not significantly affected by leaf detachment, revealing the important role of ear photosynthesis to grain filling. However, in our study grain weight was similarly reduced (35-40%) irrespective of the experimental approaches used to inhibit ear photosynthesis (DCMU and organ shading). The study by Maydup *et al.* (2010) in bread wheat also reported similar reduction in grain weight (approximately 30%) as a result of the inhibition of ear photosynthesis by DCMU and organ shading.

Photosynthetic contribution of the ear and the culm to grain filling: $\delta^{13}C$ comparison.

In a less invasive manner, the $\delta^{13}C$ approach aims to assess the relative contribution of different photosynthetic organs that are active in providing assimilates to the grains during grain filling. The $\delta^{13}C$ approach avoids the unwanted compensatory mechanisms and chemical effect of current methods derived from a plant part-specific photosynthesis limitation. Bearing this in mind, the $\delta^{13}C$ approach showed on average a higher relative contribution from the awns compared to the peduncles (Table 6 and Fig. 2), highlighting the relative importance of ear photosynthesis compared to green culm parts (the peduncle integrates leaf blades and sheaths). Furthermore, the $\delta^{13}C$ in the WSF of the awns and peduncles used in the $\delta^{13}C$ approach reflects plant responses near the time of sampling and therefore is able to reflect subtle changes in growing conditions (Yousfi *et al.*, 2013). In that sense the $\delta^{13}C$ approach (using $\delta^{13}C$ in the WSF) may help to estimate the proportion of assimilates produced by the awns and peduncles that are ready to be transported (Brandes *et al.*, 2007; Cabrera-Bosquet *et al.*, 2009) under short-term environmental conditions.

Effect of sampling conditions on the $\delta^{13}C$ of the water-soluble fraction

Although the photosynthetic contribution of the awns to grain filling has been observed to be higher under drought stress conditions (Evans *et al.*, 1972; Motzo and Giunta, 2002), our results from 2012 showed on average a higher relative contribution from the awns (Table 6) after irrigation (82%) compared to before irrigation (50%). In fact, under the improvement in water conditions after irrigation, the increase in photosynthetic activity of the ear may account for a larger relative contribution to grain filling than before irrigation. In addition, the increase in the relative contribution of awns after irrigation may have been also related to the sampling method (samples taken prior to irrigation were immediately frozen and part of samples taken after irrigation were not frozen). Greater differences between the $\delta^{13}C$ in the WSF of the peduncle and the awns versus the $\delta^{13}C$ of mature kernels in not-frozen samples suggest that the $\delta^{13}C$ in the WSF could have been biased due to the sampling method. Besides, in the growth chamber experiment (Table S1), oven-dried leaves showed more negative $\delta^{13}C$ in the WSF (-32.5‰) compared to leaves frozen with liquid nitrogen (-31.6‰). Therefore, oven-dried samples (in 2013) and samples that

were not frozen immediately after sampling (AI 2012) shared a common denominator: metabolic activity was not stopped straight away. Thus, the fact that the metabolic activity after sampling was not halted could have led to organ respiration continuing for a while after sampling (Ocheltree and Marshall, 2004; Klumpp *et al.*, 2005; Gessler *et al.*, 2009b). Numerous studies have shown significant ^{13}C enrichment of respired CO_2 compared to the remaining substrate in leaves (Ghashghaie *et al.*, 2001; Gessler *et al.*, 2009a), shoots, roots (Kodama *et al.*, 2011), peduncles and awns (Sanchez-Bragado *et al.*, 2014b). In this sense, respiration has been observed to rely on current photo-assimilation rather than mobilized reserves (Bell and Incoll, 1990), leading to more negative $\delta^{13}C$ in the remaining WSF after dark respiration.

Indirect non-intrusive approaches supporting the key photosynthetic role of the spike

The key role of the awns (in terms of photoassimilates produced) during grain filling is also supported by the positive relationships between the relative contribution of the ear to grain filling and the proportion of awns relative to the ear (Fig. 4, R^2=0.25 P< 0.01). Furthermore, even without the presence of awns (awns·ear $^{-1}$ = 0; awnless genotypes), accumulated ear photosynthesis represented near half of the total grain weight per ear (Fig. 4). This suggests that not only are the awns important tissues for assimilating atmospheric CO_2 in the ear (Bort *et al.*, 1994; Li *et al.*, 2006), but that the glumes (and other bracts) may be also involved in atmospheric CO_2 fixation in addition to re-assimilating respired CO_2 (Bort *et al*., 1996; Maydup *et al*., 2014). However, it appears that, when present, awns are the main photosynthetic organs of the ear (Tambussi *et al.*, 2007a) that fix atmospheric CO_2 (Blum, 1985). Moreover, the potential photosynthetic contribution of the ear to grain filling is also evidenced at the canopy level. The upper part of the canopy (basically constituted by the ears) integrated from heading to maturity and assuming a photosynthetic efficiency of 2.4% (Zhu *et al.*, 2008) represented a potential production of biomass of 4.1 g·ear^{-1}. Such potential production was found to be within the range of total grain weight per ear (Fig. 5), providing an indirect support in favour of the ear as the main photosynthetic organ during grain filling under well agronomic conditions.

Conclusions

As far as we know, this is the first report where different, independent experimental approaches of intrusive and non-intrusive nature were used to assess the contribution of ear photosynthesis to grain filling. Our results from the shading treatments indicate similar contribution of the ear and the culm. Conversely, the DCMU approach assigned a higher role to culm photosynthesis, but herbicide application in the culm affected the ear, biasing the final grain weight. Nevertheless, the results from any intrusive treatment should be interpreted with caution, as unwanted compensatory mechanisms in the remaining unaffected organs could affect final grain weight. However in the approach using $\delta^{13}C$, ear photosynthesis (awns) represented on average 74% of the total assimilates going to the grain. In addition, the ear contribution to grain filling may still be underestimated because the glumes were not included in the approach using $\delta^{13}C$. Other indirect, albeit non-intrusive approaches, such as measurements of organ photosynthesis and captured integrated irradiance, from heading to maturity, also support the role of the ear as a main contributor to filling grains.

In accordance with the results obtained in this study using different approaches, the contribution of the ear to grain filling was at least comparable to that of the rest of the plant under good agronomical conditions. Moreover, genetic variability was observed with regards to the relative contribution of the ear to grain filling. In this sense the $\delta^{13}C$ approach, provides a precise tool for assessing the photosynthetic contribution of the ear to grain filling, which can help to identify potential parents in order to design crosses for breeding new lines with enhanced ear photosynthesis contribution adapted to a wide range of environments. However, some consideration should be given when applying the $\delta^{13}C$ approach, including the sampling method used, in order to take into account post-harvest respiration. Moreover, further research is needed to clarify under which particular conditions ear photosynthesis is a positive trait for improving grain yield.

SUPLEMENTARY DATA

Is composed of:

Table S1. Values of $\delta^{13}C$ in the WSF of wheat leaves in the growth chamber experiment.

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***Journal of Experimental Botany* supplementary material**

Article title: *Photosynthetic contribution of the ear to grain filling in wheat: a comparison of different methodologies for evaluation*

Authors: Rut Sanchez-Bragado, Gemma Molero, Matthew P. Reynolds and Jose Luis Araus

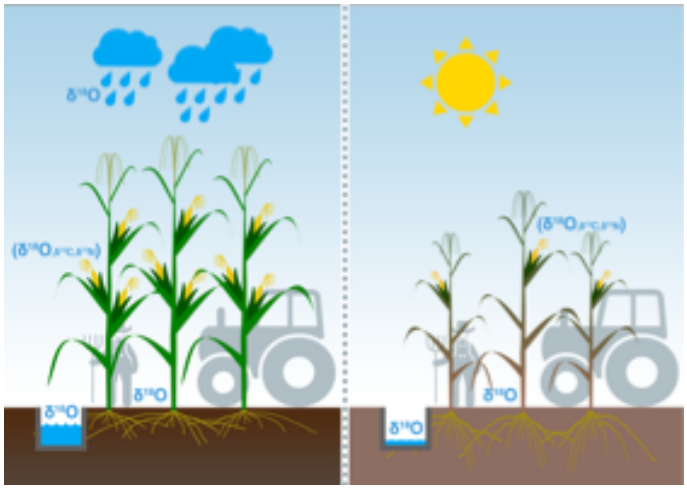
The following supplementary material is available for this article:

***Table S1**. Values of $\delta^{13}C$ in the WSF of wheat leaves in the growth chamber experiment. Values are divided into two categories; samples frozen immediately after sampling and subsequently lyophilized (frozen) and samples not frozen and oven-dried (oven) after sampling. Analysis of variance (ANOVA) for the sampling method.*

	$\delta^{13}C$ WSF leaves (‰)			Normalized values (%)		
	Oven	**Frozen**	**Frozen-Oven**	**Oven**	**Frozen**	**Frozen-Oven**
Rep. 1	-32.8	-32.1	0.7	97.9	100.0	2.1
Rep. 2	-31.8	-30.8	1.0	96.8	100.0	3.2
Rep. 3	-32.7	-31.9	0.9	97.3	100.0	2.7
Average	-32.5	-31.6	0.8	97.3	100.0	2.7

Level of significance

Oven-Frozen	0.001^{**}



CAPÍTOL 4

Factors que impedeixen la utilització dels isòtops d'oxigen com a indicador del rendiment de gra al blat de moro

CHAPTER 4

Factors preventing the performance of the oxygen isotope ratios as indicator of grain yield in maize

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* A punt per la re-submissió a *Planta*

RESUM CAPÍTOL 4

La composició isotòpica de l'oxigen ($\delta^{18}O$) s'ha proposat com una eina per integrar el fenotipejat i la transpiració de la fulla en plantes C_4 com el blat de moro. En aquest sentit, la $\delta^{18}O$ de les fulles pot ser un indicador de la variabilitat ambiental i genètica degut als processos d'evaporació, però aquesta senyal es pot veure modificada des de la "font" fins els teixits. L'objectiu d'aquest estudi va ser avaluar la importància relativa dels factors transpiratoris que afecten a la $\delta^{18}O$ dels teixits de les plantes de blat de moro. Es van realitzar dos experiments amb règims diferents d'aigua, un amb dos varietats en condicions semi-controlades, i l'altra al camp utilitzant 100 genotips, aquest últim durant dos anys consecutius. La $\delta^{18}O$ de la matèria orgànica de la base de la fulla es va correlacionar fortament amb la $\delta^{18}O$ de l'aigua de la tija, cosa que ens indica que aquest paràmetre podria ser un bon indicador de la font d'aigua en mostrejos molt grans. En comparació a les fulles es va observar una baixada de la $\delta^{18}O$ a les sedes i els grans, però no a la matèria orgànica de la tija. Això es pot interpretar com una evidencia d'intercanvi amb l'aigua font cap al "destí" que no està enriquida, tot i que això es dona principalment en teixits del "destí". El rendiment (GY) i les variables fisiològiques no van mostrar patrons clars entre les parcel·les elementals respecte a la $\delta^{18}O$, però si que es van correlacionar bé entre assajos. Aquests resultats indiquen que la resposta de la $\delta^{18}O$ a diferents factors ambientals és prou gran com per superar l'efecte de les conversions metabòliques a l'interior dels teixits "destí". Aquests resultats ajudaran a interpretar la $\delta^{18}O$ com una eina tant per la selecció de genotips com per la caracterització ecofisiològica per estudiar l'adaptació del blat de moro i altres conreus a la sequera, doncs el treball ofereix una idea de les relacions que pot haver-hi entre els òrgans "font-destí" i la transpiració.

Factors preventing the performance of the oxygen isotope ratios as indicator of grain yield in maize

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Running title: Oxygen isotopes in maize from source to sink

ABSTRACT

Oxygen isotope composition ($\delta^{18}O$) has been proposed as a phenotyping tool to integrate leaf transpiration in C_4 crops, such as maize. Within this context we hypothesize $\delta^{18}O$ in leaves may reflect primarily environmental and genetic variability in evaporative processes, but this signal may become dampened in the way from source to sink tissues. The aim of this study was to assess the relative importance of transpirative or translocation-related factors affecting $\delta^{18}O$ in plant tissues of maize. We performed two water-regime experiments, one with two varieties under semi-controlled conditions, and another in the field with 100 genotypes during two consecutive years. The $\delta^{18}O$ in organic matter at the leaf base was strongly correlated with the $\delta^{18}O$ in stem water, indicating that it could be a good proxy for source water in extensive samplings. Compared to leaves, we observed a ^{18}O-depletion in silks and grains, but not in stem soluble organic matter. We interpret this as evidence of exchange with unenriched water from source to sink, but mainly occurring within sink tissues. Although grain yield (*GY*) and physiological variables did not show clear intra-trial patterns against $\delta^{18}O$, they were generally well correlated with $\delta^{18}O$ across trials. Such results indicate that the response of $\delta^{18}O$ to environmental factors is large enough to overcome the effect of metabolic conversions within the sink tissues. This finding will eventually help to interpret $\delta^{18}O$ as a genotype selection and ecophysiological tools for the adaption of maize and other crops to drought, offering insight into source-sink relationships and transpiration processes.

Key words: stable isotopes, translocation, corn, drought-tolerance, stem water, organic matter, leaf, grain

INTRODUCTION

Maize is one of the three most important crops worldwide (FAOSTAT 2011). Maize productivity is already challenged by abiotic stresses such as drought and heat (Holzkamper et al. 2013), and the impact of these stresses is expected to increase even more in the future because of climate change, especially in tropical areas (Edmeades et al. 1989; Cairns et al. 2013). In that sense, plant breeding primarily tries to understand and utilize genetic variation for traits that impact yield which consequently can lead to yield improvement in agricultural drought conditions. Thus, is it important to study genetic variation for drought tolerance for yield improvement at different stages of crop development (Cooper *et al.*, 2014) .In this context, the use of natural abundance of stable isotopes in plant tissues has been proposed as a phenotyping tool to obtain time-integrated measures of physiological responses to abiotic stress (Araus *et al.*, 2003, 2013*a*). In particular, carbon isotope composition has been extensively applied in C_3 crops as a tool to determine water use efficiency for plant breeding (Farquhar and Richards, 1984; Araus *et al.*, 2002; Rebetzke *et al.*, 2002; Condon *et al.*, 2004), and to monitor environmental responses over a wide range of spatial and temporal scales (Zhao *et al.*, 2001; Araus *et al.*, 2003, 2014). In contrast, carbon isotopes in C_4 crops such as maize or sorghum, despite having physiological significance, show a narrow range of variation and hence limited applicability (Hubick *et al.*, 1990; Monneveux *et al.*, 2007). As an alternative, the analysis of the oxygen isotope composition ($\delta^{18}O$) in plant tissues has been proposed as a trait when selecting C_4 crops better adapted to drought stress (Cabrera-Bosquet *et al.*, 2009*a*). Unlike for $\delta^{13}C$, there are no particular differences for $\delta^{18}O$ fractionation between C_3 and C_4 crops. Previous studies have demonstrated $\delta^{18}O$ is sensitive to temperature, air humidity (Barbour and Farquhar, 2000; Helliker and Ehleringer, 2002), salinity (Yousfi *et al.*, 2012) and soil moisture (Yakir *et al.*, 1990; Ferrio *et al.*, 2007). Evaporative enrichment increases $\delta^{18}O$ in the leaf water compared to the source water and is imprinted on the newly produced assimilates. According to (Dongmann *et al.*, 1974), such enrichment depends mostly on leaf temperature and the ratio of ambient to intercellular vapor pressures (ea/ei). Thus, within the same environmental conditions, plants with higher stomatal conductance (g_s) are expected to show smaller enrichment in $\delta^{18}O$ in the leaves (Farquhar and Lloyd, 1993). Therefore,

due to sensitivity of $\delta^{18}O$ to changes in g_s, and leaf temperature and its subsequent relationship against *GY*, $\delta^{18}O$ has been proposed as a predictor of *GY* and integrative indicator of genetic differences in g_s (Barbour et al. 2000). Besides, $\delta^{18}O$ of organic matter has been used to evaluate plant response under different treatments and genotypic variability on yield in wheat (Barbour et al. 2000; Barbour 2007; Araus et al. 2013). However, there is still a lack of knowledge regarding the relative importance of different processes that may alter the $\delta^{18}O$ within different plant organs. Indeed, although the use of $\delta^{18}O$ in crop improvement strategies has been proposed for maize (Cabrera-Bosquet *et al.*, 2009*a*) and other crops such as wheat (Barbour et al. 2000; Cabrera-Bosquet et al. 2011), its implementation in breeding programs is less advanced compared to carbon isotopes. This is due to the fact that oxygen isotope values in plant matter do not always match to what is predicted from theoretical models in response to different environmental conditions (Barbour, 2007; Farquhar et al. 2007).

In C_3 cereals such as wheat or barley, whereas $\delta^{13}C$ in kernels reflects better the stress conditions experienced by the crop (particularly during the reproductive stage), the $\delta^{13}C$ in the leaves (even in the flag leaf) is less clearly correlated with *GY* (Austin et al. 1990). Therefore, and following what is found for $\delta^{13}C$ in C_3 cereals, in C_4 cereals such as maize, $\delta^{18}O$ of mature kernels, should record more reliably environmental conditions compared with leaves. This is because in maize, water stress is usually applied just before or during flowering, when vegetative part is already developed (Cabrera-Bosquet *et al.*, 2009*b*). However, although genotypic correlations between $\delta^{18}O$ in mature kernels and *GY* have been observed (Cabrera-Bosquet *et al.*, 2009*b*, 2011), maize is particularly susceptible to drought around flowering (Birch *et al.*, 2008); therefore plants experience more water stress around flowering that during most of the grain filling (Edmeades *et al.*, 1993). For example, during flowering rice panicles has been observed to be more sensitive to drought compared to leaves, as rice panicles don't have adaptive mechanisms to modulate their water loss (Pinheiro *et al.*, 2000). Thus, in such study $\delta^{13}C$ of soluble carbohydrates in the spike peduncle of rice during flowering stage, was found to be a great indicator of plant performance in terms of grain yield and/or grain number. Similarly, maize might have analogous characteristics during female flowering analyzing the

extruded silks during female flowering. In this sense, silks might be a good indicator of tolerance to drought during flowering, as silks have high percentage of water on its structure compared to leaves. Factors such the silking-to-anthesis interval, pollen viability, or silk extrusion, will determine the final kernel number per ear, and thus the productivity of the plant (Barker *et al.*, 2005). In addition, silks may provide information about the reproductive stage (female flowering), in contrast to grains, which provide information about the full cycle performance in the field. Within this context, the use of $\delta^{18}O$ in the silks might be a clear indicator of tolerance to drought. Thus, to study $\delta^{18}O$ in different plant tissues since leaves, grains or silks might be of special interest, as integrated information of different crop stage development will be observed.

However, the mechanisms controlling the transfer of oxygen isotope signal from autotrophic organs (e.g. leaves) to heterotrophic tissues (e.g. inflorescences, fruits) are of particular importance for physiological interpretations based on oxygen isotope composition in plant tissues. Therefore, $\delta^{18}O$ in plant tissues can be influenced by three main factors (Barbour et al. 2000) which are mainly represented in Fig. 1 and explained in detail as follows: The first factor is the isotopic composition of water source taken up by the plant (Roden and Ehleringer, 1999). This factor, however, does not constitute a major problem provided the plant uses a known source of water. The second factor to be considered is the enrichment in ^{18}O in the leaves due to evaporation in comparison to source water (Gonfiantini *et al.*, 1965; Pande *et al.*, 1995). This is governed by the differential diffusion of $H_2{}^{18}O$ and $H_2{}^{16}O$ isotopologues in the leaf boundary layer (Pande *et al.*, 1995), together with the regulation of leaf gas exchange (Farquhar 1989; Barbour and Farquhar 2000). Although evaporative enrichment mechanism of leaves and the resulting imprint of the isotope signal in the newly produced assimilates are well known (Barbour *et al.*, 2004), different processes may complicate the response to environmental conditions. Due to a *Péclet* effect there is a gradient of $\delta^{18}O$ in the water from the xylem to the substomatal cavity, which is proportional to transpiration (Farquhar and Lloyd, 1993). Furthermore, an additional evaporative gradient along the xylem has been described from proximal to distal areas of the leaf blade (Gan et al. 2003), which is particularly strong in elongated leaves (Helliker and Ehleringer, 2000; Farquhar and Gan, 2003; Gan *et al.*, 2003). This gradient is not only observed in water, but

is also transmitted to organic matter, as observed by Farquhar and Gan (2003) in maize. The third factor influencing $\delta^{18}O$ in plant tissue is the fractionation during biochemical reactions involved in the synthesis of organic matter (Farquhar and Lloyd, 1993). The $\delta^{18}O$ of organic matter such as cellulose, lignin, starch and other substances in different plant organs could become affected compared to $\delta^{18}O$ of the original organic substrates, depending on the water media in which such post-photosynthetic transformations take place (Yakir *et al.*, 1990; Barbour *et al.*, 2004). However, there is scarce information about the mechanisms involved in the alteration of $\delta^{18}O$ of assimilates throughout subsequent biosynthesis and during transport within the plant, which may limit its applicability as selecting trait. For example, despite the strong physiological signal recorded in the leaves (see e.g. Barbour et al. 2001), the performance of grain $\delta^{18}O$ for assessing genotype differences in yield and adaptation to water stress in crops is often limited (Ferrio et al. 2007; Araus et al. 2013).

In this context, we hypothesize that the $\delta^{18}O$ in leaves reflect primarily environmental and genetic variability in evaporative processes, but this signal may become dampened in the way from source to sink tissues. This could prevent the adoption of $\delta^{18}O$ as a tool to assess transpirative conditions in maize under field conditions. Hence, the aim of this study was to assess under controlled and under field conditions the importance of transpirative or translocation-related factors affecting $\delta^{18}O$ in plant tissues of maize. Final objective is to determine the causes why oxygen isotope ratios of different tissues are not good indicators of grain yield in maize exposed to different growing conditions The results obtained are highly relevant for studies which use $\delta^{18}O$ of different organic matter pools, both as an ecophysiological tool and in plant breeding.

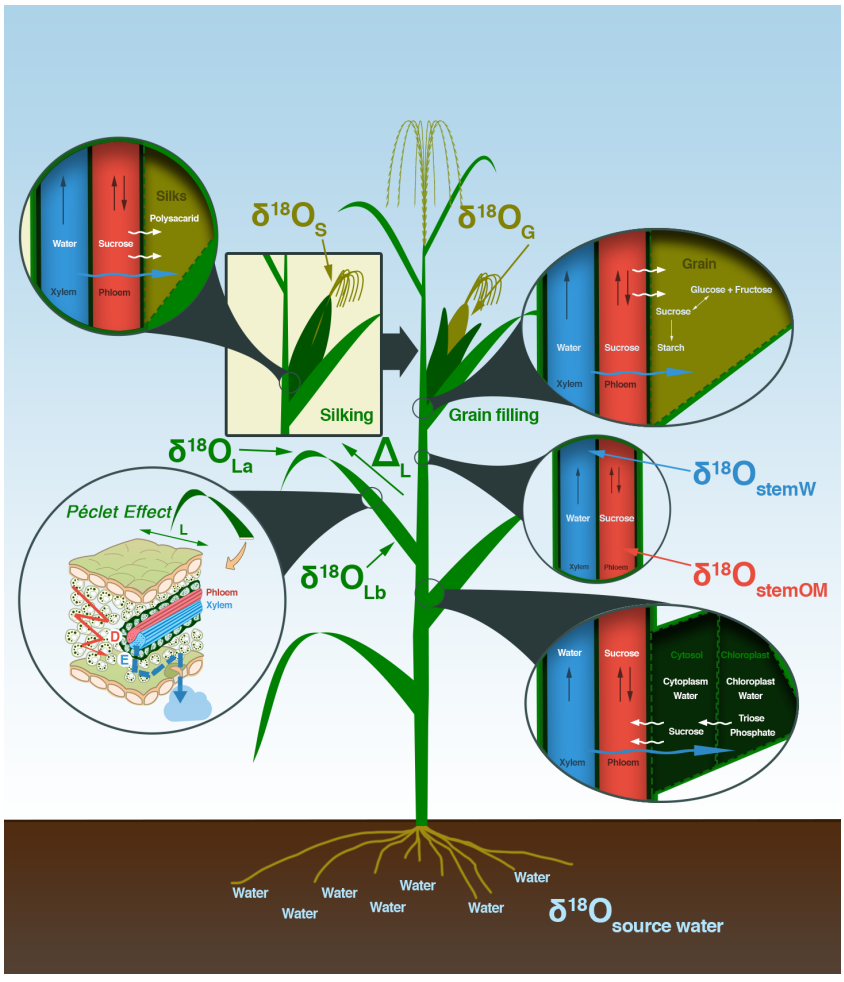

Fig. 1*. Illustration of a maize plant showing the organs samples in this study and the metabolic conversions of loading, transport and unloading of assimilates from source (leaves) to sink tissue (silks and grains), during silking and grain filling. Leaves, silks, grains, stem water and soluble organic matter of two representative plants per plot were collected for oxygen analysis.* $\delta^{18}O_{stemOM}$, $\delta^{18}O_{S}$ *and* $\delta^{18}O_{G}$ *represent the oxygen isotope composition in the stem soluble organic matter, silks and mature kernels respectively.* $\delta^{18}O_{stemW}$ *is the oxygen isotope composition of stem water which is representative of source water.* $\delta^{18}O_{La}$ *and* $\delta^{18}O_{Lb}$ *stand for the oxygen isotope composition of the leaf in the apex and the base, respectively; subjected to isotopic enrichment above source water at whole leaf (*Δ_L*). E, transpiration and D, self-diffusivity.*

MATERIAL AND METHODS

Pot trial

An experiment under controlled conditions was performed to track stable isotope signatures through different plant tissues, as well as to determine the optimum sampling strategy for field trials. Briefly, two commercial temperate maize varieties (*Kermes* and *Guadiana*) were grown outdoors as microcrops in 1 m^3 containers (9 plants per plot). Containers were filled with a sand:soil mix (3:1 v/v) and washed to eliminate excess nitrogen, as described in detail in Ferrante et al. 2010. The experiment was carried out at the facilities of the University of Lleida in Spain (41°37' N, 0°37' E, 167 m asl). The experimental design was a randomized block design, consisting of 3 blocks of 8 containers each. Plants were sown on the 17th July 2009 (Pot experiment 2009). Two applications of 50 kg N ha^{-1} were done at the 4/5-leaves and 6/7-leaves stages, plus an additional application of 7.2 l ha^{-1} of micronutrient solution at the 5/6 leaves stage (Manvert NPK 0-17-19 + microelements, Biovert S.A., corbins, Lleida). All plots were watered every second day to field capacity until two weeks before anthesis, when watering was stopped in half of the plots. Leaf parameters (leaf temperature -T_L-, stomatal conductance -g_s-, transpiration -*E*- and assimilation rates -*A*-) were determined three times during the first week of anthesis (10th, 16th and 17th September) with an ADC LCi infrared gas analyzer (ADC Bioscientific, England). Measurements were taken during midday (11-13h solar time), and in the central portion of the leaf blade. Plants were harvested on the 18th September, collecting a piece of the stem base for water distillation, one leaf per plot, adjacent to the central cob (base and appex), and silks, when available (first plot was still not flowering at the time of harvest). In total, 24 leaves and 16 silks were sampled.

Field trials

In this study a total of 300 single cross maize hybrids were used, consisting of 292 lines (plus eight hybrids of the same set as controls), selected in order to represent the genetic diversity within tropical and subtropical maize breeding programs (Wen *et al.*, 2011), crossed to the common tester CML-539. Hybrids were divided in two groups according to their maturity (based on prior experiments), early (n=150) and late (n=150). A total set of four trials were sown

including two water regimes; well watered control (early-WW and late-WW) and drought stress (early-DS and late-DS) applied at anthesis stage (as described by (Cairns *et al.*, 2013). In order to ensure drought stress at anthesis, irrigation was stopped before flowering. The field experiments were conducted during dry season at CIMMYT experimental station at Tlaltizapán, Morelos, Mexico (18°41' N, 99°07' W, 940 m asl) during 2010 and 2011. Plants were sown on 25 November 2009 and 1 December 2010 (Field trials 2010 and 2011, respectively). Drought stress was applied at flowering by stopping irrigation two weeks prior to anthesis until 30 days after anthesis. The total precipitation during the crop cycle in 2010 was 124.2 mm (Weber et al. 2012) and 45.0 mm in 2011 (Zia *et al.*, 2013). The maximum average temperatures were 37 ºC and 32 ºC, the minimum average temperatures 5ºC and 13ºC for 2010 and 2011, respectively. The averaged relative humidity was 56.5% for 2010 and 62.5% for 2011. Auxiliary irrigations were provided on the drought trials totaling 521 mm ha^{-1} and 639 mm ha^{-1} (2010 and 2011, respectively) for early genotypes and 489.3 mm ha-1 and 639 mm ha-1 (2010 and 2011, respectively) for late genotypes. In the well-watered trials auxiliary irrigation was applied in the early (848.1 and 1584 mm ha^{-1}) and the late (935.1 and 1342 mm ha^{-1}) genotypes. Experimental design was laid out as alpha-lattice with two replications in 5 m long plots consisting of 2 rows with a plot size of 7.5 m^2 with and a plant density of 66,000 plants ha^{-1}. Two seeds per hill were sown and then after emergence were thinned to one. The soil is classified as Isothermic Udic Pellustert with a pH of 7.9 and the texture is clay loam soil developed from calcareous subsoil. The field capacity is 36% and the permanent wilting point 21%. Appropriate fertilization and weed, disease and pest control were implemented to avoid yield limitations. All plots (in 2010 and 2011) received an application of 80 kg N ha^{-1} (as urea), 80 kg P ha-1 (as calcium triple superphosphate) and 25 kg K ha^{-1} (as potassium chloride) before planting. A second application of N was performed 35 days after sowing.

For leaf and silks collection, a subset of 50 genotypes for each phenology group was randomly selected, including two replicates per water regime (See detailed list of genotypes in Supplementary Material). The sampling took place during anthesis, in February 2010 and February 2011. In 2010 one sample from the leaves and silks per plant (two plants per plot) were collected. In 2011, one section of stem basis and one sample from the silks was collected per plant

from 2 plants per plot for the set of 50 early genotypes. T_{leaf} and g_s were determined at anthesis and maturity with a hand-held infrared thermometer (Mikron Infrared, USA) and a portable porometer (SC-1; Decagon devices, Washington, USA), respectively, as detailed in Zia et al. (2013). Plants were harvested at two dates respectively for water stress and well watered trials; on 14th and 15th April in 2010 and 26th and 27th April in 2011.

Plant growth and yield

Anthesis to silking interval (*ASI*) was measured as the number of days between the time at which 50% of the plants had shed pollen to the time 50% of silks emerged. Ear yield was determined in t ha^{-1} at 10% moisture level (Betrán *et al.*, 2003). Plants were harvested manually and for the 100 genotypes of the selected subset, grain yield (*GY*), biomass and yield components (hundred kernel weight -*HKW*-, kernel number per ear (Kernel·ear^{-1}) were determined.

Isotopic composition in stem water

To determine source water variations in the pot experiment (2009), a portion of the stem base was harvested, and immediately placed in sealed tubes and frozen with liquid nitrogen. Samples were kept frozen until water extraction using a cryogenic vacuum distillation line (Dawson and Ehleringer, 1993). $\delta^{18}O$ and $\delta^{2}H$ were measured in extracted water from stems in each of the 24 plots, although samples from the first block were discarded due to problems during distillation.

In the field trial in 2011, variations in source water were determined from pressed stem juice. Stem base segments were pressed with a high-pressure press, in order to obtain a liquid extract. An aliquot of this extract was passed through a 22-25 µm filter ("Miracloth", Merck KGaA, Darmstadt, Germany) and transferred to eppendorf tubes (for analysis of $\delta^{18}O$ in stem juice organic matter) and 2 ml glass vials with crimp cap (for analysis of $\delta^{18}O$ and $\delta^{2}H$ of water isotopes in stem juice). Eppendorf tubes were subsequently placed in an oven and dried at 60ºC until the solid residual was dry (48h-72h). As a reference, samples from irrigation water were placed directly in crimp-cap glass vials. Glass vials were sealed and sterilized in a water bath at 100ºC for two hours, to prevent fermentation processes, and kept cool until isotope analysis.

Stable isotope analyses

Leaves (base and apex), silks and grains were dried at 60 °C for 24h. The dried plant material was ground with a ball mill to obtain a homogenous powder and an aliquot of ca. 1 mg was transferred into silver capsules for the analysis of $\delta^{18}O$. Bulk leaves, silks and the dried extract of stem juice were all analyzed at the Centre for Systems Biology (ZBSA, University of Freiburg) in a high temperature conversion/elemental analyser (TC/EA; Finnigan MAT GmbH, Bremen, Germany), coupled to an isotope ratio mass spectrometer (Delta Plus or Delta Plus XP, Finnigan MAT GmbH, Bremen, Germany) by a Conflo II interface (Finningan MAT GmbH, Bremen, Germany). The precision for measurements was better than 0.2‰. For the bulk grains, the ^{18}O:^{16}O ratios 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 two secondary standards (IAEA 601 and IAEA 602) calibrated against Vienna Standard Mean Oceanic Water (VSMOW): the analytical precision was ~0.25‰. Analyses were conducted at Iso-Analytical Limited (Crewe, CheshireCW2 8UY, UK). The $\delta^{18}O$ of leaf base, leaf apex, silks, mature kernels and stem juice organic matter will be referred as $\delta^{18}O_{Lb}$, $\delta^{18}O_{La}$, $\delta^{18}O_S$, $\delta^{18}O_G$, and $\delta^{18}O_{stemOM}$, respectively.

Water isotopes ($\delta^{18}O$, $\delta^{2}H$) in distilled water (pot experiment 2009) and stem juice extracts (stem water, field experiment 2011) were determined by laser spectroscopy at the Serveis Científico-Tècnics of the Universitat de Lleida, using a Picarro L2120i, coupled to a high-precision vaporizer A0211. All samples were centrifuged at 12,000 g in order to remove any suspended solid, and the supernatant transferred to a glass vial with 250 ml insert. They were expressed in delta (δ) notation (‰) relative to V-SMOW (i.e. isotopic composition of oxygen, $\delta^{18}O$, and hydrogen, $\delta^{2}H$). Raw values were calibrated against three internal laboratory references (calibrated against IAEA standards VSMOW2, SLAP2 and GISP). Overall uncertainty (determined as the standard error of repeated analyses (N=20) of a reference sample not included in the calibration) was 0.05‰ and 0.17‰, for $\delta^{18}O$ and $\delta^{2}H$, respectively. The potential presence of organic contaminants was checked using the post-processing software Picarro ChemCorrect 1.2.0, giving in all cases negative results. For simplicity, both

distilled xylem water in the pot trial and pressed stem juice in the field trials will be referred as $\delta^{18}O_{stemW}$.

Large amounts of sugars reduce the performance of the vaporizer, thus juice samples were diluted to 50% with distilled water of known isotopic composition prior to injection. In preliminary tests, this proportion was found to be a good compromise of reducing sugar accumulation in the vaporizer while keeping an acceptable measuring accuracy. The precision for measurements, after considering the dilution effect, was better than 0.2‰ for $\delta^{18}O$ and 0.7‰ for $\delta^{2}H$. Original values of the sample were then recalculated from measured values and distilled water values using volume balance:

$$\delta^{18}O_{\text{sample}} = 2 * (\delta^{18}O_{\text{measured}} - 0.5 * \delta^{18}O_{\text{distilled water}}) \quad (1)$$

The magnitude of enrichment along the leaf lamina was determined as the difference between $\delta^{18}O_{La}$ and $\delta^{18}O_{Lb}$, denoted as $\delta^{18}O_{La\text{-}Lb}$. Such differences ($\delta^{18}O_{La\text{-}Lb}$) were correlated with either $\delta^{18}O_{La}$ and $\delta^{18}O_{Lb}$. The main purpose of such correlations was to test the following alternatives:

- In case a high correlation between $\delta^{18}O_{La\text{-}Lb}$ vs $\delta^{18}O_{La}$ it would suggest that differences within leaf apex and leaf base are mainly governed by leaf length (due to evaporative processes)
- Alternatively a high igh correlations between $\delta^{18}O_{La\text{-}Lb}$ vs $\delta^{18}O_{Lb}$ would suggest that differences within leaf apex and leaf base are mainly governed Péclet effect in leaf base.

Statistical analysis

Genotype and treatment effects were assessed by means of Analysis of Variance (ANOVA). In the 2009 trial, water regime, genotype and their interaction were included as fixed factors, including 3 blocks and two randomized replicates per block. In the 2010 trial, water regime, genotype, phenology and their interaction were included as fixed factors, with 2 blocks (nested to water regime and phenology) as replicates. In the 2011, water regime, genotype, and their interaction were included as fixed factors, with 2 blocks (nested to water regime) as replicates. Means were compared by Tukey's HSD test on a combination of water treatments and phenology. A bivariate correlation

procedure was applied to analyse the relationships between the measured traits. All Statistical analyses were performed using the SPSS 18.0 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using the Sigma-Plot 10.0 program (SPSS Inc.).

RESULTS

Oxygen isotope composition and ecophysiological traits across environments

Mean values of the different tissues analyzed and the agronomic and physiological variables measured in the pot experiment (2009) and the two consecutive field trials (2010 and 2011) are shown in Table 1. In the pot experiment in 2009, significant differences in the $\delta^{18}O$ in the leaf apex ($\delta^{18}O_{La}$), leaf base ($\delta^{18}O_{Lb}$) and stem water ($\delta^{18}O_{stemW}$) were not observed between well watered pots and drought stress pots. In 2010, all trials followed a similar trend, with $\delta^{18}O$ in the leaves being more enriched in comparison to the silks ($\delta^{18}O_S$), whereas mature grains ($\delta^{18}O_G$) exhibited the lowest values. As expected, isotopic enrichment increased from the base to the apex within the leaf, with higher values in the drought stressed trials compared to the well-watered controls. In 2011 both $\delta^{18}O_S$ and $\delta^{18}O_{stemW}$ exhibited significantly lower values in well watered (WW) than in the drought stressed (DS) trials. Conversely, $\delta^{18}O$ in the stem soluble organic matter ($\delta^{18}O_{stemOM}$) showed higher values in WW than in DS trials (2011).

Table 1. *Mean values of oxygen isotope composition of stem water ($\delta^{18}O_{stemW}$), stem soluble organic matter ($\delta 1^{8}O_{stemOM}$), and bulk organic matter in the leaf lamina apex ($\delta^{18}O_{La}$), the base ($\delta^{18}O_{Lb}$), leaf average ($\delta^{18}O_{L}$) silks ($\delta^{18}O_{S}$) and mature kernels ($\delta^{18}O_{G}$). Mean values of agronomical parameters and measurements of grain yield (GY), stomatal conductance (g_s), leaf temperature (T_L), hundred kernel weight (HKW), kernel number per ear (Kernel·ear-1) and anthesis to silking interval (ASI). Each value represents the means, with SE in parentheses, 2 genotypes with 6 replicates under well watered (WW) and drought stress (DS) conditions in 2009 and 50 genotypes per two replicates of early and late phenology (in 2010 field trial) and early phenology (in 2011 field trial), under well watered (WW) and drought stress applied at anthesis (DS). Values with different superscripted letters are significantly different according to Tukey's HSD test ($P < 0.05$), performed across treatments for each field trial.*

Trial		**Pot trial 2009**				**Field trial 2010**								**Field trial 2011**			
Genotypes		***Guadiana / Kermes***				**Early**				**Late**				**Early**			
Treatment		**DS**		**WW**		**DS**		**WW**		**DS**		**WW**		**DS**		**WW**	
$\delta^{18}O_{La}$	(‰)	33.5	(0.7)^{a}	33.3	(0.7)^{a}	32.7	(0.2)^{bc}	31.8	(0.1)^{a}	33.1	(0.2)^{c}	32.2	(0.2)^{ab}	-	-	-	-
$\delta^{18}O_{Lb}$	(‰)	29.3	(0.4)^{a}	29.7	(0.2)^{a}	30.2	(0.1)^{c}	29.8	(0.1)^{ab}	29.4	(0.2)^{a}	30.1	(0.2)^{c}	-	-	-	-
$\delta^{18}O_{L}$	(‰)	31.2	(0.3)^{a}	31.6	(0.3)^{a}	31.4	(0.1)^{b}	30.8	(0.1)^{a}	31.3	(0.2)^{ab}	31.2	(0.2)^{ab}	-	-	-	-
$\delta^{18}O_{S}$	(‰)	29	(0.2)^{a}	29.1	(0.2)^{a}	27.7	(0.1)^{b}	26.6	(0.1)^{a}	28.4	(0.1)^{c}	27.7	(0.1)^{b}	31.3	(0.1)^{b}	29.9	(0.1)^{a}
$\delta^{18}O_{G}$	(‰)	-	-	-	-	26.9	(0.1)^{b}	26.4	(0.1)^{a}	27.3	(0.1)^{c}	26.8	(0.1)^{b}	-	-	-	-
$\delta^{18}O_{stemOM}$	(‰)	-	-	-	-	-	-	-	-	-	-	-	-	33.9	(0.2)^{a}	36.2	(0.3)^{b}
$\delta^{18}O_{stemW}$	(‰)	-7.6	(0.3)^{a}	-7.6	(0.3)^{a}	-	-	-	-	-	-	-	-	-6.2	(0.1)^{a}	-7.2	(0.1)^{b}
GY	(T·Ha^{-1})	-	-	-	-	3.4	(0.1)^{b}	6.7	(0.1)^{c}	3.1	(0.1)^{a}	6.8	(0.1)^{c}	2	(0.1)^{a}	6.8	(0.1)^{b}
g_s (mmol H_2O m^{-2} s^{-1})		183.4	(21.7)^{a}	202.6	(20.9)^{a}	80.4	(2.2)^{b}	114.4	(4.3)^{c}	76.5	(2.8)^{b}	56.4	(2.5)^{a}	156	(2.4)^{a}	173.9	(2.1)^{b}
T_L	(°C)	33.7	(0.4)^{a}	33.5	(0.3)^{a}	33.6	(0.2)^{a}	33.4	(0.1)^{a}	34.2	(0.2)^{b}	36.3	(0.2)^{c}	35	(0.1)^{a}	33.1	(0.1)^{b}
HKW	(g)	-	-	-	-	17.1	(0.3)^{a}	26.9	(0.2)^{d}	18.0	(0.2)^{b}	25.4	(0.2)^{c}	16.2	(0.2)^{a}	27.0	(0.3)^{b}
Kernel·ear^{-1}		-	-	-	-	300.3	(4.9)^{a}	388.5	(5.2)^{c}	322	(5.2)^{b}	438.8	(6.7)^{d}	227	(5.0)^{a}	321.8	(4.8)^{b}
ASI	(days)	-	-	-	-	2.1	(0.1)^{ab}	2.6	(0.2)^{c}	1.8	(0.1)^{a}	2.4	(0.1)^{b}	3.4	(0.2)^{a}	2.5	(0.2)^{b}

The level of drought stress varied across experiments (Table 1). In the pot trial in 2009, the mild stress applied did not cause significant differences among treatments for the variables measured. In 2010, *GY* under drought stress was reduced by 49% and 54% relative to the WW control in the early and late experiments, respectively. In 2011, *GY* under drought stress was reduced by 71% relative to the WW control. Drought stress also significantly reduced the number of kernels per ear and *HKW* in both field trials. In the field trials, g_s was significantly reduced under drought stress. However, significant differences were observed only in averaged T_L between WW and DS, for the late phenology trials in 2010 and the early phenology trial in 2011.

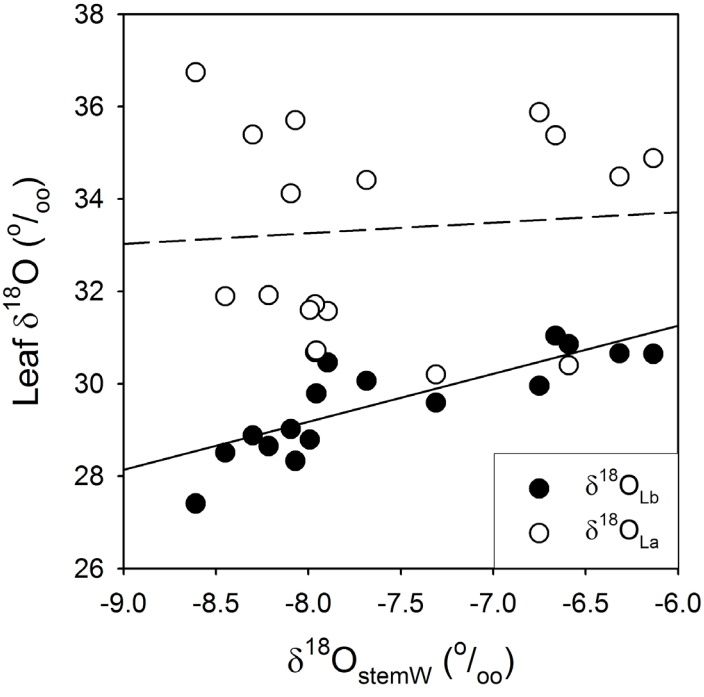

Fig. 2*. Linear correlation (N=17) between the oxygen isotopic composition of stem water (δ18OstemW) and the δ18O of bulk organic matter in the leaf apex ($\delta^{18}O_{La}$, r=0.075, P=0.751) and the leaf base ($\delta^{18}O_{Lb}$, r=0.775, P=0.0003), for the pot trial 2009.*

Correlations between oxygen isotope composition and ecophysiological variables

In the pot experiment 2009 (Fig. 2), the isotopic composition of oxygen in the stem water ($\delta^{18}O_{stemW}$) was positively correlated (pooling WW and DS pots together) with $\delta^{18}O_{Lb}$ (r= 0.775, N=17 $P < 0.001$). In contrast, no correlation was observed between $\delta^{18}O_{stemW}$ and $\delta^{18}O_{La}$. Apex-base difference ($\delta^{18}O_{La-Lb}$) was subsequently considered as a proxy for leaf water enrichment. On the other hand, E in the pot trial was negatively correlated with $\delta^{18}O_{La}$ (r=-0.515, N=24, P=0.0099, Fig. 3a), but not with $\delta^{18}O_{Lb}$ (r=0.228, N=24, P=0.283, Fig. 3b). Similarly, $\delta^{18}O_{La}$ showed a significant negative correlation with g_s both in the pot trial and across field trials (r=-0.447, N=24, P=0.029 and r=-0.204, N=400; $P <$ 0.01, respectively, Fig. 3a), but no consistent correlations were found between g_s and $\delta^{18}O_{Lb}$ (Fig. 3b). Except for the late DS trial, T_L showed no significant correlations with $\delta^{18}O_{La}$, but was positively correlated with $\delta^{18}O_{Lb}$ in the pot trial and across field trials (Fig. 3a,b). The oxygen isotope composition of bulk silks ($\delta^{18}O_S$) showed contrasting correlations with leaf variables (g_s, T_L, E) in the pot trial and across field trials (Fig. 3c), whereas the oxygen isotope composition of bulk grains ($\delta^{18}O_G$) showed similar patterns to leaf values ($\delta^{18}O_{La,}$ $\delta^{18}O_{Lb}$) across field trials (Fig. 3d).

Correlations between oxygen isotope composition in different plant parts

$\delta^{18}O_{La}$ was strongly correlated with $\delta^{18}O_{Lb}$ in all trials in 2010 ($P < 0.001$) with the exception of the early phenology trials in the WW regime (Table 2). Conversely no correlation was observed between $\delta^{18}O_{La}$ and $\delta^{18}O_{Lb}$ in the pot experiment in 2009. Even though $\delta^{18}O_G$ was weakly correlated with $\delta^{18}O_{La}$, $\delta^{18}O_{Lb}$ and $\delta^{18}O_S$ ($P < 0.05$), a clear trend was not observed within trials in 2010. Moreover in 2011 δ18OstemW was positively correlated with the $\delta^{18}O_S$ in the DS trial ($P < 0.001$), whereas no relationship was observed against to $\delta^{18}O_{stemOM}$. In the WW trial, significant associations to $\delta^{18}O_{stemW}$ were not observed.

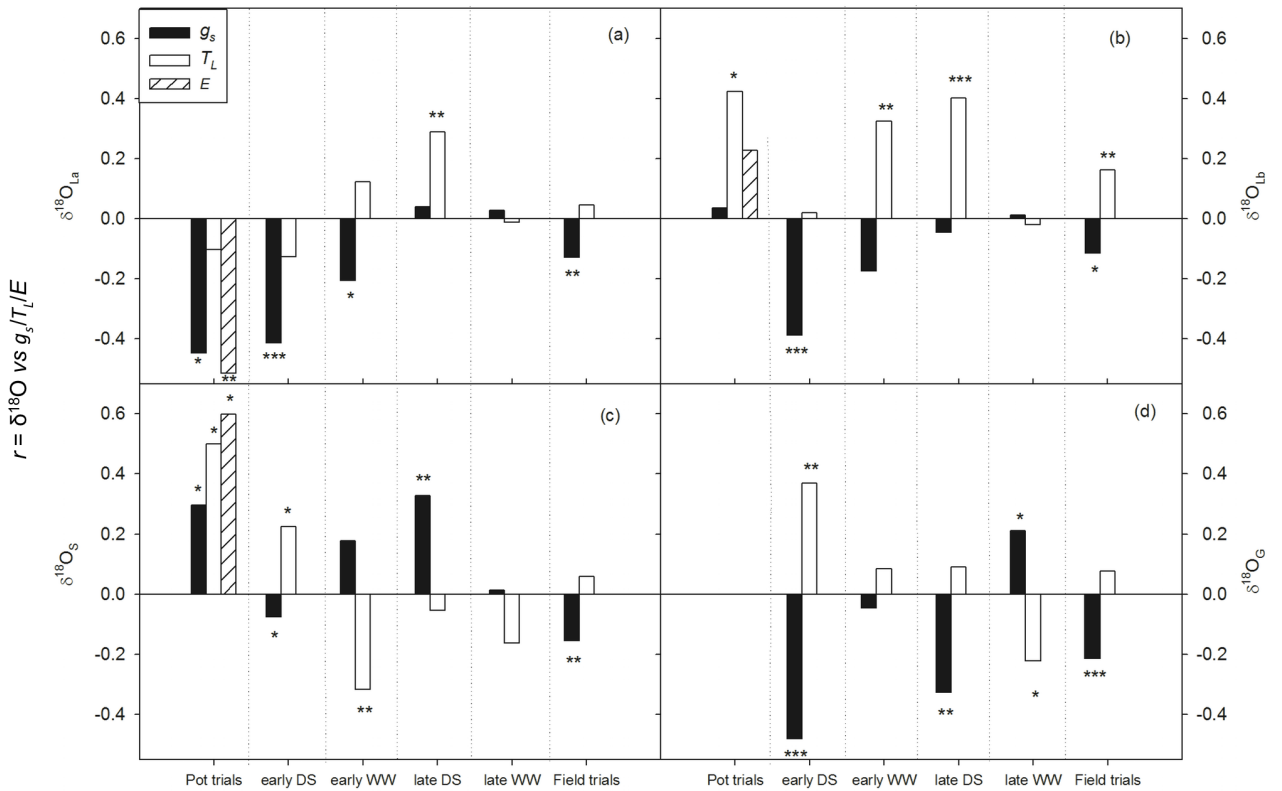

Fig. 3. *Pearson correlation of the relationship between leaf-level physiological variables (g_s, stomatal conductance; T_L, leaf temperature; E, transpiration rates) against oxygen isotope composition of bulk organic matter in the (a) leaf lamina apex ($\delta^{18}O_{La}$), (b) the base ($\delta^{18}O_{Lb}$), (c) silks ($\delta^{18}O_S$) and (d) mature kernels ($\delta^{18}O_G$).*

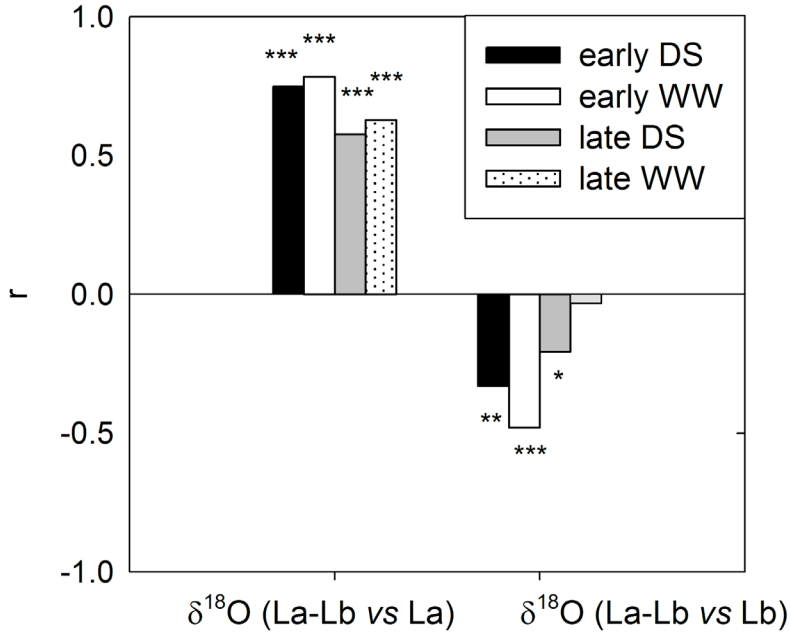

Fig. 4*. Determination coefficients of the relationship between the enrichment along the leaf lamina ($\delta^{18}O_{La-Lb}$) against bulk organic matter in the leaf apex ($\delta^{18}O_{La}$) and the leaf base ($\delta^{18}O_{Lb}$). Level of significance: ***, P < 0.001; **, P < 0.01; * P < 0.05. Data from field trial 2010.*

In the field trials performed in 2010 (Fig. 4), $\delta^{18}O_{La-Lb}$ was linearly correlated with $\delta^{18}O_{La}$ at all growing conditions (P < 0.001). However, weaker correlations were observed between the $\delta^{18}O_{La-Lb}$ versus $\delta^{18}O_{Lb}$ (P < 0.001; P < 0.01; P < 0.05) for the four trials.

Table 2. *Pearson correlation of the relationship between oxygen isotope composition of stem water ($\delta^{18}O_{stemW}$), stem soluble organic matter ($\delta^{18}O_{stemOM}$), and bulk organic matter in the leaf lamina apex ($\delta^{18}O_{La}$), the base ($\delta^{18}O_{Lb}$), silks ($\delta^{18}O_{S}$) and mature kernels ($\delta^{18}O_{G}$). The 2 genotypes with 6 replicates under well watered (WW) and drought stress (DS) conditions in 2009 and 50 genotypes per two replicates of early and late phenology (in 2010 field trial) and early phenology (in 2011 field trial), under well watered (WW) and drought stress applied at anthesis (DS) were considered. Level of significance ***, P < 0.001; **, P < 0.01; * P < 0.05; ns, not significant.*

Trial	**Pot trial 2009**		**Field trial 2010**				**Field trial 2011**	
Genotypes	***Guadiana/Kermes***		**Early**		**Late**		**Early**	
Treatment	**DS**	**WW**	**DS**	**WW**	**DS**	**WW**	**DS**	**WW**
$\delta^{18}O_{La}$ *vs* $\delta^{18}O_{Lb}$	-	0.323^{ns}	0.378^{***}	0.169^{ns}	0.680^{***}	0.756^{***}	-	-
$\delta^{18}O_{La}$ *vs* $\delta^{18}O_{S}$	-	-0.009^{ns}	0.058^{ns}	0.112^{ns}	0.175^{ns}	0.048^{ns}	-	-
$\delta^{18}O_{La}$ *vs* $\delta^{18}O_{G}$	-	-	0.202^{*}	0.195^{ns}	0.035^{ns}	0.013^{ns}	-	-
$\delta^{18}O_{La}$ *vs* $\delta^{18}O_{stemW}$	-	0.341^{ns}	-	-	-	-	-	-
$\delta^{18}O_{Lb}$ *vs* $\delta^{18}O_{S}$	-	-0.220^{ns}	0.045^{ns}	0.011^{ns}	0.279^{**}	0.150^{ns}	-	-
$\delta^{18}O_{Lb}$ *vs* $\delta^{18}O_{G}$	-	-	0.252^{*}	-	-	0.017^{ns}	-	-
$\delta^{18}O_{Lb}$ *vs* $\delta^{18}O_{stemW}$	0.864^{***}	0.636^{ns}	-	-	-	-	-	-
$\delta^{18}O_{S}$ *vs* $\delta^{18}O_{stemW}$	0.281^{ns}	0.298^{ns}	-	-	-	-	0.345^{***}	0.145^{ns}
$\delta^{18}O_{S}$ *vs* $\delta^{18}O_{G}$	-	-	-	0.180^{ns}	-	-	-	-
$\delta^{18}O_{stemOM}$ *vs*	-	-	-	-	-	-	0.077^{ns}	-
$\delta^{18}O_{stemOM}$ *vs* $\delta^{18}O_{S}$	-	-	-	-	-	-	0.091^{ns}	0.019^{ns}

***Table 3**. Pearson correlation of the relationship between the grain yield (GY) and the oxygen isotope composition of stem water ($\delta^{18}O_{xylem}$), stem soluble organic matter ($\delta^{18}O_{stemOM}$), and bulk organic matter in the leaf lamina apex ($\delta^{18}O_{La}$), the base ($\delta^{18}O_{Lb}$), silks ($\delta^{18}O_{S}$) and mature kernels ($\delta^{18}O_{G}$). 50 genotypes per two replicates of early and late phenology (in 2010 field trial) and early phenology (in 2011 field trial), under well watered (WW) and drought stress applied at anthesis (DS) were considered. Level of significance ***, P < 0.001; **, P < 0.01; * P < 0.05; ns, not significant.*

Trial	**Field trial 2010**					**Field trial 2011**		
Genotypes	**early**		**late**		**Across trials**	**early**		**Across trials**
Treatment	**DS**	**WW**	**DS**	**WW**		**DS**	**WW**	
GY vs								
$\delta^{18}O_{La}$	-0178^{ns}	0.066ns	0.049^{ns}	0.019^{ns}	-0.218***	-	-	
$\delta^{18}O_{Lb}$	-0.141^{ns}	0.067^{ns}	-0.044^{ns}	-0.037^{ns}	0.048^{ns}	-	-	
$\delta^{18}O_{S}$	0.052^{ns}	-0.062^{ns}	0.097^{ns}	0.132^{ns}	-0.346***	-0.353***	-0.087^{ns}	-0.591***
$\delta^{18}O_{G}$	0.030^{ns}	0.045^{ns}	0.018^{ns}	0.136^{ns}	-0.288***	-	-	
$\delta^{18}O_{stermW}$	-	-	-	-	-	-0.338**	-0.118^{ns}	-0.562***
$\delta^{18}O_{stemOM}$	-	-	-	-	-	-0.364***	-0.238*	-0.365***

Correlations between oxygen isotope composition and grain yield

The relationship between *GY* and oxygen isotope composition of different tissues, depending on water regime, phenology group and trial are presented in Table 3. In the field experiment in 2010, *GY* did not exhibit significant intra-trials (genotypic) correlations with $\delta^{18}O_{La}$, $\delta^{18}O_{Lb,}$ $\delta^{18}O_S$ and $\delta^{18}O_G$. However, $\delta^{18}O_{La}$, $\delta^{18}O_S$ and $\delta^{18}O_G$ were significantly correlated across-trials with GY (r=-0.218, *N*=400, *P*<0.001; r=-0.347, *N*=400 *P*<0.001; r=-0.288, *N*=400, *P*<0.001, respectively). Still *GY* was negatively and significantly associated with $\delta^{18}O_{stemOM}$ in 2011, although the correlation coefficient was higher in DS (*r* = -0.354; *N*=100, *P* < 0.001) compared to WW (*r* = -0.238, *N*=100, *P* < 0.05). In addition, negative and significant relationships were observed for *GY* with $\delta^{18}O_S$ (*r*=-0.353, *N*=100, *P*<0.001) and $\delta^{18}O_{stemW}$ (*r*=-0.338; *N*=100, P < 0.001) in the DS trial. Moreover, *GY* across trials was well correlated against $\delta^{18}O_{stemOM}$, (*r*=0.356; *N*=200, P < 0.001) and $\delta^{18}O_{stemW}$ (*r*=0.562; *N*=200, P < 0.001). Thus, in order to assess the role of different plant tissues (independent variables) analysed ($\delta^{18}O_{stemOM}$, $\delta^{18}O_S$, $\delta^{18}O_{stemW}$), which contribute to *GY* (dependent variable), a stepwise regression analysis was performed for each of the two water regimes (DS and WW) and all treatments together (DS+WW) in the field trial 2011 (Table 4). For both DS and WW trials, the independent first variable chosen by the model was $\delta^{18}O_{stemOM}$ (*r*=0.364, *N*=100, *P* < 0.001; *r*=0.238, *N*=100, *P* < 0.001, respectively). In the DS trials, the second variable chosen was $\delta^{18}O_S$, followed by $\delta^{18}O_{stemW}$. Conversely including DS and WW treatments together, the first variable chosen was $\delta^{18}O_S$

***Table 4**. Stepwise analysis for the whole set of 50 genotypes per two replicates in field trial in 2011 of the early phenology in drought stress (DS), well watered (WW) and DS and WW treatments together conditions (DS+WW) with GY as a dependent variable, and stem soluble organic matter ($\delta^{18}O_{stemOM}$), bulk silks ($\delta^{18}O_s$) and stem water ($\delta^{18}O_{stemW}$) as independent variables. Level of significance: ***, P < 0.001*

Treatment	Variable chosen	*r*	R^2	Significance
DS	$\delta^{18}O_{stemOM}$	0.364	0.123	***
	$\delta^{18}O_{stemOM}$, $\delta^{18}O_S$	0.486	0.220	***
	$\delta^{18}O_{stemOM}$, $\delta^{18}O_S$, $\delta^{18}O_{stemW}$	0.531	0.259	***
WW	$\delta^{18}O_{stemOM}$	0.238	0.057	***
WW+DS	$\delta^{18}O_S$	0.591	0.345	***
	$\delta^{18}O_S$, $\delta^{18}O_{stemW}$	0.676	0.451	***
	$\delta^{18}O_S$, $\delta^{18}O_{stemW}$, $\delta^{18}O_{stemOM}$	0.487	0.479	***

DISCUSSION

Evaporative enrichment of leaf water

The isotopic enrichment along the leaf ($\delta^{18}O_{La-Lb}$) was well correlated with the $\delta^{18}O$ in the leaf apex ($\delta^{18}O_{La}$), but not with the base ($\delta^{18}O_{Lb}$) (Fig. 4). This indicates that the main source of variation for leaf $\delta^{18}O$ is the evaporative enrichment along the leaf blade, rather than source water. Gan et al. (2003) demonstrated in maize leaves, that the *Péclet* effect associated with longitudinal flow was larger than the radial *Péclet* effect (associated to a gradient perpendicular to flow). Hence, $\delta^{18}O_{La}$ is the result of accumulated enrichment of xylem water occurring along the leaf blade, and thus the difference between apex and base constitutes a good proxy for leaf-water enrichment. The consistent negative correlation found between $\delta^{18}O_{La}$ and both E and g_s (Fig. 3), further supports a major role of the *Péclet* effect in determining $\delta^{18}O$ in longitudinal leaves.

Moreover, $\delta^{18}O$ of primary assimilates synthesized in the leaf will be subjected to exchange with i) xylem water entering the leaf usually through an upstream flow (metabolic processes) and ii) water which has been isotopically enriched during transpiration (environmental processes). In our pot experiment in 2009, $\delta^{18}O_{stemW}$ correlated with $\delta^{18}O_{Lb}$ (Fig. 2) suggesting in this experiment mainly metabolic processes (i; mentioned above) were affecting $\delta^{18}O$ in the primary assimilates of the leaf base. In contrast, no correlations were observed between $\delta^{18}O_{stemW}$ and $\delta^{18}O_{La}$ (Fig. 2) indicating that environmental processes (ii; mentioned above) are mostly affecting the $\delta^{18}O$ in the leaf apex, as a consequence of leaf transpiration leading to evaporative enrichment of leaf water (Gonfiantini et al. 1965; Yakir et al. 1990; Farquhar et al. 1993). A high significance of leaf evaporative enrichment was also supported by the results obtained in the field trial 2010, where $\delta^{18}O_{La}$ was significantly more enriched in comparison to $\delta^{18}O_{Lb}$ at all growing conditions (Table 1).

In agreement with more severe drought conditions in 2011 than in 2010, we found greater $\delta^{18}O$ in the silks for this season, particularly in the DS trial (+3.5‰ and +3.3‰, for DS and WW, respectively, see Table 1). In contrast, the $\delta^{18}O$ of stem soluble organic matter ($\delta^{18}O_{stemOM}$) appears to be highly enriched under well-watered conditions (36.2‰). This trend goes against the presumably higher leaf evaporative enrichment in drought-stressed plants (Farquhar and Lloyd, et

al. 1993; Cernusak et al. 2003). This apparent contradiction may be a consequence of a greater turnover time of non-structural sugars in the stem of drought-stressed plants, leading to greater exchange rates with stem water, as compared to well-watered plants, resulting in $\delta^{18}O$ values closer to those of leaf-exported sugars (Song *et al.*, 2014; Gessler *et al.*, 2014). This effect could have been enhanced by a greater basipetal allocation of sugars under drought to promote root development (Palta and Gregory, 1997), thus increasing the relative size of the sugar pool at the stem base and decreasing the divergence in $\delta^{18}O$ between both organs (Gessler *et al.*, 2014).

Oxygen isotopes from source to sink

In the field trial 2010, $\delta^{18}O_S$ and $\delta^{18}O_G$ exhibit lower average values compared to both $\delta^{18}O_{Lb}$ and $\delta^{18}O_{La}$ (Table 1), indicating that the oxygen signal in silks and grain might have exchanged oxygen isotopes with the non-enriched ^{18}O source water (with low values of $\delta^{18}O$). In addition, $\delta^{18}O_G$ exhibit lower values compared to $\delta^{18}O_S$, suggesting that the proportion of oxygen atoms exchanged with unenriched source water was higher during grain filling, as compared to silk formation. Since sink strength rises during grain filling, turnover time of carbon reserves increases, thereby providing extra chances to ^{18}O from sucrose to exchange oxygen atoms with un-enriched water (Barbour and Farquhar, 2000). Results from the 2011 trial partially support this observation, showing a positive trend between $\delta^{18}O_{stemW}$ and $\delta^{18}O_S$, although only significant for the DS trial. In contrast, the clear lack of correlation between $\delta^{18}O_{stemW}$ and $\delta^{18}O_{stemOM}$ suggests that assimilates may exchange oxygen atoms with water after unloading of assimilates from the phloem into sink tissue, rather than before unloading. Such a correlation would have indicated that oxygen water has been exchanged with assimilates in the phloem, before entering the silks. Sternberg et al. (1986) found similar results in castor bean, where oxygen atoms from sucrose synthesized in the leaf and transported in the phloem were exchanged with un-enriched ^{18}O water during cellulose synthesis after unloading from the phloem.

Physiological maturity in kernels is reached approximately 55-65 days after silking (Farnham *et al.*, 2003), indicating that the two organs might develop at different temperature and water status. Indeed, maize is particularly susceptible to drought around flowering (Birch *et al.*, 2008). Differences in water status

during development are to be expected in the present study not only for well watered but also for water stressed trials, since irrigation was resumed just after flowering and, therefore, plants experienced more water stress around flowering than during most of the grain filling. However, relatively small differences in $\delta^{18}O_S$ and $\delta^{18}O_G$ were observed. Therefore, low differences between $\delta^{18}O$ values in the silks compared to grains ($\delta^{18}O_{S-G}$) might be partly attributed to small differences in temperature and water conditions during silking and grain filling. However, in that case we would expect greater differences with extended phenology (i.e. larger ASI), but we did not find any significant correlation between ASI and $\delta^{18}O_{S-G}$ (data not shown). However, in the analysis performed during 2011 field trial, first variable chosen by the stepwise analysis accounting for variations in GS across DS and WW conditions was $\delta^{18}O_S$ (Table 4). Such results suggest that oxygen isotopic signal was better recorded in the silks compared to grains. Such better performance on silks compared to grains was also supported by correlations of $\delta^{18}O$ in all tissues against GY in field trials 2010 (in Table 3), where $\delta^{18}O$ of silks showed higher correlation coefficient compared to grains and leaves. On the other hand, we found a wider range of variation in the $\delta^{18}O$ of silks, as compared to grains, in agreement with the "dampening" effect generally found on the way from source to sink tissues (see e.g. Offermann et al. 2011; Gessler *et al*. 2014). Hence, isotopic results may reflect that grains are located farther than silks in the source-to-sink chain of transport and metabolic conversions. During grain filling, phloem transport of sugars to maize kernels proceeds via the pedicel, where sucrose is cleaved to glucose and fructose (See Fig. 1) and resynthesized into sucrose before being converted to starch (Felker and Shannon, 1980; Griffith *et al.*, 1987). Starch is known to be the main compound (70%) in the maize kernel (Nelson and Pan, 1995; James *et al.*, 2003). As a consequence of sucrose hydrolysis and subsequent resynthesis, extra chances to exchange oxygen with water are provided (Barbour and Farquhar, 2000). Such exchange associated with metabolic conversions might have affected the $\delta^{18}O$ in the present study as assimilates experience a high number of metabolic conversions during their way from the leaves to the reproductive organs and finally the grains (Farquhar and Lloyd, 1993). However, it has to be noted again that leaves and grains develop at different time scales, which makes interpretation and comparison of $\delta^{18}O$ between different tissues more complex.

Implications for plant breeding and crop ecophysiology

In our study, we did not find consistent intra-trial (genotypic) correlations between $\delta^{18}O$ and either *GY* (Table 3) or physiological variables (Fig. 3). An explanation of such uncoupling between $\delta^{18}O$ and leaf physiological processes might be a consequence of the high number of metabolic conversions affecting the $\delta^{18}O$ of assimilates transported from source to sink tissues. Conversely, *GY* and physiological variables were well correlated with $\delta^{18}O$ across trials in 2009, 2010 and 2011. This indicates that the range of response of $\delta^{18}O$ against environmental factors is much higher than that caused by metabolic conversions and, indeed, the $\delta^{18}O$ in all tissues analyzed was well correlated with g_s (Fig. 3). Furthermore, $\delta^{18}O$ in the grain showed even higher correlation against g_s (Fig. 3d) than leaves and silks, indicating that leaf-level conditions can still be recorded in growing kernels. Up to now, the use of $\delta^{18}O$ has been proposed for breeding as a predictor of grain yield under well watered conditions in wheat (Barbour et al. 2000). Although studies testing $\delta^{18}O$ in maize under water stress conditions are more scarce, our results showing the negative correlation within leaf and kernel $\delta^{18}O$ vs g_s are consistent with the theroetical basis (Pande *et al.*, 1995). In a study performed with maize by Cabrera-Bosquet et al. (2009b), under severe stress conditions genotypes showing higher $\delta^{18}O$ were the most productive, whereas under well-watered conditions those genotypes with lowest $\delta^{18}O$ showed the highest yield. In our case, however, we only found significant negative trends between *GY* and $\delta^{18}O$ in both DS and WW, probably due to the relatively mild stress level achieved in our trials. Thus, intra-trials $\delta^{18}O$ comparison for genotype selection should be interpreted with caution, because depending on water regime, genotypes might present different plant response.

CONCLUSIONS

Our results suggest that the $\delta^{18}O$ in sink tissues still reflects physiological responses at the leaf level, and thus might be applied as phenotyping tools integrating environmental factors throughout the crop cycle. However, at the genotype level, *GY* was only marginally correlated with $\delta^{18}O_{stem}OM$, suggesting that there is still a lack of knowledge on mechanism controlling $\delta^{18}O$ transfer from autotrophic to heterotrophic tissues. Depletion of $\delta^{18}O$ by exchange with water in the silks and the grains suggests a source-sink gradient related to metabolic conversions. According to our observations, exchange with un-

enriched source water is likely to occur within the sink tissues, rather than during phloem transport. This finding will eventually help to discard plant tissues, which are more susceptible to post-photosynthetic fractionation processes and which may help to understand the use of $\delta^{18}O$ as a genotype selection tool for the adaptation of maize and other crops to drought. Additionally, our findings may help to interpret source-sink relationships. Nevertheless, although $\delta^{18}O$ the grains and leaves were linearly correlated with *GY*, the silks were the only tissue related with *GY* in linear regression approach (stepwise analysis across trials). Therefore, silks could be reflecting evapo-transpirative demand during female flowering. This is not trivial since silks extrusion has been related to final kernel number per ear, and thus the productivity of the plant. Thereby, $\delta^{18}O$ in the silks might help to select potential lines in maize better adapted to drought. However, further analyses are required to help understanding $\delta^{18}O$ transfer during retranslocation of carbohydrates from source to sink organs, and to use the $\delta^{18}O$ of different organic matter pools for interpreting ecophysiological studies and selection in plant breeding.

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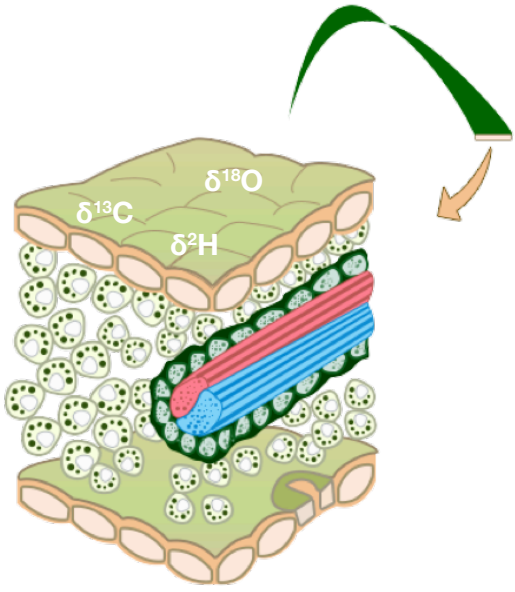

CAPÍTOL 5

Estudi Comparatiu del comportament de la $\delta^{13}C$, la $\delta^{18}O$ i la $\delta^{2}H$ com a eina de fenotipejat per a l'adaptació del blat dur a diferents condicions d'aigua i de nitrogen

CHAPTER 5

Comparative performance of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ for phenotyping durum wheat adaptation to different water and nitrogen conditions

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RESUM CAPÍTOL 5

La sequera i la baixa fertilitat poden afectar molt al creixement de les plantes i per tant al rendiment del gra. Les noves eines de fenotipejat poden ajudar a la millora del rendiment dels cereals, a la seva adaptació a la sequera i a la manca de nitrogen. En aquest estudi es va avaluar la composició isotòpica de δ^2H com una eina fenotípica per a la millora del blat cultivat sota diferents condicions de reg i règims de nitrogen. El rendiment es va comparar amb la δ^2H i la de $\delta^{18}O$ i la de $\delta^{13}C$. L'estudi es va realitzar al camp en condicions d'ambient mediterrani en un conjunt de genotips (cultivars moderns i varietats locals) durant dos anys consecutius. La composició isotòpica es va analitzar en la matèria seca , la fracció soluble en aigua i l'aigua extreta dels diferents teixits. Pel que respecta als resultats, el contingut de N sota un estrès hídric combinat amb diferents règims de nitrogen, el paràmetre δ^2H va ser el millor alhora de predir el rendiment (GY), més que els altres dos isòtops,. La δ^2H i la $\delta^{13}C$ van correlacionar negativament amb la conductància estomàtica. La $\delta^{18}O$ analitzada en grans va correlacionar molt menys que els altres dos isòtops amb el rendiment. Aquest estudi demostra la utilitat de la δ^2H, ja sigui de forma independent o combinada amb la de la $\delta^{13}C$ i esporàdicament amb la de la $\delta^{18}O$ com una eina potencial per a la selecció de genotips per un elevat rendiment potencial. També pot ser útil sota una amplia gamma de condicions de creixement ja que pot integrar la fotosíntesi i la transpiració al llarg de l'evolució del conreu.

Comparative performance of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ for phenotyping durum wheat adaptation to different water and nitrogen conditions

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Summary

- Drought and low fertility can substantially affect plant growth and grain yield. Novel phenotyping tools may help breeding for yield potential and adaptation to drought and low nitrogen stresses simultaneously. This study evaluated the hydrogen isotope composition (δ^2H) as a phenotypic trait for wheat breeding under different water and nitrogen conditions. δ^2H performance was compared with those of oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) isotope composition.
- The study was performed under field conditions in a Mediterranean-type dryland environment. A set of genotypes (cultivars and landraces), were evaluated during two consecutive years. Isotope composition was analyzed in dry matter, water soluble fraction and water from different tissues.
- δ^2H performed better than the other two isotopes predicting grain yield (GY) and N content under water stress but contrasting N regimes. δ^2H similarly than $\delta^{13}C$ correlated negatively with stomatal conductance. When analyzed in kernels $\delta^{18}O$ performed much poorer than the other two isotopes predicting GY.
- This study illustrates the usefulness of δ^2H independently or combined with $\delta^{13}C$ and marginally with $\delta^{18}O$ as a potential tool for genotype selection for yield potential under a wide range of growing conditions providing time-integrated records of the photosynthetic and evaporative performance of the plant during crop development.

Keywords: carbon, oxygen, hydrogen, isotope composition, wheat, drought, nitrogen.

INTRODUCTION

Durum wheat (*Triticum turgidum* var. *durum*) is among the main crops cultivated in the Mediterranean basin (FAOSTAT 2012). Indeed, countries located in the Mediterranean basin are often simultaneously exposed to high evapotranspiration (Lobell et al. 2008) and low nitrogen availability (Oweis *et al.*, 1998; Sadras, 2004). Breeding for drought and low fertility resistance in wheat (Araus *et al.*, 2002) is a way of improving yield and stability of durum wheat. However, there is still a lack of accurate phenotyping tools which limits breeding for adaptation to drought and low nitrogen stresses simultaneously (Araus *et al.*, 2008).

The signature of stable carbon isotopes in plant matter provides time integrated information on plant water status through the crop cycle (Araus *et al.*, 2003). Carbon isotope composition ($\delta^{13}C$) of plant dry matter, frequently expressed as discrimination from surrounding air ($\Delta^{13}C$) has been used as a tool for screening genotypes of C3 crops with high transpiration efficiency (*TE*) (Farquhar & Richards, 1984; Richards *et al.*, 2002) over the period in which assimilates are deposed (Araus *et al.*, 2002, 2008). However, the use of $\delta^{13}C$ as a tool for screening genotypes with higher grain yield (*GY*) is not straightforward as relationship within $\delta^{13}C$ and *GY* depends on the water conditions experienced by the crop. Whereas negative relationship between $\delta^{13}C$ and *GY* (or positive between $\Delta^{13}C$ and *GY*) has been widely reported under well-watered and moderated stress conditions (Araus *et al.*, 1998, 2003; Fischer *et al.*, 1998; Monneveux *et al.*, 2005; Lopes & Reynolds, 2010); under very severe drought conditions, positive relationship between $\delta^{13}C$ and *GY* has been observed (Rebetzke *et al.*, 2002; Condon *et al.*, 2002), being genotypes with conservative strategy (high *WUE* associated to high $\delta^{13}C$) the most productive (Voltas *et al.*, 1999; Araus *et al.*, 2003; Condon *et al.*, 2004). In C_3 plants ^{13}C discrimination mainly occurs within two steps during CO_2 uptake,: (i) CO_2 diffusion from the air to the internal gas space through the boundary layer and stomata and (ii) carboxilation reaction by Rubisco (Farquhar *et al.*, 1982). The $\delta^{13}C$ imprinted in the assimilates may be further affected by post-photosynthetic reactions (Badeck *et al.*, 2005) and transport. On the other hand, the effect of nitrogen (N) fertilization on $\delta^{13}C$ still remains unclear and contradictory results have been reported. Thus, by increasing N supply, $\delta^{13}C$ in wheat has been observed either to decrease (Shangguan et al. 2000), increase (Cabrera-Bosquet *et al.*, 2007) or not be affected (Hubick *et al.*, 1990).

Alternatively, within the recently years interest is growing on using oxygen isotope composition $\delta^{18}O$ in plant matter as it integrates evaporative conditions during crop cycle (Barbour *et al.*, 2000a; Barbour, 2007a). Such growing interest lies on the fact that $\delta^{18}O$ is only affected by stomatal conductance (g_s), whereas $\delta^{13}C$ is affected by g_s and photosynthetic CO_2 fixation (Barbour et al. 2000; Farquhar et al. 2007). Thus ^{18}O is enriched in leaves or other transpiring organs with regard the source water (Gonfiantini *et al.*, 1965; Farquhar, 1989; Pande *et al.*, 1995). Even so, the use of $\delta^{18}O$ is not straightforward as miscellaneous factors can affect the use of this trait for breeding(Barbour & Farquhar, 2000). Thus for example $\delta^{18}O$ of photoassimilates may be affected by the isotopic composition of water source taken up by the plant (Yakir *et al.*, 1990a; Roden *et al.*, 2000; Williams *et al.*, 2005), by the plant height and leaf length or by plant the post-photosynthetic fractionation during biochemical reactions involved in the synthesis of organic matter (Farquhar & Lloyd, 1993) and its subsequent transport within the plant (Offermann *et al.*, 2011b). Nonetheless, despite the factors above explained which may affect $\delta^{18}O$ of organic matter, $\delta^{18}O$ has been used to evaluate plant response under different treatments and genotypic variability on yield in wheat (Barbour *et al.*, 2000a; Barbour, 2007a; Araus *et al.*, 2013a). Genotypic correlations between $\delta^{18}O$ and *GY* have been found to be negative under well water conditions (Cabrera-Bosquet et al. 2009a) or positive under severe drought conditions (Cabrera-Bosquet et al. 2009b). Furthermore, studies combining effect of N supply (Cernusak *et al.*, 2007) and water regime on $\delta^{18}O$ (Cabrera-Bosquet et al. 2009) are still scarce. Hence, there is still a lack of knowledge regarding the relative importance of different processes, which may alter the $\delta^{18}O$ within different plant organs.

Hydrogen isotopic composition ($\delta^{2}H$) has been used, like $\delta^{18}O$, in plant ecology to assess growing conditions or the source of water used by plants (Epstein *et al.*, 1977; Schwendenmann *et al.*, 2015). Similarly to $\delta^{18}O$, $\delta^{2}H$ of plants is not only influenced by plant physiological behavior such as stomatal conductance but also by effects of climate in transpiration and by $\delta^{2}H$ of source of water (Sternberg *et al.*, 1984). Thus, high correlation between oxygen and hydrogen isotopic composition of organic matter may indicate source (i.e. water) effect (Epstein *et al.*, 1977), while the lack of correlation would suggest additional oxygen and/or hydrogen isotope fractionation effect associated with biochemistry (Sternberg *et al.*, 1986b). However differently of

$\delta^{18}O$ in terrestrial plants, $\delta^{2}H$ of organic matter is also affected by photosynthetic metabolism and has been proposed as a proxy to assess CAM metabolism in plants (Sternberg *et al.*, 1984). In fact, photosynthesis has a major impact on the $\delta^{2}H$ of plant organic matter (Yakir 1992). However, even when the mechanisms related to photosynthetic metabolism affecting $\delta^{2}H$ in plant are insufficiently understood (Yakir, 1992), these appear clearly different than those determining $\delta^{13}C$. It has been reported that recent autotrophically-produced cellulose in leaves might be depleted in ^{2}H compared to available water (Yakir *et al.*, 1990a). Conversely, during heterotrophic metabolism ^{2}H become enriched as large proportion of hydrogens are exchanged with water (Ziegler 1989). Hence, photosynthesis produces depletion in ^{2}H of carbohydrates with carbon-bound hydrogen (fractionation factor around -170‰), whereas post-photosynthetic metabolism has the opposite effect (+150‰) (Yakir, 1992). Therefore, final $\delta^{2}H$ in leaf carbohydrates will be a balance within both opposite processes (Yakir *et al.*, 1990b).

Combination of $\delta^{2}H$ and $\delta^{18}O$ could be useful approach in characterizing genotypic mechanisms and pattern of water uptake and loss and metabolic processes at various levels in vegetative parts and seeds of wheat plants. In addition combination of $\delta^{2}H$ and $\delta^{13}C$ can provide information on the metabolic functioning of a photosynthetic tissue, and autotrophic or heterotrophic carbohydrates translocation. Thus, a comprehensive understanding is required of how genotypic variability for C, O and H isotope composition of the photosynthetic and transpiring tissues (e.g. the flag leaf and ear for durum wheat) and the sink (grains) is affected by water and nitrogen simultaneously.

The objective of this study was to compare of $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ analyzed in dry matter and water soluble fraction of leaves and ears, as well as in mature kernels to assess genotypic differences in durum wheat performance under a combination of different water and nitrogen conditions. To the best of our knowledge there are not studies reporting on the role of $\delta^{2}H$ as a phenotypic trait for breeding and therefore studies comparing the performance of these three stable light isotopes ($\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$) assessing genotypic differences in grain yield are absent. Thus a set modern cultivars and landraces, were evaluated together during two consecutive years under Mediterranean conditions. The reason to compare cultivars and landraces is worthwhile as it could provide clues on how past advances in breeding have been related to physiological mechanisms (such as water uptake, transpiration, photosynthesis).

Material and Methods

Germplasm used and experimental conditions

Nine durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf. Husn.) cultivars were used: five historical Spanish landraces (*Blanqueta, Griego de Baleares, Negro, Jerez 37* and *Forment de Artes*) and five modern Spanish commercial varieties delivered after 1990 (*Anton, Bolo, Don Pedro, Regallo* and *Sula*). The selection criteria for the landraces were based on their phenology, selecting for greatest similarity to the phenology of modern cultivars. The field experiments were conducted during the spring growing seasons of 2010 and 2011 at the experimental field of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) in Aranjuez, central Spain (40º03'N, 3º31'E, 500 m above sea level) with experimental conditions explained elsewhere (Sanchez-Bragado *et al.*, 2014a). Two water treatments (support irrigation, WW, and rain-fed, WS) combined with two nitrogen regimes (fertilized, HN, and non-fertilized, LN) were assayed. The trials were planted on 30 December 2010 and 18 November 2011 in plots with 6 rows 0.20 m apart, accounting for a total area of 7.1 m^2 (5 m length and 1.2 m width) per plot. The total accumulated precipitation during 2010 and 2011 seasons was 275.4 and 126.1 mm respectively. For both years sprinkler irrigation was applied to irrigation plots around initiation of booting (beginning of April) and grain filling (around May 15th and 30th) with an approximated amount of 60 mm for each date. Environmental conditions during the growing seasons are detailed in Fig. 1. Prior to sowing, all trials received 60 kg ha^{-1} of phosphorous as superphosphate (18%) and 60 kg ha^{-1} potassium as potassium chloride (60%). Further, the HN plants were dressed with nitrogen applied at the beginning of tillering (January 27th in 2010 and January in 2011) and jointing (March 20th in 2010 and March in 2011) using a dose of 45 kg ha^{-1} and 105 kg ha^{-1} of urea (46%), respectively. The LN plants were not N fertilized, relying exclusively on the N availability in the soil before sowing. The arrangement of water and nitrogen treatments was carried out according to a split-split plot design with three replicates. Experiment plots were kept free of weeds, insect pests and diseases by recommended chemical measures (Sanchez-Bragado et al. 2014).

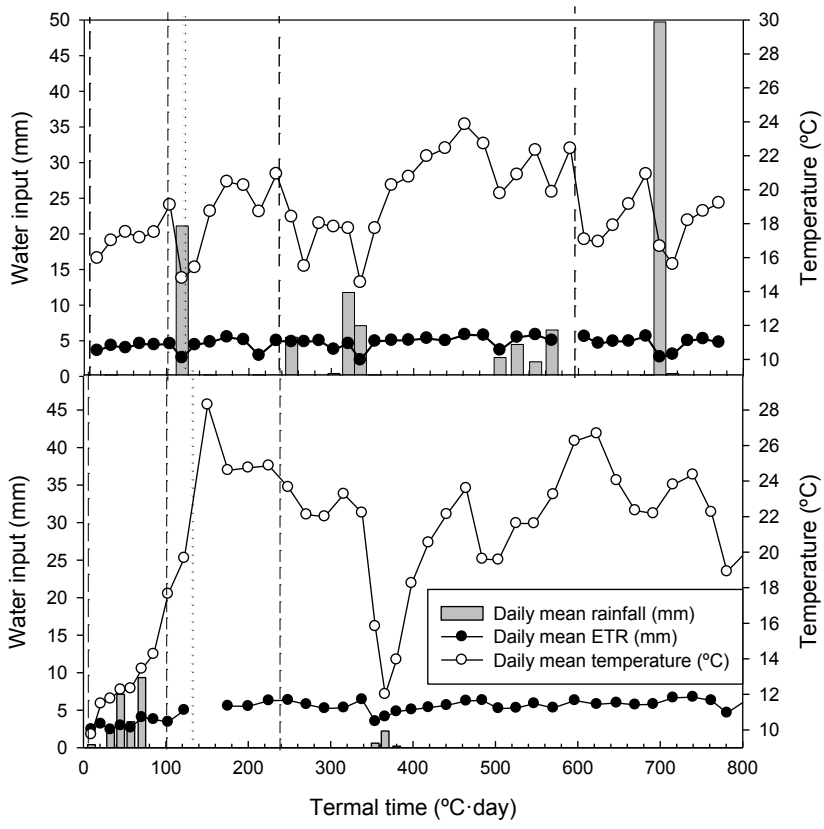

Fig. 1 *Daily mean precipitation (mm), evapotranspiration (mm) and air temperature (ºC) during the growing season from flowering to physiological maturity expressed as thermal time (ºC·day) during 2010 crop season (upper panel) and 2011 crop season (lower panel). Vertical dotted lines symbolize sampling dates and vertical dashed lines represent dates of irrigation.*

Days to anthesis were recorded as the number days from sowing to 50% of ears showing extruded anthers along their head lengths (Simmons et al. 1995). Sampling was performed around seven days after anthesis (7th May) in 2010 and two weeks after anthesis (18th April) in 2011. In 2010 genotype *Foment de Artes* was discarded due to late phenology. Also, in 2011 old landraces under supply irrigation treatment were discarded due to lodging. In crop season 2010 roots were collected from the upper layer (0-10cm) with a soil auger, further rinsed with distilled water and then placed in inside a paper envelope. Thereafter, five representative flag leaves and ears were collected per plot, oven dried together with collected roots at 70ºC for 48 hours,

weighed and finely ground for hydrogen, oxygen and carbon isotope analysis (on total dry matter). In 2011, flag leaves and developing grains from five representative tillers were collected and immediately frozen for further water extraction (see below). Stomatal conductance (g_s) was measured with a leaf porometer (Decagon; http://www.decagon.com) in five flag leaves per plot. At maturity, the central four rows of each plot were harvested and grain yield (*GY*) recorded. Subsequently, mature kernels were processed as explained below for isotope analysis. In addition, phenology was recorded throughout the cycle using the Zadoks scale (Zadoks *et al.*, 1974). Harvest was performed manually and by machine in 2010 and 2012, respectively.

Carbon isotope analyses

Carbon isotope analyses of mature grains as well as the total dry matter (DM) and water soluble fraction (WSF) of the flag leaf blades and ears were performed using an Elemental Analyzer (Flash 1112 EA; ThermoFinnigan, Bremen, Germany) coupled with an Isotope Ratio Mass Spectrometer (Delta C IRMS, ThermoFinnigan, Bremen, Germany) operating in continuous flow mode in order to determine the stable carbon ($^{13}C/^{12}C$) isotope ratios of the same samples. Samples of approximately 1 mg of total dry matter for mature grains, 0.7 mg for flag leaves and ears and reference materials were weighed into tin capsules, sealed, and then loaded into an automatic sampler (ThermoFinnigan) before EA-IRMS analysis. The $^{13}C/^{12}C$ ratios of plant material were expressed in δ notation (Coplen, 2008): $\delta^{13}C = (^{13}C/^{12}C)_{sample} / (^{13}C/^{12}C)_{standard} - 1$, where 'sample' refers to plant material and 'standard' to international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose and USGS 40 L-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB) with an analytical precision (SD) of 0.10‰. Measurements were carried out at the Scientific Facilities of the University of Barcelona. The $\delta^{13}C$ of flag leaf (DM), ears (DM), roots and mature kernels will be referred as $\delta^{13}C_{flag}DM$, $\delta^{13}C_{ear}DM$, $\delta^{13}C_{roots}$ and $\delta^{13}C_{grain}$, respectively.

Oxygen isotope analyses

The $^{18}O:^{16}O$ ratios of the same mature grains as well as the total dry matter (DM) and water soluble fraction (WSF) of flag leaf blades and ears 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). Samples of 1 mg of total dry matter for mature grains, flag leaves, ears and roots and reference materials were weighed into silver capsules, sealed and oven-dried at 60ºC for not less than 72 h to remove moisture and loaded into an automatic sampler. Results were expressed as $\delta^{18}O$ values, using two secondary standards (IAEA 601 and IAEA 602) calibrated against Vienna Standard Mean Oceanic Water (VSMOW), and the analytical precision was ~0.25‰. Analyses were conducted at Iso-Analytical Limited (Crewe, CheshireCW2 8UY, UK). The $\delta^{18}O$ of flag leaf (DM), ears (DM), roots and mature kernels will be referred as $\delta^{18}O_{flag}DM$, $\delta^{18}O_{ear}DM$, $\delta^{18}O_{roots}$ and $\delta^{18}O_{grain}$ respectively.

Hydrogen isotope analyses

The $^{2}H:^{1}H$ ratios of the same mature grains as well as the total dry matter (DM) and water soluble fraction (WSF) of the flag leaf blades and ears were determined by an on-line pyrolysis technique using a Thermo-Chemical Elemental Analyser (TC/EA Thermofisher Scientific Inc) coupled with an IRMS (Delta plus xp). Samples of 0.15 mg of total dry matter for mature grains, flag leaves, ears, roots and reference materials were weighed into silver capsules, sealed and oven-dried at 60ºC for not less than 72 h to remove moisture and loaded into an automatic sampler. Results were expressed as $\delta^{2}H$ values, using international secondary standards of known $^{2}H/^{1}H$ ratios (IAEA CH7 polyethylene foil, IAEA 602, 5α-androstane, Coumarine and Icosanoic) calibrated against Vienna Standard Mean Oceanic Water (VSMOW), and the analytical precision was ~0.5‰. The $\delta^{2}H$ of flag leaf (DM), ears (DM), roots and mature kernels will be referred as $\delta^{2}H_{flag}DM$, $\delta^{2}H_{ear}DM$, $\delta^{2}H_{roots}$ and $\delta^{2}H_{grain}$ respectively.

Water-soluble fraction

The protein-free water-soluble fraction (WSF) of the flag leaves and ears were extracted from the same dry samples tested for carbon, deuterium and oxygen isotopes, as described previously (Cabrera-Bosquet et al. 2011; Yousfi et al. 2013). Aliquots of 40 µl (carbon), 20 µl (hydrogen), 100 µl (oxygen) of supernatant containing protein-free WSF were transferred into tin capsules for carbon analysis and into silver capsules for hydrogen and oxygen analyses. The capsules containing the aliquots were oven dried at 60ºC. The $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ of flag leaf (WSF) and ears (WSF) will be referred as $\delta^{13}C_{flag}WSF$, $\delta^{13}C_{ear}WSF$, $\delta^{2}H_{flag}WSF$, $\delta^{2}H_{ear}WSF$, $\delta^{18}O_{flag}WSF$ and $\delta^{18}O_{ear}WSF$, respectively.

Isotopic composition in stem water

To determine source water variations in the field experiments 2010 and 2011, a portion of the stem base was harvested in the field. In 2010 field trial, variations in source water were determined from pressed stem juice. Stem base segments were pressed with a high-pressure press, in order to obtain a liquid extract. Subsequently liquid extracted was transferred to 2 ml glass vials with crimp cap. Glass vials were sealed and sterilized in a water bath at 100ºC for two hours, to prevent fermentation processes, and kept cool until isotope analysis. In 2011 field trial, a portion of the stem base was placed immediately after sampling in sealed tubes and subsequently frozen in a freezer at -20ºC. Thereafter, water was extracted from the stem base using a cryogenic vacuum distillation line (Dawson & Ehleringer, 1993). The δ^2H and $\delta^{18}O$ of water extracted from the stem will be referred as δ^2H_{stemW} and $\delta^{18}O_{stemW}$, respectively.

Isotopic composition in grain and flag leaf water

In the field trial in 2011, developing grains a flag leaf were collected and placed in sealed tubes and frozen immediately after sampling. Thereafter, water was extracted from the developing grains and flag leaves using a cryogenic vacuum distillation line (Dawson & Ehleringer, 1993) and measured together with stem water samples. The δ^2H and $\delta^{18}O$ of water extracted from developing grains and flag leaf will be referred as δ^2H_{flagW}, δ^2H_{earW}, $\delta^{18}O_{flagW}$and $\delta^{18}O_{earW}$, respectively.

Hydrogen and oxygen analysis in water

Water isotopes ($\delta^{18}O$, δ^2H) in distilled water from stem base, flag leaves and developing grains (experiment 2011) and stem juice extracts (stem water, experiment 2010) were determined by laser spectroscopy at the Serveis Científico-Tècnics of the Universitat de Lleida, using a Picarro L2120i, coupled to a high-precision vaporizer A0211. All samples were centrifuged at 12,000 g in order to remove any suspended solid, and the supernatant transferred to a glass vial with 250 ml insert. They were expressed in delta (δ) notation (‰) relative to V-SMOW (i.e. isotopic composition of oxygen, $\delta^{18}O$, and hydrogen, δ^2H). Raw values were calibrated against three internal laboratory references (calibrated against IAEA standards VSMOW2, SLAP2 and GISP). Overall uncertainty, determined as the standard error of repeated analyses, (N=20) of a reference sample not included in the calibration, was 0.05‰ and 0.17‰, for $\delta^{18}O$ and δ^2H, respectively. The potential presence of organic contaminants was checked using the post-processing software Picarro ChemCorrect 1.2.0 giving in some cases

positive results. Consequently, data was thereafter corrected to avoid undesired effects of organic contaminants. For simplicity, distilled stem water and pressed stem juice will be referred as $\delta^{18}O_{stemW}$.

Large amounts of sugars reduce the performance of the vaporizer, thus juice samples were diluted to 50% with distilled water of known isotopic composition prior to injection. In preliminary tests, this proportion was found to be a good compromise of reducing sugar accumulation in the vaporizer while keeping an acceptable measuring accuracy. The precision for measurements, after considering the dilution effect, was better than 0.2‰ for $\delta^{18}O$ and 0.7‰ for $\delta^{2}H$. Original values of the sample were then recalculated (equation 1) from measured values and distilled water values using volume balance:

$$\delta^{y}X = 2 * (\delta^{y}X_{measured} - 0.5 * \delta^{y}X_{distilled\ water}) \quad (1)$$

Statistical analysis

Grain yield, agronomic components and isotopic data were subjected to one way analyses of variance (ANOVA) using the general lineal model to calculate the effects of water regime, nitrogen supply, genotype and their interaction on the studied parameters. Means were compared by Tukey's HSD test and were performed on a combination of water treatments and nitrogen supply. A bivariate correlation procedure was constructed to analyse the relationships between the measured traits. Statistical analyses were performed using the SPSS 18.0 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using the Sigma-Plot 10.0 program (SPSS Inc.).

RESULTS

Hydrogen, oxygen and carbon isotope composition across environments and genotypes

Mean values of stable of hydrogen (δ^2H), oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) isotope composition within different tissues are shown in Table 1. Hydrogen isotopic composition in mature kernels (δ^2H_{grain}) showed the most enriched (less negative) values (-31.0‰ on average across genotypes) compared to the ear (δ^2H_{ear}=-73.5‰ on average across genotypes and fractions), flag leaf (δ^2H_{flag}=-98.5‰) and roots (δ^2H_{roots}=-67,5‰). Conversely, the most enriched values for oxygen and carbon isotope composition were exhibited by the water-soluble fraction in the ear ($\delta^{18}O_{ear}$WSF=30.5‰ and $\delta^{13}C_{ear}$WSF=-23.8‰, on average respectively). Hydrogen isotope composition of the water from basal stem (δ^2H_{stemW}) was depleted (-45.9‰) compared to δ^2H_{grain} (-31.0‰), but enriched compared to δ^2H_{flag}, δ^2H_{ear} and δ^2H_{roots} either in WSF and DM (Table 1). On the contrary, oxygen isotope composition in the stem water ($\delta^{18}O_{stemW}$) displayed the most depleted value (-6.2‰) regardless tissues and fraction analyzed (Table 1). Among the waters of the different plant parts analyzed (Table 2), δ^2H_{stemW} and $\delta^{18}O_{stemW}$ exhibited the most depleted values, whereas hydrogen and oxygen isotopic composition of water extracted from flag leaf showed the most enriched values (δ^2H_{flagW}= 9.2‰ and $\delta^{18}O_{flagW}$=11.3‰, respectively).

***Table 1**. Mean values and ANOVA of grain yield (GY), stomatal conductance (g_s) and leaf temperature (T_{leaf}), stable isotope composition (‰) of hydrogen (δ^2H), oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) of dry matter (DM) and the water-soluble fraction (WSF) of different plant parts (flag leaf, ears and roots) sampled at mid grain filling plus mature kernels (grains) and the water from the basal part of the stem (stem water) under support irrigation (WW), water stress condition (WS), fertilized conditions (HN), non-fertilized condition (LN), modern cultivars (cultivars) and old landraces (landraces). δ13CDM and $\delta^{13}C$ WSF, $\delta^{18}O$ WSF and δ^2H WSF, were measured in 108 plots (five cultivars and four landraces, four growing conditions and three replicates), whereas $\delta^{18}O$ DM and δ^2H DM were measured in 48 plots (two cultivars and two landraces, four growing conditions and three replicates). Each value represents the mean ± SD. Mean values across plant tissues with different letters are significantly different according to the Tukey's honestly significant difference test (P<0.05)*

	Cultivars	Landraces	WW	WS	+N	-N
Hydrogen						
δ^2H_{roots} DM	-64.1 ± 17.7a	-69.9 ± 19.6a	-61.5 ± 22.4a	-72.4 ± 12.7b	-69.3 ± 16.9a	-64.9 ± 20.5a
δ^2H_{stemW}	-46.4 ± 7.2a	-45.3 ± 5.7a	-46.3 ± 7.6a	-45.6 ± 5.6a	-47.1 ± 7.3a	-44.8 ± 5.7a
δ^2H_{flag} DM	-109.6 ± 8.4a	-110.2 ± 8.4a	-115.1 ± 6.4a	-104.5 ± 6.5b	-106.4 ± 9.1a	-113.6 ± 5.3b
δ^2H_{flag} WSF	-87.1 ± 5.9a	-90.1 ± 6.4b	-89.8 ± 6.7a	-87.1 ± 5.5a	-87.8 ± 6.9a	-89.1 ± 5.5b
δ^2H_{spike} DM	-85.9 ± 8.2a	-90.9 ± 10.1b	-94.4 ± 6.9a	-82.4 ± 7.7b	-84.9 ± 9.6a	-92.0 ± 8.0a
δ^2H_{spike} WSF	-57.0 ± 6.1a	-61.8 ± 7.9a	-62.9 ± 5.8a	-55.5 ± 6.9b	-56.7 ± 8.1a	-61.7 ± 5.5b
δ^2H_{grain}	-32.2 ± 7.8a	-29.6 ± 9.0a	-35.0 ± 6.3a	-27.1 ± 8.4b	-26.3 ± 7.3a	-35.8 ± 6.6b
Oxygen						
$\delta^{18}O_{roots}$ DM	28.1 ± 7.5a	30.8 ± 8.8a	29.6 ± 9.0a	29.3 ± 7.6a	32.3 ± 9.4a	26.7 ± 5.9b
$\delta^{18}O_{stemW}$	-6.3 ± 1.0a	-6.1 ± 0.6a	-6.4 ± 0.9a	-6.0 ± 0.8a	-6.4 ± 0.9a	-6.0 ± 0.7a
$\delta^{18}O_{flag}$ DM	30.8 ± 1.7a	31.0 ± 1.1a	30.3 ± 1.0a	31.4 ± 1.6b	31.1 ± 1.6a	30.7 ± 1.3a
$\delta^{18}O_{flag}$ WSF	30.7 ± 2.2a	30.0 ± 2.3a	28.9 ± 1.9a	32.3 ± 0.9b	29.6 ± 2.7a	31.2 ± 1.3a
$\delta^{18}O_{spike}$ DM	26.6 ± 2.4a	26.1 ± 2.6a	26.5 ± 2.0a	26.2 ± 2.9a	26.7 ± 2.2a	26.0 ± 2.7a
$\delta^{18}O_{spike}$ WSF	30.9 ± 1.1a	29.9 ± 0.98a	30.2 ± 0.9a	30.7 ± 1.3a	30.3 ± 1.2a	30.7 ± 1.1a
$\delta^{18}O_{grain}$	30.4 ± 0.7a	30.6 ± 0.9b	30.4 ± 0.7a	30.6 ± 0.9a	30.3 ± 0.6a	30.7 ± 0.9a
Carbon						
$\delta^{13}C_{flag}$ DM	-25.9 ± 0.9a	-25.5 ± 0.9b	-26.2 ± 0.7a	-25.2 ± 0.8b	-25.3 ± 0.8a	-26.2 ± 0.8b
$\delta^{13}C_{flag}$ WSF	-27.0 ± 1.1a	-27.2 ± 1.1a	-27.9 ± 0.9a	-26.3 ± 0.7b	-27.2 ± 1.4a	-26.9 ± 0.7a
$\delta^{13}C_{spike}$ DM	-24.5 ± 1.0a	-25.0 ± 0.9b	-25.4 ± 0.7a	-24.1 ± 0.8b	-24.5 ± 1.0a	-25.0 ± 0.9b
$\delta^{13}C_{spike}$ WSF	-23.3 ± 0.9a	-24.4 ± 1.0b	-24.5 ± 0.9a	-23.1 ± 0.8b	-23.6 ± 1.1a	-24.0 ± 1.1a
$\delta^{13}C_{grain}$	-24.6 ± 0.9a	-23.9 ± 1.0b	-25.0 ± 0.6a	-23.5 ± 0.7b	-24.0 ± 1.1a	-24.6 ± 0.8b
g_s (mmol·H_2O·m·s)	184.7 ± 78.1a	170.2 ± 59.0a	222.8 ± 56.2a	133.8 ± 52.8b	163.6 ± 84.5a	193.1 ± 49.0b
T_{leaf} (ºC)	28.4 ± 1.4a	27.9 ± 1.3b	28.1 ± 1.5a	28.3 ± 1.3a	28.3 ± 1.5a	28.1 ± 1.3a
GY (Mg ha^{-1})	1.9 ± 0.1 a	1.5 ± 0.1 b	2.1 ± 0.1 a	1.3 ± 0.1 b	1.8 ± 0.1 a	1.6 ± 0.1 a

Overall δ^2H was lower in landraces compared to cultivars (Table 1), although significant differences were only observed in $\delta^2H_{flag}WSF$ (-90.1‰ and -87.1‰ for landraces and cultivars, respectively) and $\delta^2H_{ear}DM$ (-90.9‰ and -85.9‰ for landraces and cultivars, respectively). A similar trend was observed by $\delta^{13}C$, showing landraces lower $\delta^{13}C$ in the ear DM and WSF compared to cultivars (Table 1). However, within the two growing cycles (Table 1 and Table 2) $\delta^{13}C$ in mature kernels ($\delta^{13}C_{grain}$) was higher in landraces (-23.9‰ and -23.8‰ for 2010 and 2011, respectively) compared to modern cultivars (-24.9‰ and -25.3‰ for 2010 and 2011, respectively). On the contrary, $\delta^{18}O$ did not show significant differences between cultivars and landraces regardless of the plant organ and fractions considered, with the exception of $\delta^{18}O_{grain}$ (Table 1).

Moreover, significant differences within supplementary irrigation (WW) and rain-fed (WS) conditions (Tables 1 and S1) were mainly detected by δ^2H and $\delta^{13}C$. Concerning $\delta^{18}O$, only the flag leaf either DM or WSF showed significant differences when the two water regimes were compared in 2010. Overall, water stress trended to increase δ^2H, $\delta^{18}O$ and $\delta^{13}C$ irrespective of tissue, fraction or water analyzed, with the exception of $\delta^2H_{roots}DM$ (-61.5‰ and -72.4‰ for WW and WS conditions, respectively)(Table 1 and Table 2). Furthermore, when N fertilization conditions were compared, δ^2H and $\delta^{13}C$ showed higher isotope composition in fertilized plots compared to non-fertilized plots, although not significant differences were observed in $\delta^2H_{roots}DM$, δ^2H_{stemW} and $\delta^{13}C_{flag}WSF$ and $\delta^{13}C_{ear}WSF$. By contrast, $\delta^{18}O$ did not exhibit significant differences within fertilization conditions with the exception of the roots.

***Table 2**. Mean values and ANOVA of grain yield (GY), stomatal conductance (g_s) and leaf temperature (T_{leaf}), hydrogen isotope composition (‰) in the flag leaf water (δ^2H_{flagW}), grain water (δ^2H_{grainW}), stem water (δ^2H_{stemW}) and irrigation water in WW plots ($\delta^2H_{sourceW}$). Oxygen isotope composition (‰) in the flag leaf water ($\delta^{18}O_{flagW}$), grain water ($\delta^{18}O_{grainW}$), stem water ($\delta^{18}O_{stemW}$), irrigation water in WW ($\delta^{18}O_{sourceW}$), dry matter (DM) ofthe flag leaf ($\delta^{18}O_{flag\ DM}$) and mature kernels ($\delta^{13}C_{grain}$). Carbon isotope composition of the dry matter in the flag leaf ($\delta^{13}C_{flag\ DM}$) and mature kernels ($\delta^{13}C_{grain}$). $\delta^{13}C$ analysis was performed on nine durum wheat genotypes and three replicates grown under a two different water conditions (support irrigation, WW vs rain-fed, WS, including all levels of nitrogen). For water extracted from flag leaves, $\delta^{18}O$ and δ^2H were measured in a subset of 2 cultivars and 2 landraces (with three replicates) under fertilized conditions and two water regimes (18 plots). $\delta^{18}O$ and δ^2H were measured in extracted water from stem, developing grains and dry matter in a subset of 5 cultivars and 5 landraces (with three replicates) under fertilized conditions and two water regimes (45 plots) during crop cycle 2011. Each value represents the mean ± SE. Mean values across plant tissues with different letters are significantly different according to the Tukey's honestly significant difference test ($P<0.05$).*

	Modern C.	Old LC.	WW	WS
Deterium				
δ^2H_{flagW}	7.0 ± 4.6a	13.8 ± 3.8a	-8.1 ± 0.4a	17.9 ± 2.4b
δ^2H_{grainW}	-20.4 ± 1.5a	-5.5 ± 3.9b	-26.2 ± 0.9a	-9.7 ± 2.3b
δ^2H_{stemW}	-43.7 ± 0.4a	-43.2 ± 1.2a	-43.0 ± 0.6a	-43.8 ± 0.6a
$\delta^2H_{sourceW}$			-45.0	
Oxygen				
$\delta^{18}O_{flagW}$	10.3 ± 2.3a	13.5 ± 1.4a	3.0 ± 0.5a	15.5 ± 1.0b
$\delta^{18}O_{grainW}$	5.2 ± 0.6a	10.6 ± 1.2b	2.8 ± 0.2a	9.3 ± 0.7b
$\delta^{18}O_{stemW}$	-5.6 ± 0.1a	-5.6 ± 0.2a	-5.6 ± 0.1a	-5.6 ± 0.1a
$\delta^{18}O_{sourceW}$			-6.1	
$\delta^{18}O_{flag}$ DM	30.5 ± 0.4a		27.7 ± 0.1a	33.3 ± 0.2b
$\delta^{18}O_{grain}$	31.3 ± 0.2a		30.2 ± 0.1a	32.3 ± 0.1b
Carbon				
$\delta^{13}C_{flag}$ DM	-27.6 ± 0.2		-28.8 ± 0.1a	-26.5 ± 0.1b
$\delta^{13}C_{grain}$	-25.3 ± 0.1a	-23.8 ± 0.1b	-26.3 ± 0.1a	-24.4 ± 0.1b
g_s (mmol·H_2O·$m^{-2}s^{-1}$)	69.5 ± 50.5		110.0 ± 36.3	29.1 ± 22.5
T_{leaf} (ºC)	37.7 ± 0.5		35.6 ± 0.4	35.9 ± 0.6
GY (Mg·Ha^{-1})	3.1 ± 0.2b	1.6 ± 0.1a	4.5 ± 0.1a	1.7 ± 0.1b

Grain yield and water status

With regard to yield (Table 1 and Table 2), *GY* was much higher in 2011 (on average 3.1 $Mg \cdot ha^{-1}$) compared to 2010 (on average 1.7 $Mg \cdot ha^{-1}$). Moreover, cultivars and WW crops showed higher *GY* compared to landraces and WS crops in the two growing cycles (Table 1 and Table 2). Conversely, not significant differences were observed in *GY* between fertilization conditions (Table 1). Moreover, cultivars showed higher canopy temperature (T_{leaf}) and stomatal conductance (g_s) compared to landraces. Furthermore, g_s was higher under WW conditions (222.8 $mmol \cdot H_2O \cdot m^{-2}s^{-}1$) compared to WS conditions (56.2 $mmol \cdot H_2O \cdot m^{-2}s^{-}1$). However, g_s decreased as response to nitrogen (Table 1).

Correlations of δ^2H, $\delta^{18}O$ and $\delta^{13}C$ from different plant parts with GY, g_s and N status

δ^2H, $\delta^{18}O$ and $\delta^{13}C$ on DM, WSF and water from the different tissues together with the composition of these three isotopes in mature kernels from 2010, were correlated against GY and g_s and N concentration of the flag leaf and the spike (Table 3). In general δ^2H and $\delta^{13}C$ in the different tissues and water analyzed were negatively correlated against GY ($P<0.05$) and g_s ($P<0.01$) within all growing conditions (Table 3, Table S2, Table 4). However, whereas δ^2H and $\delta^{13}C$ were positively related with total nitrogen content in the flag leaf (N-flag, $P<0.01$) and ear (N-ear, $P<0.01$) under WS conditions (across N fertilization levels), under HN conditions (and both water regimes combined) such correlation was negative (Table 3). Conversely, δ18O on DM and WSF exhibited weaker correlations versus GY, g_s, N-flag and N-ear, being marginally significant in the flag leaf and stem water (Tables 3 and S2). However, in 2011 $\delta^{18}O_{leaf}$ DM was strongly related to GY when all growing conditions were combined (Table 4). In addition, $\delta^{18}O_{flagW}$ and δ^2H_{flagW} in 2011, showed a positive and strong relationship with GY (Table 4, $r=0.82$, $P<0.001$ and $r=0.77$, $P<0.001$, respectively). In a lesser extent but still significant $\delta^{18}O_{grainW}$ and δ^2H_{grainW} were also correlated against GY (Table 4).

Fractionation of hydrogen, oxygen and carbon isotope composition across plant parts

In order to estimate potential differences in fractionation of hydrogen, oxygen and carbon isotope signatures within the plant, correlation analysis was performed within each plant component (DM, WSF and water) among $\delta^{18}O$, $\delta^{13}C$ and $\delta^{2}H$ (Fig. 2). The highest relationship was observed in the flag leaf WSF (left column, Fig. 2) between $\delta^{18}O$ *vs* $\delta^{13}C$ (R^2=0.75, P<0.001). However, in the ear WSF, $\delta^{2}H$ was the best correlated with $\delta^{13}C$ (R^2=0.55, P<0.001). The weakest correlations were observed by $\delta^{2}H$ *vs* $\delta^{18}O$ in the flag leaf WSF (R^2=0.16, P<0.001) and ear WSF (R^2=0.21, P<0.001). In mature kernels $\delta^{2}H$ and $\delta^{13}C$ were strongly related (R^2=0.48, P<0.001), whereas $\delta^{18}O$ did not correlated either with $\delta^{2}H$ or $\delta^{13}C$. Likewise, in order to estimate fractionation of hydrogen, oxygen and carbon isotope composition within water extracted from different plant tissues, correlation analysis was performed in Table 4. $\delta^{2}H_{flagW}$ was strongly related with $\delta^{2}H_{grainW}$ (R=0.66, P<0.001) whereas no correlation was observed against $\delta^{2}H_{stemW}$. Similarly, $\delta^{18}O_{flagW}$ was positively correlated with $\delta^{18}O_{grainW}$ (R=0.67, P<0.001) but not with $\delta^{18}O_{stemW}$. Furthermore, $\delta^{2}H_{flagW}$ was strongly correlated against $\delta^{18}O_{flagW}$ (R=0.99, P<0.001) and $\delta^{18}O_{grainW}$ (R=0.71, P<0.01), whereas $\delta^{2}H_{grainW}$ was better related with $\delta^{18}O_{grainW}$ (R=0.99, P<0.001) than with $\delta^{18}O_{flagW}$ (R=0.61, P<0.01).

***Table 3**. Linear regression of the relationship between the carbon ($\delta^{13}C$) oxygen ($\delta^{18}O$) and deuterium ($\delta^{2}H$) isotope compositions in the water soluble fraction (WSF) and dry matter (DM) of the flag leaf and ear as well as in mature kernels (grains) against the grain yield (GY), stomatal conductance (g_s), and total nitrogen content in the flag leaf (N-flag) and in the ear (N-ear). The nine genotypes and three replications per genotype were considered, accounting for a total of 54 values under rainfed water stress conditions (WS, this page) including fertilized and non-fertilized conditions and under fertilized conditions (HN, next page) including the two water conditions. For the $\delta^{18}O$ and $\delta^{2}H$ of dry matter (flag leaf, ear and roots) only two cultivars and two landraces were considered (24 plots). Analysis were performed in samples from the 2010 crop season. Level of significance: $P < 0.001$; $P < 0.01$; $P < 0.05$; not significant, $P > 0.05$.*

(WS)	GY			g_s			N-flag			N-ear		
	Pearson	Sig.	n	Pearson	Sig.	n	Pearson	Sig.	n	Pearson	Sig.	n
Hydrogen												
$\delta^{2}H_{roots}$ DM	-0.085	0.693	24	0.247	0.246	24	-0.043	0.842	24	-0.164	0.444	24
$\delta^{2}H_{stemW}$	-0.132	0.341	54	**0.430**	**0.001**	54	**0.340**	**0.012**	54	0.064	0.645	54
$\delta^{2}H_{flag}$ DM	**-0.485**	**0.019**	23	**-0.729**	**0.000**	23	**0.472**	**0.023**	23	**0.730**	**0.000**	23
$\delta^{2}H_{flag}$ WSF	**-0.319**	**0.019**	54	**-0.562**	**0.004**	24	**0.579**	**0.003**	24	**0.725**	**0.000**	54
$\delta^{2}H_{spike}$ DM	**-0.417**	**0.042**	24	**-0.452**	**0.001**	54	**0.398**	**0.003**	54	**0.845**	**0.000**	24
$\delta^{2}H_{spike}$ WSF	**-0.514**	**0.000**	54	**-0.564**	**0.000**	54	**0.725**	**0.000**	54	**0.490**	**0.000**	54
$\delta^{2}H_{grain}$	**-0.444**	**0.001**	54	**-0.607**	**0.000**	54	**0.391**	**0.003**	54	**0.359**	**0.008**	54
Oxygen												
$\delta^{18}O_{roots}$ DM	-0.246	0.247	24	-0.147	0.493	24	-0.093	0.664	24	0.137	0.522	24
$\delta^{18}O_{stemW}$	-0.219	0.112	54	**0.313**	**0.021**	54	**-0.299**	**0.028**	54	0.162	0.242	54
$\delta^{18}O_{flag}$ DM	-0.217	0.114	54	-0.215	0.118	54	0.001	0.993	54	0.172	0.214	54
$\delta^{18}O_{flag}$ WSF	**-0.458**	**0.002**	44	-0.001	0.996	44	-0.255	0.094	44	-0.222	0.147	44
$\delta^{18}O_{spike}$ DM	-0.263	0.055	54	0.242	0.078	54	-0.090	0.515	54	-0.034	0.805	54
$\delta^{18}O_{spike}$ WSF	**-0.444**	**0.001**	54	0.175	0.205	54	-0.198	0.151	54	-0.111	0.424	54
$\delta^{18}O_{grain}$	-0.237	0.084	54	0.106	0.445	54	-0.179	0.195	54	-0.201	0.146	54
Carbon												
$\delta^{13}C_{flag}$ DM	**-0.319**	**0.019**	54	**-0.687**	**0.000**	54	**0.337**	**0.013**	54	**0.451**	**0.001**	54
$\delta^{13}C_{flag}$ WSF	**-0.336**	**0.013**	54	**-0.393**	**0.003**	54	0.052	0.709	54	0.211	0.126	54
$\delta^{13}C_{spike}$ DM	**-0.390**	**0.004**	54	**-0.459**	**0.000**	54	**0.376**	**0.005**	54	**0.370**	**0.006**	54
$\delta^{13}C_{spike}$ WSF	**-0.486**	**0.000**	53	**-0.293**	**0.033**	53	**0.278**	**0.044**	53	**0.339**	**0.013**	53
$\delta^{13}C_{grain}$	**-0.332**	**0.014**	54	**-0.734**	**0.000**	54	**0.468**	**0.000**	54	**0.395**	**0.003**	54

(HN)	GY			g_s			%N Flag			%N Spike		
	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N
Hydrogen												
δ^2H_{roots} DM	0.208	0.341	23	0.384	0.071	23	**0.463**	**0.026**	23	**0.469**	**0.024**	23
δ^2H_{stemW}	-0.165	0.246	51	-0.129	0.367	51	-0.176	0.218	51	**0.313**	**0.025**	51
δ^2H_{flag} DM	**-0.632**	**0.001**	24	**-0.789**	**0.000**	24	**-0.564**	**0.004**	24	-0.065	0.762	24
δ^2H_{flag} WSF	**-0.354**	**0.009**	54	**-0.452**	**0.001**	54	**-0.360**	**0.007**	54	0.155	0.264	54
δ^2H_{spike} DM	**-0.555**	**0.005**	24	**-0.758**	**0.000**	24	-0.337	0.108	24	0.198	0.354	24
δ^2H_{spike} WSF	**-0.313**	**0.021**	54	**-0.391**	**0.003**	54	-0.057	0.682	54	0.140	0.311	54
δ^2H_{grain}	**-0.521**	**0.000**	54	**-0.507**	**0.000**	54	**-0.463**	**0.000**	54	-0.161	0.246	54
Oxygen												
$\delta^{18}O_{roots}$ DM	-0.013	0.953	23	0.057	0.794	23	-0.119	0.588	23	-0.119	0.588	23
$\delta^{18}O_{stemW}$	**-0.318**	**0.023**	51	**0.300**	**0.033**	51	-0.244	0.084	51	**0.395**	**0.004**	51
$\delta^{18}O_{flag}$ DM	**-0.305**	**0.025**	54	**-0.396**	**0.003**	54	**-0.537**	**0.000**	54	-0.186	0.177	54
$\delta^{18}O_{flag}$ WSF	**-0.590**	**0.000**	49	**-0.786**	**0.000**	49	**-0.492**	**0.000**	49	**-0.289**	**0.044**	49
$\delta^{18}O_{spike}$ DM	-0.140	0.311	54	-0.182	0.189	54	0.012	0.930	54	-0.029	0.836	54
$\delta^{18}O_{spike}$ WSF	-0.120	0.388	54	**-0.292**	**0.032**	54	-0.006	0.965	54	-0.065	0.642	54
$\delta^{18}O_{grain}$	-0.181	0.190	54	-0.009	0.950	54	-0.128	0.357	54	-0.089	0.524	54
Carbon												
$\delta^{13}C_{flag}$ DM	**-0.608**	**0.000**	54	**-0.699**	**0.000**	54	**-0.631**	**0.000**	54	-0.065	0.642	54
$\delta^{13}C_{flag}$ WSF	**-0.638**	**0.000**	54	**-0.798**	**0.000**	54	**-0.605**	**0.000**	54	-0.123	0.377	54
$\delta^{13}C_{spike}$ DM	**-0.436**	**0.001**	54	**-0.671**	**0.000**	54	**-0.283**	**0.038**	54	-0.082	0.556	54
$\delta^{13}C_{spike}$ WSF	**-0.354**	**0.009**	54	**-0.634**	**0.000**	54	-0.215	0.118	54	-0.076	0.583	54
$\delta^{13}C_{grain}$	**-0.818**	**0.000**	54	**-0.835**	**0.000**	54	**-0.574**	**0.000**	54	**-0.290**	**0.033**	54

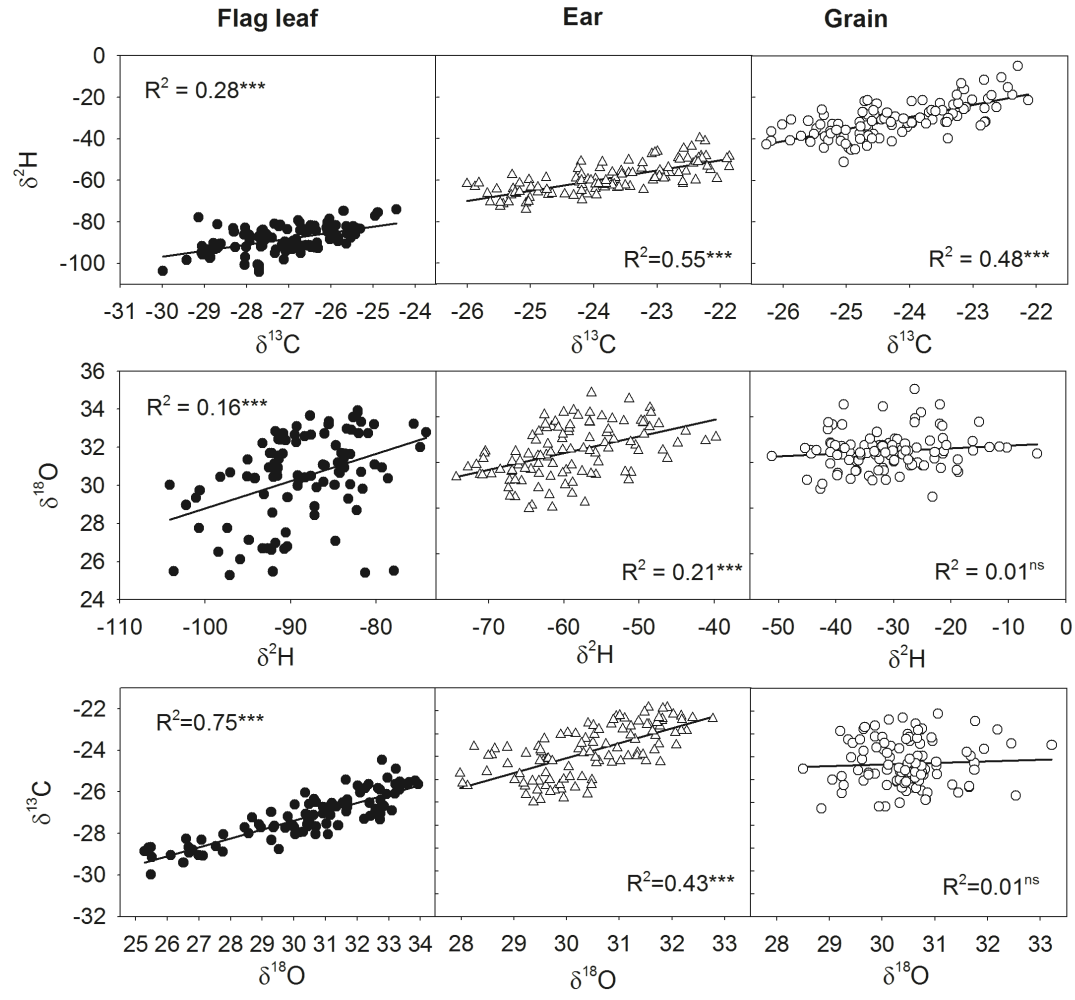

Fig. 2 *Linear regression of the relationship between the carbon ($\delta^{13}C$) oxygen ($\delta^{18}O$) and deuterium ($\delta^{2}H$) isotope compositions of the water soluble fraction (WSF) within the flag leaf (left column, closed circles), ear (middle column, open triangles) and mature kernels (right column, open circles). The nine genotypes and three replications per genotype were considered, accounting for a total of 108 plot values under all growing conditions of the 2010 crop season. Level of significance: $P < 0.001$; $P < 0.01$; $P < 0.05$; not significant, $P > 0.05$.*

***Table 4**. Linear regression of the relationship between hydrogen isotope composition of the flag leaf water (δ^2H_{flagW}), grain water (δ^2H_{grainW}) and stem water (δ^2H_{stemW}); oxygen isotope composition of the flag leaf water (δ18Oflag water), grain water ($\delta^{18}O_{grainW}$), and stem water ($\delta^{18}O_{stemW}$), and the dry matter of the flag leaf ($\delta^{18}O_{flag}$ DM) and mature kernels ($\delta^{18}O_{grain}$). Carbon isotope composition of the dry matter in the flag leaf ($\delta^{13}C_{flag}$ DM) and mature kernels ($\delta^{13}C_{grain}$). $\delta^{18}O$ and δ^2H of the water extracted from the flag leaf, were analyzed in a subset of 2 cultivars and 2 landraces (with three replicates) under fertilized conditions and two water regimes (18 plots). $\delta^{18}O$ and δ^2H were measured in water from the stem, developing grains and dry matter in a subset of 5 cultivars and 5 landraces (with three replicates) under fertilized conditions and two water regimes (45 plots). Analyses were performed in samples from the 2011 crop season. Level of significance: P < 0.001; P < 0.01; P < 0.05; not significant, P > 0.05.*

	δ^2H_{flagW}			δ^2H_{grainW}			δ^2H_{stemW}			$\delta^{18}O_{flagW}$			$\delta^{18}O_{grainW}$			$\delta^{18}O_{stemW}$			GY ($Mg \cdot Ha^{-1}$)		
	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N
Hydrogen																					
δ^2H_{flagW}				**0.657**	**0.003**	18	-0.133	0.599	18	**0.989**	**0.000**	18	**0.704**	**0.001**	18	-0.001	0.996	18	**-0.776**	**0.000**	18
δ^2H_{grainW}	**0.657**	**0.003**	18				-0.256	0.093	44	**0.613**	**0.007**	18	**0.989**	**0.000**	44	-0.212	0.168	44	**-0.536**	**0.000**	44
δ^2H_{stemW}	-0.133	0.599	18	-0.256	0.093	44				-0.118	0.642	18	-0.266	0.081	44	**0.815**	**0.000**	45	0.121	0.430	45
Oxygen																					
$\delta^{18}O_{flagW}$	**0.989**	**0.000**	18	**0.613**	**0.007**	18	-0.118	0.642	18				0.674	0.002	18	0.042	0.869	18	**-0.821**	**0.000**	18
$\delta^{18}O_{grainW}$	**0.704**	**0.001**	18	**0.989**	**0.000**	44	-0.266	0.081	44	**0.674**	**0.002**	18				-0.200	0.193	44	**-0.623**	**0.000**	44
$\delta^{18}O_{stemW}$	-0.001	0.996	18	-0.212	0.168	44	**0.815**	**0.000**	45	0.041	0.869	18	-0.200	0.193	44				-0.074	0.629	45
$\delta^{18}O_{flag}$ DM	**0.956**	**0.000**	12	**0.721**	**0.000**	29	-0.316	0.088	30	**0.969**	**0.000**	12	**0.824**	**0.000**	29	0.108	0.571	30	**-0.929**	**0.000**	29
$\delta^{18}O_{grain}$	**0.899**	**0.000**	12	**0.728**	**0.000**	29	**-0.437**	**0.016**	30	**0.905**	**0.000**	12	**0.797**	**0.000**	29	-0.067	0.726	30	**-0.842**	**0.000**	29
Carbon																					
$\delta^{13}C_{flag}$ DM	**0.891**	**0.000**	12	**0.692**	**0.000**	29	-0.278	0.137	30	**0.920**	**0.000**	12	**0.781**	**0.000**	29	0.164	0.386	30	**-0.867**	**0.000**	29
$\delta^{13}C_{grain}$	**0.873**	**0.000**	12	**0.596**	**0.001**	29	**-0.371**	**0.043**	30	**0.915**	**0.000**	12	**0.719**	**0.000**	29	0.149	0.432	30	**-0.938**	**0.000**	29

Variation range of hydrogen, oxygen and carbon isotope composition

The variation range and analytical error for the different isotopes analyzed was assessed (Table 5). δ^2H showed the highest observed range (δ=-300 to 20 ‰) whereas $\delta^{18}O$ showed the lowest observed range (δ=-30 to 5 ‰). $\delta^{13}C$ exhibited an intermediate range within δ^2H and $\delta^{18}O$ (δ=-30 to -5 ‰). In addition, δ^2H showed the highest analytical error calculated from the standards (δ^2H error standard = 1.73‰) and samples (δ^2H error samples = 4.85‰) compared to $\delta^{18}O$ and $\delta^{13}C$. $\delta^{13}C$ showed the lowest analytical standard ($\delta^{13}C$ error standard = 0.13‰) and samples error $\delta^{13}C$ error samples = 0.14‰). Relative mass differences of δ^2H, $\delta^{18}O$ and $\delta^{13}C$ in different tissues are presented in Fig. 3. δ^2H showed the largest relative mass differences in all tissues compared to $\delta^{18}O$ and $\delta^{13}C$. Further, δ^2H showed the highest relative mass differences within all analyzed tissues (excluding the roots) which ranged from 120‰ to 40‰ compared to $\delta^{18}O$ (from 27‰ to 32‰) and $\delta^{13}C$ (from 25‰ to 30‰).

Table 5*. Relative abundance of most common stable isotopes in ecological studies (elaborated from Dawson et al., 2002; Mateo et al., 2003; Sulzman, 2007), the observed range δ, the analytical error subtracted from the literature, the analytical error calculated in our study as the standard deviation within the standards (Analytical Error patterns) and within samples samples (Analytical Error samples) and the primary standard (Primary Standard) used for the isotope analyses.*

Element	Nomenclature	Observed range δ (‰)	Analytical Error literature (‰)	Analytical Error standard (‰)	Analytical Error samples (‰)	Primary Standard
Hydrogen	δ^2H	-300 to +20	4-7	1.73	4.85	SMOW[a]
Carbon	$\delta^{13}C$	-35 to -5	0.1	0.13	0.14	PDB[b]
Oxygen	$\delta^{18}O$	-30 to +5	0.05-0.3	0.22	0.28	SMOW[a]

[a] Standard Mean Oceanic Water

[b] Pee-Dee Belemnite

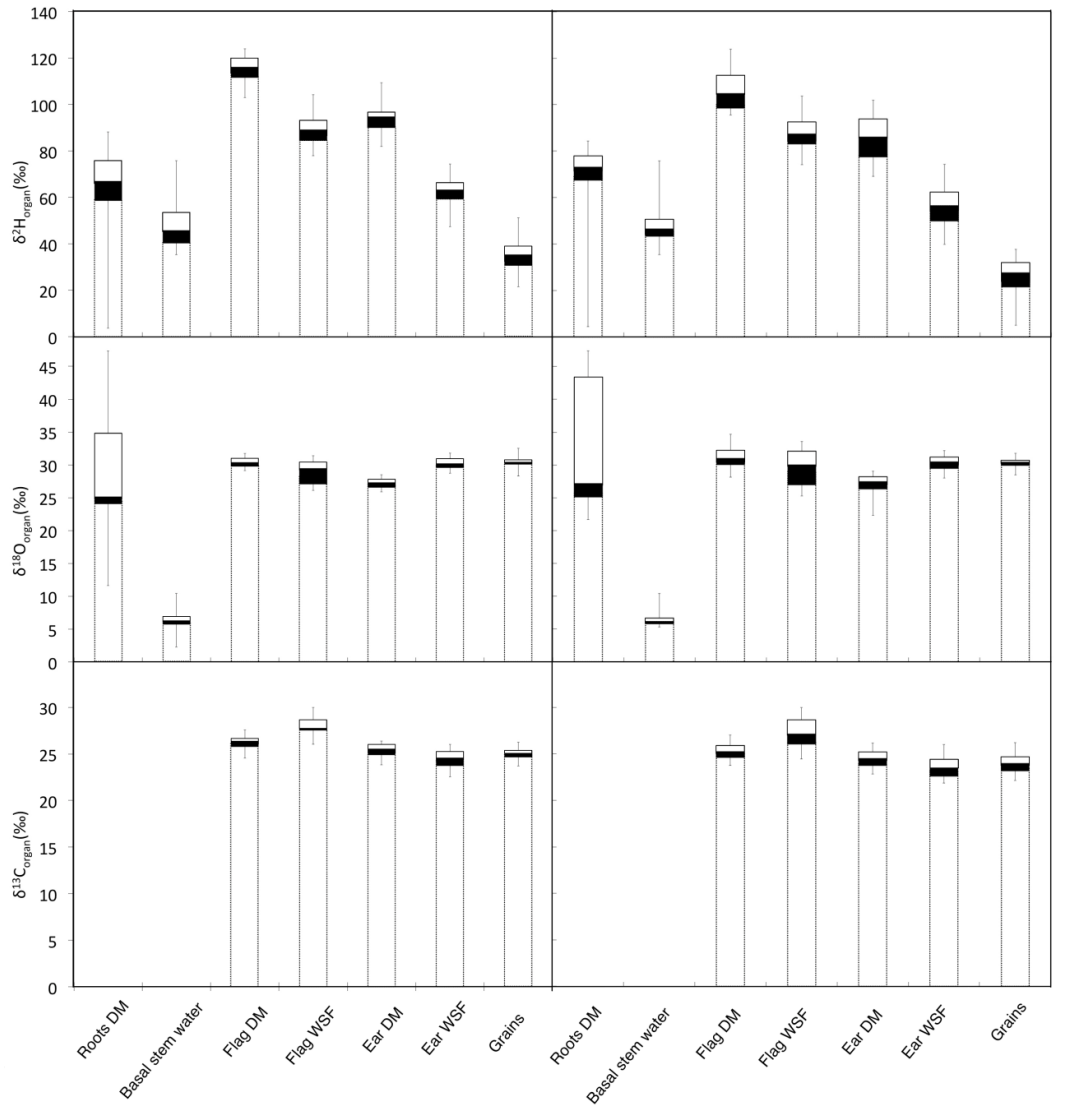

Fig. 3 *Stable isotope composition (‰) of deuterium (δ^2H), oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) of dry matter (DM) and the water-soluble fraction (WSF) of different plant parts (flag leaf, ears and roots) sampled at mid grain filling plus mature kernels (grains) and stem water of nine durum wheat genotypes and three replicates grown (upper part) under WW conditions (support irrigation, including both nitrogen fertilization levels) and (lower part) HN conditions (N fertilization, including the two water regimes) during crop cycle 2010. Spread of data denoted by box whiskers plot: box limits represent 25 and 75 percentiles, line within box median; whisker ends 1 and 99 percentile.*

DISCUSSION

Relationship between carbon isotope composition versus GY

Grain yield (*GY*) recorded within 2010 and 2011, was in the range reported by previous studies in the Mediterranean basin under dry rainfed and low supplementary irrigation conditions (Araus *et al.*, 1998, 2003, 2013b). In agreement with previous studies (Condon *et al.*, 1987; Araus *et al.*, 1998, 2003; Fischer *et al.*, 1998; Monneveux *et al.*, 2005; Lopes & Reynolds, 2010) carbon isotope composition ($\delta^{13}C$) was negatively correlated with *GY* not just across water regimes (including WW and WS plots) but also through genotypes within a growing condition (Tables 3 and 4). Correlations of $\delta^{13}C$ with *GY* across N fertilization levels and a given water regime were also negative under WS, whereas under WW conditions they were positive for the flag leaf and the ear and negative for the kernels (Tables…). Such dependence of the slope of the relationships between $\delta^{13}C$ and *GY* across N fertilization levels on the water regime and/or the organ may be related to the effect of N fertilization at different levels. The positive relationships under WW conditions may be the consequence of a N fertilization improving yield through a larger green biomass more exposed to suffer water stress (and then experiencing a higher $\delta^{13}C$). On the contrary the negative relationship between $\delta^{13}C$ and *GY* may be consequence of a positive effect of N fertilization on root development and water uptake. According to our results, in trials under drought conditions with mean yield up to 2 $Mg \cdot ha^{-1}$ (Araus *et al.*, 2003) not significant relationships between $\delta^{13}C$ and GY has been reported (Araus *et al.*, 2003) or even have moved to positive (Voltas *et al.*, 1999) indicating that higher WUE (and thus higher $\delta^{13}C$) increases yield under stress (Farquhar & Richards, 1984; Araus *et al.*, 2003, 2013a; Condon *et al.*, 2004).

Relationship between oxygen isotope composition versus GY

Isotope composition in the grain ($\delta^{18}O_{grain}$) and *GY* were not significantly correlated (neither in WS or HN conditions, Table 3) in 2010, whereas in 2011, such relationship was strongly significant (Table 4). Not consistent correlations found in our results within 2010 and 2011 crop season may be related to differences in environmental conditions and its subsequent yield performance within both years. Environmental range and *GY* was much lower in 2010 (2.1 $Mg \cdot ha^{-1}$ and 1.3 $Mg \cdot ha^{-1}$ for WW and WS trials, respectively) compared to 2011 (4.5 $Mg \cdot ha^{-1}$ WW to 1.7 $Mg \cdot ha^{-1}$ for WW and WS trials, respectively). In fact, in study by Barbour *et al.*, (2000b) in wheat, correlations of

$\delta^{18}O_{grain}$ against *GY* and g_s were not consistent within the three seasons they analyzed. In the mentioned study $\delta^{18}O_{grain}$ was only well correlated against *GY* in the year with highest precipitation and lowest solar radiation. Such findings indicate that $\delta^{18}O_{grain}$ might not reflect evaporative conditions under narrow environmental ranges and with mild to severe drought. Thus, as a consequence of high levels of remobilization under more severe water conditions, preservation of evaporative conditions imprinted in the $\delta^{18}O$ grains might be low or even non-existent (Barbour *et al.*, 2000b). Likewise correlations disparity found during two growing seasons between $\delta^{18}O_{grain}$ and *GY* could be due to the proportion of remobilized photo-assimilates (Barbour *et al.*, 2000a). Triose phosphates formed from photosynthesis during the day are converted to sucrose for transport (Barbour & Farquhar, 2000). Thus, the main exchange of water with carbonyl oxygen occurs during the formation of triose phosphate molecules, since two of the three oxygen atoms present in the molecule belong to carbonyl groups (Sternberg *et al.*, 1986b; Barbour *et al.*, 2000b). In fact $\delta^{18}O_{flag}$WSF and *GY*, as well as $\delta^{18}O_{ear}$WSF and *GY* were correlated under WS conditions (Table 3), indicating that the signal of evaporative conditions are still preserved in leaves assimilates, but $\delta^{18}O$ of the dry matter of the same organs or the other (such as grains or roots) did not correlate. In addition, besides $\delta^{18}O$ fractionation associated to biochemical reactions in the synthesis of organic matter (Farquhar & Lloyd, 1993) and its subsequent transport (Offermann *et al.*, 2011a), $\delta^{18}O$ of organic matter may be also influenced by source of water (Epstein *et al.*, 1977; Yakir *et al.*, 1990b; Roden *et al.*, 2000; Williams *et al.*, 2005; Barbour, 2007b). This is not straightforward, since $\delta^{18}O$ of source water (water from the base of the stem) is in addition subjected to evaporative enrichment in the leaf during transpiration (Farquhar *et al.*, 1993) and during grain formation (Pande *et al.*, 1994). In addition, it has been reported that $\delta^{18}O$ of water in developing grains exhibits a biphasic enrichment compared to stem water (Pande *et al.*, 1994). Such biphasic enrichment may be linked to developmental metabolism of the grain and rapid loss of water together with oxidative metabolism during later stages of maturation (Pande *et al.*, 1995). Such biphasic enrichment in the grains may be therefore affecting both $\delta^{2}H$ and $\delta^{18}O$ as observed in our results where water from developing grains showed higher $\delta^{2}H$ and $\delta^{18}O$ compared to stem water (Table 2). Consequently, the enrichment of water in the grain could be an additional factor that may hinder the ability of $\delta^{18}O_{grain}$ (but not in the $\delta^{2}H_{grain}$ as was strongly correlated with *GY*) to register environmental conditions on its dry matter in the grains. Moreover the $\delta^{2}H$ and $\delta^{18}O$ of water in the flag leaf are much higher than the water from developing

grains and stem (Table 2), which also agrees with the widely reported strong evaporation processes taking place in the leaf (Farquhar & Gan, 2003; Barbour *et al.*, 2004). In addition δ^2H and $\delta^{18}O$ from the water of a photosynthetic and transpiring organs such as the flag leaf were strongly correlated with *GY* (Table 4). In short, strong correlation between *GY* and δ^2H and $\delta^{18}O$ from water in leaves is suggesting that leaf water is mainly reflecting evaporative enrichment and thus environmental conditions, with the additional advantage (at least in the case of $\delta^{18}O$) of avoiding fractionation associated to biochemical reactions in the synthesis of organic matter (Farquhar & Lloyd, 1993).

Relationship between hydrogen isotope composition versus GY

δ^2H and $\delta^{18}O$ have been associated to transpiration (Yakir & Deniro, 1990) and both can be subjected to post-photosynthetic fractionation exchange processes (Yakir, 1992). However and differently as for $\delta^{18}O$ it has been reported that photosynthesis could have a major impact in δ^2H (Sternberg *et al.*, 1984). In addition a large hydrogen isotopic fractionation has been described in the past from leaf water to organic matter (Sternberg *et al.*, 1986b), as is evidenced by the higher δ^2H of grains compared with all other plant parts (including the ear), as well as from the δ^2H of roots compared with the flag leaf (Table 1). These results suggest that δ^2H was exposed to post-photosynthesis enrichment especially in a mostly heterotrophic organs (such as the grains in our study compared to more depleted δ^2H in autotrophic organs such as the leaves (Yakir *et al.*, 1990b). In addition δ^2H showed larger relative mass differences within different organs compared to $\delta^{18}O$ and $\delta^{13}C$ (Fig. 3). However, in spite of the above mentioned fractionation processes, δ^2H from either grains or the different photosynthetic organs, was well correlated with *GY* (Table 3) even better than $\delta^{13}C$ in WS conditions, which suggests that original δ^2H in organic matter (such as grains) was not hindered due to fractionation processes. In fact, stepwise regression analysis performed for each of the two water regimes (WW and WS) in 2010, to assess which isotope composition in the grain ($\delta^{13}C_{grain}$, $\delta^{18}O_{grain}$ and δ^2H_{grain}) better explained variability in GY (dependent variable); the first variable chosen by the model under WS conditions was δ^2H_{grain} (r=0.444 $P \leq$ 0.001, data no shown).

Relationship between carbon, oxygen and hydrogen isotope composition

High correlation between oxygen and hydrogen isotopic composition has been reported to indicate ultimate source of plant isotopic signal (Epstein *et al.*, 1977),

whereas absence of correlation between oxygen and hydrogen isotopic composition would indicate additional biochemical effects (Sternberg *et al.*, 1986a). Keeping this in mind, in 2010 growing season, the absence of significant correlations between $\delta^{18}O_{grain}$ and $\delta^{2}H_{grain}$ (Fig. 2) together with the lack of relationship between $\delta^{18}O_{grain}$ *vs GY* suggests that $\delta^{18}O_{grain}$ is more sensitive during grain formation (than $\delta^{2}H_{grain}$) to biochemical reactions (Farquhar & Lloyd, 1993) or to exchange with isotopic composition of source water (Barbour, 2007a). Likewise, $\delta^{18}O_{flag}$ and $\delta^{13}C_{flag}$ in the WSF were significantly and strongly correlated suggesting that both isotopes are able to reflect environmental conditions on autotrophic organs (leaves and ears) probably governed by changes on transpiration and stomatal conductance as previously reported in durum wheat (Cabrera-Bosquet *et al.*, 2009a, 2011). Moreover, $\delta^{2}H_{flag}$ *vs* $\delta^{13}C_{flag}$ were better related than $\delta^{2}H_{flag}$ *vs* $\delta^{18}O_{flag}$ (Fig. 2) suggesting that $\delta^{2}H$ in the leaves is not only affected by changes on transpiration and stomatal conductance but also by photosynthetic reactions (Yakir *et al.*, 1990b). Moreover, the increase from the basal to the apical part of the plant (root-flag leaf with regard the ear) in $\delta^{2}H$ and $\delta^{18}O$ of DM and WSF may be due in part to that effect of a progressive enrichment in $\delta^{2}H$ and $\delta^{18}O$ of the water within the plant associated to the evaporative demand and changes on soil water availability (Sanchez-Bragado *et al.*, 2014b). However an additional effect may be the progressive increase of drought conditions (typical of Mediterranean basin) during the final stages of crop growth as found in the $\delta^{13}C$ (Condon & Richards, 1992), which also increases from the flag leaf to the ear (Badeck *et al.*, 2005) (Table 1). Besides, an additional factor which may increase the composition of the three isotopes in the ear compared for example with the flag leaf could be due to lower g_s of the inflorescence (Araus *et al.*, 1993; Tambussi *et al.*, 2005).

Genotypic variability of carbon, oxygen and hydrogen isotope composition of cultivars and landraces

Likewise, *GY* was higher in cultivars compared to landraces for both growing cycles. Cultivars have been observed to have a shorter duration to reach heading (Araus *et al.*, 2013a) compared to landraces (Araus *et al.*, 2002), escaping from incipient mild stress produced during reproductive stage (Araus *et al.*, 2007). In fact, enriched values of $\delta^{13}C_{grain}$, $\delta^{2}H$ and $\delta^{18}O$ of grain water were observed in landraces compared to cultivars (Table 1), evidencing that landraces have been exposed to an extended stress episode resulting in lower *GY* compared to cultivars (Araus *et al.*, 2007). In a study by

Condon *et al.*, (2004), genotypes with enriched $\delta^{13}C_{grain}$, tend to be conservative in their growth rate, especially if enriched $\delta^{13}C_{grain}$ was the consequence of a lower stomatal conductance (g_s). Indeed, in our results cultivars tended to show higher g_s compared with landraces, but not significant differences were observed. Conversely, T_{leaf} was higher in modern cultivars compared to landraces suggesting that the tall canopies of landraces contribute to a more efficient convective cooling than in the case of cultivars. This might be the reason why landraces showed depleted values in $\delta^{2}H_{flag}$ WSF compared to cultivars, whereas $\delta^{18}O_{flag}$ WSF and $\delta^{13}C_{flag}$ WSF did not show genotypic variability.

Water and nitrogen effects on plant growth and water status

In our study a significant increase in $\delta^{13}C$ and $\delta^{2}H$ values in plants grown under fertilized conditions was observed compared to not fertilized plots. Likewise, $\delta^{18}O$ showed a similar trend although not significant differences were observed. The increase of $\delta^{13}C$ with increase of N fertilization has been reported (Farquhar, 1989) as a consequence of a reduction in the ratio C_i/C_a, due to either an increase of photosynthetic capacity or decrease of g_s (Farquhar & Richards, 1984; Condon *et al.*, 2004). However, photosynthesis has been also observed to decrease with N fertilization increase (Cabrera-Bosquet *et al.*, 2009a), due to low g_s values (which subsequently causes a high $\delta^{13}C$). In fact in our study, fertilized plots showed lower g_s compared to not fertilized plots (Table 1). In addition, $\delta^{13}C$ and $\delta^{2}H$ were positively related with total nitrogen in the flag leaf (%N flag) and negatively correlated with g_s under WS conditions (Table 3). Such results indicate that with increase of nitrogen supply, biomass is therefore increased, forcing the plants to compete for water resources and exacerbating water stress in the plants. On the contrary, in HN conditions (including two water regimes) correlations between $\delta^{13}C$ and $\delta^{2}H$ and total nitrogen in the flag leaf were in some cases negative (Table S2). Such results suggest that under WW conditions N fertilization has not a negative effect increasing water stress but rather the opposite. It has been reported that providing there is water available in the soil (i.e. under irrigation conditions) an adequate N fertilization may improve not only growth but also the water status of the crop by contributing to a better growth of roots. Overall, these findings suggest that $\delta^{2}H$ and $\delta^{13}C$ are exposed at least in part to a similar source of variation. The imprint in $\delta^{2}H$ in leaf carbohydrates has been postulated to be a balance within photosynthetic and post-photosynthetic effects (Yakir *et al.*, 1990a). Although any change in metabolic activities would

produce a large effect in δ^2H (Yakir, 1992), δ^2H is also sensitive to changes in g_s as observed by strong correlations between δ^2H and g_s (Table 3). Such positive relationship suggests that under the environmental conditions of our study both isotopes are mainly reflecting changes on g_s conductance. This is in spite the fact the signature of both isotopes in the plant is affected (probably in a different way) by the photosynthetic metabolism (Farquhar, 1989; Yakir, 1992) and further post-photosynthetic fractionations of diverse nature.

Summarizing, δ^2H performs better than the other two isotopes predicting *GY* and N content under water stress but contrasting N regimes (Table 3). δ^2H similarly than $\delta^{13}C$ correlated negatively with g_s. δ^2H in addition to $\delta^{13}C$ was able to detect genetic variability within a wide range of environmental conditions. Differently than the other two isotopes $\delta^{18}O$ of kernels performed very poorly (lost all its capacity) for predicting GY in a repetitive manner (e.g. through years and growing conditions), compared with the $\delta^{18}O$ analyzed in the photosynthetic organs (particularly in the WSF) (Table 3). That means postphotosynthetic metabolism, together with translocation (plus charge-discharge) processes from the photosynthetic organs to the kernels are fractionating ^{18}O of assimilates and disturbing the environmental signal of the $\delta^{18}O$. This study illustrates the usefulness of combined measures of $\delta^{13}C$ and δ^2H (and marginally $\delta^{18}O$) at the plant matter level, which may provide time-integrated records of the photosynthetic and evaporative performance of the plant during crop development. Although, further research is required to understand mechanisms related to photosynthetic metabolism affecting δ^2H and involved in δ^2H fractionation of different organic matter pools, the use of δ^2H may potentially help plant breeders to select genotypes better adapted simultaneously to water and nitrogen limited conditions.

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New Phytologist **Supporting Information**

Article title: *Comparative performance of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ for phenotyping durum wheat adaptation to different water and nitrogen conditions*

Authors: Rut Sanchez-Bragado, Maria Dolors Serret, Jordi Bort, José Luis Araus

The following Supporting Information is available for this article:

Table S1*. Mean values and ANOVA of grain yield (GY) stable isotope composition (‰) of deuterium ($\delta^{2}H$), oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) on dry matter (DM) and the water-soluble fraction (WSF) of different plant parts (flag leaf, ears and roots) sampled at mid grain filling) plus mature kernels (grains) and the stem water (water), grain yield (GY), leaf temperature (Tleaf) and stomatal conductance (g_s) under a combination of support irrigation and N fertilization (WW+HN), support irrigation and without N fertilization (WW-LN), rainfed and N fertilization (WS+HN) and rainfed without N fertilization (WS-LN). The set of genotypes included five modern cultivars and four old landraces grown during the 2010 crop cycle. For each growing condition analyses where performed on nine durum wheat genotypes and three replications, except for $\delta^{18}O$ and $\delta^{2}H$ on dry matter where only two modern cultivars and two landraces were analysed. Each value represent the mean ± SE. Mean values across plant tissues with different letters are significantly different according to the Tukey's honestly significant difference test ($P<0.05$).*

	WW+HN	WW-LN	WS+HN	WS-LN
Deterium				
δ^2H_{roots} DM	-62.5 ± 6.5a	-60.5 ± 7.0a	-75.4 ± 2.3a	-69.4 ± 4.6a
δ^2H_{stemW}	-46.7 ± 1.8a	-45.8 ± 1.3a	-47.4 ± 1.1a	-43.8 ± 0.9a
δ^2H_{flag} DM	-112.7 ± 2.2b	-117.4 ± 1.0a	-100.0 ± 1.5c	-109.5 ± 1.0b
δ^2H_{flag} WSF	-90.0 ± 1.3ab	-89.7 ± 1.3a	-85.7 ± 1.2b	-88.5 ± 0.8ab
δ^2H_{spike} DM	-91.5 ± 1.7ab	-97.4 ± 1.9a	-78.3 ± 1.2c	-86.6 ± 1.5b
δ^2H_{spike} WSF	-61.2 ± 1.3a	-64.6 ± 0.8a	-52.1 ± 1.3b	-58.8 ± 1.0a
δ^2H_{grain}	-30.3 ± 0.8b	-39.7 ± 0.8a	-22.2 ± 1.4c	-31.9 ± 1.2b
Oxygen				
$\delta^{18}O_{roots}$ DM	34.0 ± 0.2a	25.6 ± 0.1a	30.8 ± 0.2a	27.9 ± 0.1a
$\delta^{18}O_{stemW}$	-6.5 ± 3.1a	-6.2 ± 1.6ab	-6.2 ± 2.5ab	-5.9 ± 1.8c
$\delta^{18}O_{flag}$ DM	30.1 ± 0.3a	30.5 ± 0.2ab	32.0 ± 0.4c	30.8 ± 0.5bc
$\delta^{18}O_{flag}$ WSF	27.5 ± 0.3a	30.3 ± 0.1b	32.2 ± 0.2c	32.4 ± 0.2c
$\delta^{18}O_{spike}$ DM	26.7 ± 0.6a	26.3 ± 0.6a	26.8 ± 0.7a	25.7 ± 1a
$\delta^{18}O_{spike}$ WSF	30.0 ± 0.2a	30.4 ± 0.1a	30.5 ± 0.2a	31.0 ± 0.3a
$\delta^{18}O_{grain}$	30.3 ± 0.1a	30.5 ± 0.2a	30.4 ± 0.1a	30.8 ± 0.2a
Carbon				
$\delta^{13}C_{flag}$ DM	-25.8 ± 0.1b	-26.7 ± 0.1a	-24.8 ± 0.1d	-25.6 ± 0.1b
$\delta^{13}C_{flag}$ WSF	-28.4 ± 0.2a	-27.4 ± 0.1b	-26.1 ± 0.2c	-26.4 ± 0.1c
$\delta^{13}C_{spike}$ DM	-25.2 ± 0.1b	-25.7 ± 0.1a	-23.8 ± 0.1d	-24.4 ± 0.1c
$\delta^{13}C_{spike}$ WSF	-24.3 ± 0.2a	-24.6 ± 0.2a	-22.8 ± 0.1b	-23.3 ± 0.2b
$\delta^{13}C_{grain}$	-24.9 ± 0.1a	-25.2 ± 0.1a	-23.1 ± 0.1c	-24.0 ± 0.1b
g_s (mmol·H_2O·m·s)	232.4 ± 65.7c	213.2 ± 44.0c	94.7 ± 20.3a	172.9 ± 45.8b
T_{leaf} (ºC)	28.2 ± 1.6a	27.9 ± 1.3a	28.3 ± 1.3a	28.3 ± 1.4a
GY (Mg Ha^{-1})	2.3 ± 0.1c	1.9 ± 0.1b	1.3 ± 0.1a	1.4 ± 0.1a

***Table S2**. Linear regressions of the relationships between the carbon (δ13C) oxygen ($\delta^{18}O$) and deuterium ($\delta^{2}H$) isotope compositions of the water soluble fraction (WSF) and dry matter (DM) of the flag leaf and the ear, and of mature kernels (grains) against the grain yield (GY), stomatal conductance (g_s), total nitrogen content of the flag leaf (%Nflag) and the ear (%Near). Relationships were assessed under (upper panel) rainfed water stress conditions (WS, including fertilized and non-fertilized plots) and (lower panel) fertilized conditions (HN, including the two water regimes). For each condition the nine genotypes and three replications per genotype were considered, accounting for a total of 54 values, except for the $\delta^{18}O$ and $\delta^{2}H$ of the ear and the flag leaf dry matter, where only two modern cultivars and two landraces were considered. For Analysis were performed in samples from the 2010 crop season. Level of significance: $P < 0.001$; $P < 0.01$; $P < 0.05$; not significant, $P > 0.05$.*

(WW)	GY			g_s			%N Flag			%N Spike		
	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N
Deuterium												
δ^2H_{roots} DM	-0.193	0.377	23	0.229	0.292	23	-0.039	0.860	23	-0.195	0.372	23
$\delta^2H_{stem\ water}$	-0.274	0.057	49	**-0.310**	**0.030**	49	-0.106	0.469	49	0.220	0.128	49
δ^2H_{flag} DM	0.059	0.784	24	-0.220	0.301	24	0.292	0.166	24	0.282	0.182	24
δ^2H_{flag} WSF	-0.063	0.655	53	**-0.270**	**0.050**	53	-0.008	0.956	53	0.059	0.676	53
δ^2H_{spike} DM	0.312	0.137	24	-0.212	0.319	24	**0.485**	**0.016**	24	**0.508**	**0.011**	24
δ^2H_{spike} WSF	**0.404**	**0.003**	53	0.235	0.090	53	**0.439**	**0.001**	53	**0.338**	**0.013**	53
δ^2H_{grain}	**0.275**	**0.044**	54	0.031	0.825	54	**0.687**	**0.000**	54	**0.624**	**0.000**	54
Oxigen												
$\delta^{18}O_{roots}$ DM	0.143	0.514	23	0.132	0.550	23	0.498	0.016	23	-0.329	0.125	23
$\delta^{18}O_{stemW}$	0.340	0.017	49	0.313	0.029	49	-0.240	0.097	49	0.203	0.162	49
$\delta^{18}O_{flag}$ DM	0.093	0.510	53	0.029	0.836	53	-0.185	0.185	53	-0.123	0.382	53
$\delta^{18}O_{flag}$ WSF	-0.217	0.115	54	**-0.271**	**0.047**	54	**-0.686**	**0.000**	54	**-0.619**	**0.000**	54
$\delta^{18}O_{spike}$ DM	-0.002	0.988	54	-0.074	0.595	54	-0.042	0.761	54	-0.032	0.820	54
$\delta^{18}O_{spike}$ WSF	0.093	0.503	54	-0.126	0.366	54	-0.047	0.737	54	-0.244	0.075	54
$\delta^{18}O_{grain}$	-0.170	0.219	54	0.079	0.572	54	-0.201	0.145	54	**-0.271**	**0.048**	54
Carbon												
$\delta^{13}C_{flag}$ DM	0.097	0.486	54	-0.214	0.120	54	**0.445**	**0.001**	54	**0.548**	**0.000**	54
$\delta^{13}C_{flag}$ WSF	-0.264	0.054	54	**-0.436**	**0.001**	54	**-0.553**	**0.000**	54	**-0.367**	**0.006**	54
$\delta^{13}C_{spike}$ DM	**0.411**	**0.002**	54	-0.084	0.544	54	**0.479**	**0.000**	54	**0.460**	**0.000**	54
$\delta^{13}C_{spike}$ WSF	**0.399**	**0.003**	54	-0.098	0.480	54	0.344	0.011	54	0.211	0.125	54
$\delta^{13}C_{grain}$	**-0.330**	**0.015**	54	**-0.384**	**0.004**	54	0.214	0.120	54	0.155	0.263	54

(LN)	GY			g_s			%N Flag			%N Spike		
	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N	Pearson	Sig.	N
Deuterium												
δ^2H_{roots} DM	-0.131	0.543	24	0.359	0.085	24	**-0.072**	**0.738**	24	**-0.115**	**0.593**	24
δ^2H_{stemW}	-0.249	0.076	52	0.050	0.727	52	-0.017	0.904	52	**-0.040**	**0.779**	52
δ^2H_{flag} DM	-0.277	0.200	23	**-0.278**	**0.199**	23	**0.094**	**0.670**	23	0.103	0.640	23
δ^2H_{flag} WSF	-0.056	0.691	53	**-0.063**	**0.654**	53	**0.190**	**0.174**	53	0.214	0.124	53
δ^2H_{spike} DM	-0.243	0.252	24	**-0.362**	**0.082**	24	**0.387**	**0.061**	24	**0.472**	**0.020**	24
δ^2H_{spike} WSF	**-0.554**	**0.000**	53	**-0.306**	**0.026**	53	**0.091**	**0.515**	53	**0.053**	**0.708**	53
δ^2H_{grain}	**-0.449**	**0.001**	54	**-0.501**	**0.000**	54	**0.113**	**0.417**	54	**-0.031**	**0.825**	54
Oxigen												
$\delta^{18}O_{roots}$ DM	0.046	0.831	24	0.265	0.210	24	-0.006	0.976	24	-0.027	0.900	24
$\delta^{18}O_{stemW}$	**-0.385**	**0.005**	52	**-0.020**	**0.886**	52	-0.107	0.449	52	**0.046**	**0.744**	52
$\delta^{18}O_{flag}$ DM	**-0.638**	**0.000**	49	**-0.277**	**0.054**	49	**-0.084**	**0.550**	53	0.163	0.243	53
$\delta^{18}O_{flag}$ WSF	**0.408**	**0.002**	54	**-0.245**	**0.074**	54	**-0.059**	**0.686**	49	**0.059**	**0.689**	49
$\delta^{18}O_{spike}$ DM	-0.463	0.000	54	0.066	0.636	54	-0.217	0.115	54	-0.057	0.682	54
$\delta^{18}O_{spike}$ WSF	0.197	0.154	54	**-0.309**	**0.023**	54	-0.022	0.872	54	-0.068	0.626	54
$\delta^{18}O_{grain}$	-0.136	0.333	53	-0.023	0.868	53	0.001	0.995	54	**-0.151**	**0.276**	54
Carbon												
$\delta^{13}C_{grain}$	**-0.524**	**0.000**	54	**-0.591**	**0.000**	54	**0.089**	**0.524**	54	0.017	0.906	54
$\delta^{13}C_{flag}$ DM	**-0.496**	**0.000**	54	**-0.596**	**0.000**	54	**-0.081**	**0.560**	54	**0.029**	**0.833**	54
$\delta^{13}C_{flag}$ WSF	**-0.518**	**0.000**	54	**-0.522**	**0.000**	54	**0.057**	**0.684**	54	0.226	0.101	54
$\delta^{13}C_{spike}$ DM	**-0.549**	**0.000**	54	**-0.418**	**0.002**	54	0.150	0.279	54	0.209	0.129	54
$\delta^{13}C_{spike}$ WSF	**-0.438**	**0.001**	53	**-0.292**	**0.034**	53	**0.296**	**0.031**	53	**0.264**	**0.056**	53

Discussió

DISCUSSIÓ GENERAL

Aquesta última secció de la tesi pretén:

- Sintetitzar els resultats obtinguts en els diferents capítols de la tesi en relació als objectius generals i discutir-los d'acord amb la literatura disponible.

Síntesi dels principals resultats obtinguts

La present tesi ha estudiat la variació dels isòtops estables del carboni, de l'oxigen i de l'hidrogen així com la seva utilitat com a eines eco-fisiològiques en la millora i gestió de cultius. Com a resultat, aquesta tesi inclou 5 capítols on el blat i el blat de moro es van exposar a diferents condicions experimentals. Per una banda, es va desenvolupar una aproximació basada en l'isòtop de carboni que permetia avaluar la contribució relativa dels diferents òrgans a l'ompliment del gra (Capítol 1 i 2). Per assegurar la veracitat d'aquesta aproximació com a eina en millora de cultius es va comparar amb altres aproximacions alternatives als isòtops estables (Capítol 3). Per altra banda, es va estudiar l'isòtop d'oxigen i les seves limitacions reals en camp (Capítol 4). Per últim es va comparar l'isòtop estable de carboni, d'oxigen i de forma novedosa un isòtop molt poc estudiat en el camp de la millora de cultius, com és l'isòtop d'hidrogen (Capítol 5).

1. Relació entre la composició isotòpica del carboni i el rendiment

Està àmpliament documentat que la composició isotòpica del carboni ($\delta^{13}C$) de la matèria orgànica, proporciona una informació integrada de l'estatus hídric de la planta durant el temps en què s'ha desenvolupat el cicle del cultiu (Araus et al., 2003). Tot i ser l'isòtop més establert com a eina de selecció de genotips amb alt rendiment potencial i/o adaptació a la sequera, la seva interpretació no és tant senzilla. En els nostres resultats, la majoria de les correlacions entre la $\delta^{13}C$ dels diferents òrgans van ser negatives contra el rendiment (GY) (Capítol 5). En aquest sentit, els resultats són esperables ja que correlació negativa entre la $\delta^{13}C$ dels òrgans superiors de la planta i el GY està àmpliament documentada (Araus et al. 1998, 2013; Fischer et al. 1998; Monneveux et al. 2005; Lopes and Reynolds 2010). A més, en els tractaments fertilitzats (quan es van unir els dos regim hídrics) es van observar unes majors correlacions, que en part es podrien explicar per un major rang del rendiment degut les diferències d'estatus hídric de les plantes (condicions barrejades de sequera i de reg suplementari). Així, un major rang en el rendiment dels tractaments fertilitzats podria haver elevat en conseqüència les correlacions entre la $\delta^{13}C$ i el GY. A més, només la $\delta^{13}C$ els grans madurs van mostrar correlacions fenotípiques negatives consistents (Taula 1) contra el rendiment. Les correlacions van ser majors quan millor eren les condicions de creixement (Figura 1 de la Discussió), degut a com es comentava al major rang de valors de rendiment. De fet es van trobar resultats similars en un experiment dut a terme en blat on les correlacions entre $\delta^{13}C$ dels grans madurs i el GY van augmentar, al millorar les condicions ambientals (Araus et al., 2003)

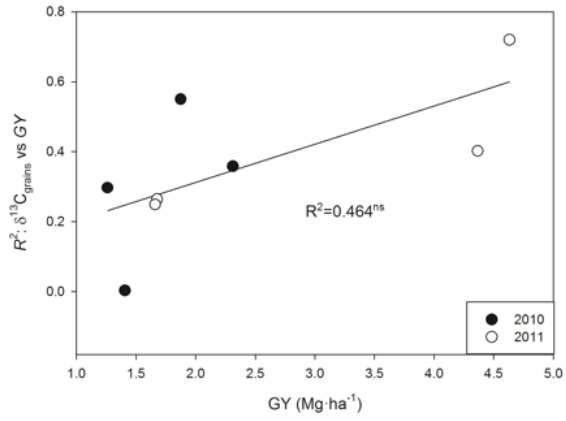

Figura 1*. Correlació entre rendiment mig de cadascuna de les 8 condicions de creixement i el valor del coeficient de correlació entre la $\delta^{13}C$ dels grans madurs i el rendiment. Són 4 les condicions de creixement que resulten de la combinació de 2 regims hídrics i 2 nivells de nitrògen durant dos anys consecutius (2010 i 2011).*

No obstant, en les correlacions genotípiques entre la $\delta^{13}C$ dels grans madurs i el GY (Taula 1 de la Discussió) es mostren més altes en els tractament fertilitzats (WW+HN i WS+HN), indicant que el N també podria estar tenint un efecte en les plantes. De fet, s'ha vist que la fertilització pot ajudar desenvolupat un sistema radicular major (Cooper et al., 1987), el qual permet a les plantes a tenir més accés a l'aigua de zones més profundes i un millor estatus hídric (disminuint així la $\delta^{13}C$ i augmentant el GY). També, la $\delta^{13}C$ s'ha acceptat àmpliament com un indicador de l'EUA (Farquhar and Richards, 1984; Farquhar, 1989). Així tenint en compte que la $\delta^{13}C$ està relacionada positivament amb la EUA (Condon et al., 2004) i negativament amb el GY, aquells genotips que tinguin un rendiment major serien aquells que tinguin una EUA menor i una major g_s (Araus et al., 2002) el que pot comportar una major us efectiu de l'aigua (Blum, 2009).

Per altra banda, tot i les correlacions negatives obtingudes en els tractament fertilitzat (Taula 2), l'efecte del N en les plantes és controvertit. Tanmateix, s'ha observat que al aplicar N, la $\delta^{13}C$ pot disminuir (Shangguan et al., 2000) o augmentar (Cabrera-Bosquet et al., 2007). En els casos en que la $\delta^{13}C$ augmenta en part podria ser perquè en absència de sequera el N es considera com el principal nutrient que limita el rendiment i el major factor que controla l'acumulació de la taxa de la biomassa (Jensen et al., 1990). En conseqüència el subministrament de N pot tenir un efecte negatiu en la g_s, degut a un estrès hídric causat per una gran biomassa. Com a conseqüència el radi del CO_2 intracel·lular (C_i) i el de l'ambient (C_a) disminueix (Cabrera-Bosquet et al., 2007), el que pot causar un augment de la $\delta^{13}C$ i de la EUA (tant instantània com integrada en el temps). En els nostres resultats, tot i que la $\delta^{13}C$ de la majoria d'òrgans es van correlacionar negativament amb el GY, el tractament fertilitzat mostrà una $\delta^{13}C$ més enriquida en tots els òrgans comparat amb el tractament que no estava fertilitzat (Taula 1, Capítol 5).

Per altra banda, la $\delta^{13}C$ de l'òrgan que mostrà correlacions genotípiques negatives més constants contra el GY en les diferents condicions de creixement van ser els grans madurs (Taula 2). A més, les correlacions negatives van ser més dèbils durant el 2010 que el 2011. La causa de les més baixes correlacions al 2010 podria estar relacionada amb el més baix rendiment d'aquest any (menor que 2.1 $Mg \cdot ha^{-1}$) comparat amb el 2011 (2.1 $Mg \cdot ha^{-1}$). Un menor GY suggereix que all 2010 el cultiu podria haver estat sotmès a un estrès mig, afectant així la força de les correlacions (Figura 1 de la disussió). De fet, en experiments sota sequera on els rendiments són molt baixos (menor de 2 $Mg \cdot ha^{-1}$), les correlacions entre la $\delta^{13}C$ i el GY poden ser inexistents (Araus et al., 2003) o inclús canviar a positives (Voltas et al., 1999; Rebetzke et al., 2002; Condon et al., 2002).

***Taula 2**. Regressió lineal fenotipica de la relació entre composicions isotòpiques del carboni ($\delta^{13}C$) de l'oxigen ($\delta^{18}O$) i de l'hidrogen ($\delta^{2}H$) en la fracció soluble en aigua (WSF) i la matèria seca (DM) de la fulla bandera i l'espiga, així com en els grans madurs (grains) contra el rendiment (GY). Es van considerar els nou genotips i tres repeticions per genotip (Capítol 5), el que representa un total de 27 valors a les fraccions solubles i als grans madurs combinats en condicions de reg suplementari i fertilitzat (WW+HN), reg suplementari i no fertilitzat (WW-LN), secà i fertilitzat (WS+HN), secà i no fertilitzat (WS-LN). Per la $\delta^{18}O$ i $\delta^{2}H$ de la matèria seca (fulla bandera, espiga i arrels) només van ser considerats dos cultivars moderns i dues varietats locals per tres repeticions (12 parcel·les). Les anàlisis es dugueren a terme la temporada 2010. Durant el 2011 la $\delta^{13}C$ de la fracció soluble en aigua l'espiga de es va analitzar a les areste. Nivells de significació: P <0,001; P <0,01; P <0,05; no és significativa, P< 0,05.*

	2010		**2011**				**2011**				**2011**				**2011**	
	Pearson	Sig.	Pearson	Sig.	Pearson	Sig.	Pearson	Sig.	Pearson	Sig.	Pearson	Sig.	Pearson	Sig.	Pearson	Sig.
Hydorgen																
δ^2H_{roots} DM	-0.183	0.589			-0.229	0.473			0.362	0.247			-0.357	0.254		
$\delta^2H_{xylem\ water}$	-0.352	0.092			-0.066	0.753			-0.169	0.399			-0.305	0.122		
δ^2H_{flag} DM	-0.205	0.523			0.459	0.133			-0.424	0.169			-0.225	0.507		
δ^2H_{flag} WSF	-0.170	0.397			0.235	0.247			-0.321	0.103			-0.249	0.210		
δ^2H_{spike} DM	0.082	0.801			0.427	0.167			-0.467	0.126			-0.177	0.582		
δ^2H_{spike} WSF	**0.427**	**0.026**			-0.060	0.772			**-0.458**	**0.016**			**-0.514**	**0.006**		
δ^2H_{grain}	-0.050	0.806			-0.014	0.946			**-0.620**	**0.001**			-0.187	0.351		
Oxygen																
$\delta^{18}O_{xylem\ water}$	-0.061	0.860			0.222	0.489			**-0.576**	**0.050**			0.119	0.713		
$\delta^{18}O_{roots}$ DM	-0.376	0.070			-0.066	0.753			-0.127	0.529			**-0.490**	**0.009**		
$\delta^{18}O_{flag}$ DM	-0.054	0.868	-0.069	0.808	0.026	0.936	**-0.742**	**0.002**	-0.228	0.476	-0.393	0.147	-0.459	0.133	-0.435	0.105
$\delta^{18}O_{flag}$ WSF	0.149	0.459			-0.032	0.874			**-0.436**	**0.042**			**-0.558**	**0.007**		
$\delta^{18}O_{spike}$ DM	-0.388	0.213			-0.007	0.982			0.009	0.977			-0.375	0.230		
$\delta^{18}O_{spike}$ WSF	0.141	0.484			0.334	0.089			-0.200	0.317			**-0.740**	**0.000**		
$\delta^{18}O_{grain}$	-0.119	0.553	-0.155	0.582	-0.139	0.489	-0.232	0.404	-0.352	0.071	-0.110	0.695	-0.299	0.130	-0.424	0.115
Carbon																
$\delta^{13}C_{spike}$ DM	-0.264	0.183			-0.174	0.385			**-0.486**	**0.010**			-0.028	0.888		
$\delta^{13}C_{spike}$ WSF	0.372	0.056	-0.082	0.773	0.141	0.482	-0.347	0.205	-0.212	0.287	-0.191	0.313	**-0.397**	**0.040**	-0.108	0.571
$\delta^{13}C_{flag}$ DM	-0.073	0.716	-0.072	0.798	0.056	0.783	-0.473	0.075	-0.327	0.096	-0.065	0.817	-0.283	0.152	-0.060	0.833
$\delta^{13}C_{flag}$ WSF	**0.484**	**0.011**	-0.292	0.290	0.293	0.138	-0.017	0.951	-0.275	0.164	-0.303	0.104	**-0.562**	**0.003**	**-0.524**	**0.003**
$\delta^{13}C_{grain}$	**-0.598**	**0.001**	**-0.634**	**0.011**	-0.234	0.240	**-0.848**	**0.000**	**-0.545**	**0.003**	**-0.514**	**0.004**	-0.050	0.804	**-0.499**	**0.005**

Per altra banda, mentre les condicions ambientals com la disponibilitat d'aigua o nitrogen poden afectar la $\delta^{13}C$ i la seva relació amb el GY, bàsicament per un efecte en la conductància estomàtica, també hi ha diferències constitutives de la $\delta^{13}C$ associades als diferents òrgans (Hubick and Farquhar, 1989; Araus et al., 1993). Doncs, en cereals C_3 com ara el blat o l'ordi, la $\delta^{13}C$ dels grans madurs s'ha demostrat que pot reflectir millor les condicions d'estrès experimentades pel cultiu (particularment durant l'estadi reproductiu), comparat amb la $\delta^{13}C$ de la fulla bandera en el que no està tant clar que correlacioni amb el GY (Austin et al., 1990). De fet, els nostres resultats ho recolzen, ja que la $\delta^{13}C$ de la fulla bandera tot i correlacionar molt be amb el GY en els tractaments de nitrogen (induït per un ampli rang ambiental) no correlacionà en condicions de reg suplementari (Taula S2, Capítol 5) ni quan es van separar les 4 condicions de creixement (Taula 2).

2. Relació entre la composició isotòpica del carboni i la fotosíntesi de l'espiga

A banda del potencial ús directe que pugui tenir l'anàlisi de la $\delta^{13}C$ (o del $\Delta^{13}C$) en els diferents teixits de la planta, com indicador de rendiment potencial o de l'adaptació a la sequera (i estressos relacionats com salinitat, calor, etc.) en blat i altres conreus C_3; la firma isotòpica del de carboni també pot servir de manera indirecta per formular altres criteris de selecció que tenen els mateixos objectius d' incrementar el rendiment i l'estabilitat del blat. En aquest context, un camí pot se seleccionar per una major fotosíntesi de l'espiga (Araus et al. 1993; Tambussi et al. 2005; Tambussi et al. 2007b; Araus et al. 2008; Parry et al. 2011). S'ha demostrat que la fotosíntesi de l'espiga en condicions de sequera pot tenir una gran contribució durant l'ompliment del gra (Tambussi et al., 2005). No obstant, tot i que s'ha estudiat àmpliament la fotosíntesi de l'espiga (Araus et al., 1993; Bort et al., 1994; Tambussi et al., 2005, 2007b; Maydup et al., 2010; Saeidi et al., 2012) la seva contribució durant l'ompliment del gra no és encara del tot clara, degut a problemes intrínsecs

a la naturalesa intrusiva de les tècniques de fenotipatge emprades fins la data. En aquest context, aquesta Tesi proposa l'ús de la composició isotòpica del carboni (abundància natural), per desenvolupar una aproximació per quantificar la contribució relativa dels diferents òrgans a l'ompliment del gra (Capítols 1 i 2). L'aproximació es va basar en les diferències constitutives de la $\delta^{13}C$ (abundància natural) dels assimilats de diferents òrgans fotosintèticament actius durant l'ompliment del gra. Per exemple, en aquest sentit la $\delta^{13}C$ de la fulla bandera s'ha observat que té un valor més baix (més negatiu) comparat amb l'espiga, mentre que la $\delta^{13}C$ dels grans madurs exhibeixen valors entre la fulla bandera i l'espiga (Araus et al., 1993). La naturalesa d'aquestes diferències constitutives entre els òrgans (p.e. fulla i espiga) està probablement associada a les diferències de la permeabilitat de l'òrgan al CO_2 atmosfèric (Farquhar, 1989; Araus et al., 1993).

No obstant, s'ha de tenir en compte els factors que puquin afectar la signatura isotòpica dels foto-assimilats. Així, la $\delta^{13}C$ dels carbohidrats exportats des dels òrgans autotròfics fins als òrgans heterotròfics podrien patir un fraccionament durant la càrrega, transport o descarrega del floema (Cernusak et al., 2009). Però, aquest fraccionament tot i haver-se demostrar majoritàriament en espècies arbòries (Damesin and Lelarge, 2003; Scartazza et al., 2004; Brandes et al., 2006; Gessler et al., 2007, 2009a) i en espècies llenyoses, en espècies herbàcies no sembla ser tant evident. De fet, diversos estudis realitzats en blat (Yoneyama et al., 1997) i altres espècies herbàcies com la mongeta i *Ricinus communis* (Badeck et al., 2005; Gessler et al., 2009b) no van poder provar que hi hagués un clar fraccionament post-fotosintètic de la $\delta^{13}C$ dels sucres entre la fulla i el floema. Per una altra banda, la respiració associada a la translocació pot tenir una efecte en la discriminació de l'isòtop de carboni, tot i que la disciminació s'ha vist que pot ser molt baixa (Bort et al., 1996; Badeck et al., 2005).

Així, assumint els punt comentats més amunt, la $\delta^{13}C$ dels grans madurs seran el resultat de la combinació de la $\delta^{13}C$ dels assimilats produïts per els diferents òrgans fotosintètics. Amb aquest mètode, l'objectiu va ser comparar la $\delta^{13}C$ dels assimilats produïts per els diferents òrgans fotosintètics amb la $\delta^{13}C$ dels grans madurs (Capítols 1, 2).

2.1 Contribució relativa de l'espiga i la fulla durant l'ompliment del gra en condicions de sequera

La fulla bandera en blat s'ha considerat tradicionalment com el principal òrgan fotosintètic durant l'ompliment del gra (Evans and Rawson, 1970; Araus and Tapia, 1987). En conseqüència, l'aproximació emprada va implicar comparar la contribució relativa de la fulla bandera amb la de l'espiga a l'ompliment del gra (Capítol 1) en un experiment amb quatre condicions de creixement (reg i sequera, fertilitzat i sense fertilitzar amb N). Paral·lelament, també es va avaluar si els genotips previs a la Revolució Verda (antigues) responien igual que les varietats modernes a l'aproximació desenvolupada. Els nostres resultats estan d'acord amb la literatura, (Tambussi et al., 2007a). Així la contribució relativa de l'espiga va augmentar amb l'estrès hídric dels grans (observat per una major $\delta^{13}C$ dels grans madurs) (Figura 1, Capítol 5). A més, es van observar diferències genotípiques en la contribució relativa de la fotosíntesi de l'espiga (Figura 2, Capítol 1). Els genotips antics, caracteritzats per tindre un índex de collita menor, mostraren una contribució fotosintètica relativa molt major que els genotips actuals. El fet de trobar una contribució de l'espiga major al disminuir l'índex de collita (Figura 3, Capítol 1), suggereix que l'ompliment del gra pot ser mantingut bàsicament per la fotosíntesi de l'espiga en els genotips antics, a part de la contribució que puguin tenir les reserves pre-antesi (Maydup et al., 2012). Per contra, la correlació negativa entre la contribució de l'espiga i el HI, sobretot en els genotips actuals (Figura 4, Capítol 1), suggeria que al augmentar la

mida relativa del 'destí' (es a dir augmentar el HI), l'ompliment del gra no es podia sustentar únicament per la fotosíntesi de l'espiga. De fet, estudis recents indiquen evidències de l'aparició d'una limitació de la 'font' en els genotips moderns (Álvaro et al., 2008; Pedro et al., 2011; Acreche and Slafer, 2011; Serrago et al., 2013). Així, l'increment del HI, acompanyat de la disminució de les reserves pre-antesi degut als gens de nanisme (Maydup et al., 2012) recolzen que els genotips moderns no poden ser nodrits únicament per la fotosíntesi de l'espiga.

Per una altra banda, la contribució relativa de l'espiga no només va estar governada per la relació "font-destí", sinó també per les condicions de creixement. De fet, al afegir el N, la contribució relativa de l'espiga va incrementar (Taula 3, Capítol 1), tant en condicions de reg suplementari com de sequera. Com s'ha comentat abans (a l'apartat 1), la fertilització nitrogenada pot causar un augment de les condicions d'estrès hídric en el cultiu (induït per un augment de biomassa). En conseqüència, la contribució relativa de l'espiga a l'ompliment del gra pot augmentar (degut a la millor adaptació que té l'espiga en sequera). De fet, en un estudi de blat dur sota bones condicions agronòmiques, es va trobar que la taxa de fotosíntesi instantània de tota l'espiga podia correlacionar millor amb el GY que la làmina de la fulla bandera (Abbad et al., 2004). No obstant això, els genotips amb major contribució de l'espiga no necessàriament tenen més alt rendiment del gra. De fet, tal com s'indica més amunt, els ecotips que tenen un HI baix tenen una contribució de l'espiga més elevada. Tal i com s'explica més en detall en el Capítol 2, el lleuger dèficit hídric en bones condicions agronòmiques i elevat adobat nitrogenat, pot causar un augment en la contribució relativa de l'espiga (Tambussi et al. 2007) però no necessàriament un augment del GY. Per tant, s'ha de tenir cura alhora d'avaluar la contribució relativa de l'espiga, sobretot en que el panel de genotips estiguin exposats ja siguin a condicions agronòmiques òptimes o alternativament sota nivells similars d'estrès hídric.

Per altra banda, en els tractaments on la correlació de la $\delta^{13}C$ de l'espiga contra el rendiment era la més alta, la correlació entre la $\delta^{13}C$ de la fulla bandera i el rendiment va ser la més baixa (Taula 2 i Taula S2, Capítol 5). Ara bé, tot i la baixa contribució fotosintètica relativa observada en la fulla bandera (Figura 1, Capítol 1) no es pretén contradir l'important rol que té junt amb les fulles més inferiors. En realitat, són les fulles les que determinen el nombre de flors fèrtils per espiga o inclús el nombre de llavors i la seva mida potencial (Slafer i Savin, 1994). De fet, sota condicions agronòmiques òptimes, els valors acumulats de CO_2 fixat per la fulla i l'espiga (incloent la respiració), han sigut comparables amb el pes total dels grans per espiga des de l'espigat fins a la maduresa fisiològica (Figura 1, Capítol 2). Així, considerant una translocació eficient vers el "destí" i poca respiració nocturna, tant la fulla bandera com l'espiga podien tenir un paper comparable i suficient per si mateix alhora de mantenir l'ompliment del gra. De fet, les mesures de llum interceptada per la lamina de la fulla bandera i l'espiga van ser similars (Figura 3, Capítol 2), mentre que la llum absorbida per la resta de la tija va representar un 40% del total rebut per la fulla bandera i l'espiga conjuntament. Aquests resultats indiquen que la fulla bandera no és necessàriament l'única font d'assimilats de la tija. Això ens indica que l'aproximació aquí presentada, té la limitació inherent de no considerar la contribució potencial d'altres òrgans fotosintètics que també poden estar desenvolupant un paper important durant l'ompliment del gra, com serien el peduncle, la beina de la fulla bandera, i les fulles inferiors. Però, tot i la potencial contribució dels altres òrgans (a part de l'espiga) a l'ompliment del gra, la producció potencial de biomassa calculada per l'espiga a partir de la irradiància interceptada a nivell de dosser, va ser de 4.1 $g \cdot espiga^{-1}$ (Figura 5, Capítol 4), assumint una eficiència fotosintètica del 2.4% (Zhu et al., 2008). Aquests valors potencials de producció (a partir de la fotosíntesi bruta de l'espiga) van ser comparables amb el pes final dels grans per espiga, proporcionant més evidències indirectes a favor de la importància de la fotosíntesi de l'espiga durant l'ompliment del gra, comparat amb la fulla bandera.

2.2 Contribució relativa de les arestes i la tija durant l'ompliment del gra en condicions agronòmiques òptimes

Així, com ja s'ha vist a l'apartat anterior, l'espiga comparada amb la fulla bandera en condicions de sequera, pot tenir un paper important com a font de foto-assimilats durant l'ompliment del gra. Però, també s'ha demostrat que l'espiga pot tenir un paper rellevant sota bones condicions agronòmiques (Araus et al., 1993; Bort et al., 1994; Tambussi et al., 2005, 2007a; Maydup et al., 2010). A més, en aquestes condicions, diferents malalties fúngiques poden afectar les fulles (Robert et al., 2005) més que les espigues (Tiedemann and Firsching, 2000). En aquest cas, es va considerar una nova aproximació metodològica per avaluar la contribució dels principals òrgans fotosintètics de l'espiga que són les glumes i les arestes (Bort et al., 1994). També, es va tenir en compte la $\delta^{13}C$ del peduncle ja és l'òrgan pel qual travessen tots els assimilats que provenen de la fulla bandera, la beina, les fulles inferiors, així com també les reserves pre-antesis que es remobilitzen als grans en creixement (Gebbing and Schnyder, 1999). De fet, s'ha documentat que durant la primera meitat del l'ompliment del gra és quan s'utilitzen les reserves pre-antesi (Wardlaw and Willenbrink, 1994; Gebbing and Schnyder, 1999). Així, el peduncle doncs també està integrant la contribució potencial de totes aquestes reserves. A més, per tenir una idea dels carbohidrats disponibles en cadascun dels òrgans analitzats, aquests es van quantificar a la part superior del peduncle, a la fulla bandera, a les arestes i a les glumes . Tots aquestes òrgans van mostrar uns valors similars. En canvi, en la part inferior del peduncle els valors de carbohidrats solubles van ser de fins a sis vegades més que els altres òrgans (Fig. 2, Capítol 2). Això possiblement estigui indicant que sota bones condicions agronòmiques, la capacitat fotosintètica de les plantes durant l'ompliment del gra, excedeix la demanda dels grans en creixement (Slafer and Andrade, 1991; Bingham et al., 2007; Dreccer et al., 2009). De totes maneres, en el cas de que aquestes reserves es remobilitzessin, la $\delta^{13}C$ de peduncle les estaria en part de reflexant.

A l'experiment del CIMMYT sota bones condicions agronòmiques, l'espiga va representar una contribució d'un 70% del total d'assimilats que van als grans (Figura 4, Capítol 2). De fet, es van obtenir resultats similars en un experiment amb marcatge del ^{13}C (Aranjuelo et al. 2011), on es va estudiar el carboni fixat per la fulla bandera durant l'inici de l'ompliment del gra. En aquest estudi, només una petita part dels sucres solubles fixats per la fulla bandera van arribar a l'espiga. La resta de sucres solubles que no van viatjar vers l'espiga es van emmagatzemar com compostos estructurals de carboni i midó que posteriorment es van respirar. Així, en aquest estudi es va concloure que el carboni sintetitzat a l'espiga és el que majoritàriament es va utilitzar per l'ompliment del gra.

Per altra banda, la re-fixació del CO_2 respirat és un element a tindre en compte (Bort et al., 1996) en aquesta aproximació, ja que s'ha vist que pot contribuir fins a un 70% de la sacarosa acumulada a les bràctees (Gebbing and Schnyder, 2001). Però, en el nostre estudi el CO_2 respirat de tots els òrgans analitzats van mostrar una $\delta^{13}C$ més enriquida en comparació amb el substrat original. Això indicaria un empobriment en ^{13}C dels substrat, el que no dona suport a un possible enriquiment de la $\delta^{13}C$ dels grans degut a la translocació de carbohidrats i a la respiració .

Per altra banda, en la nostra aproximació, tampoc es van incloure les glumes, tot i que s'ha documentat que són fotosintèticament actives i una possible font d'assimilats (Araus et al., 1993; Bort et al., 1994). A més, s'ha vist que les glumes re-fixen principalment CO_2 (Bort et al., 1996) respirat pels grans (Gebbing and Schnyder, 2001). És més, si les bràctees de l'espiga són hermèticament tancades al gas, no hi ha discriminació durant el procés de re-assimilació del CO_2 respirat (Farquhar, 1989) i per tant les diferencies entre $\delta^{13}C$ dels grans i de la fracció soluble de les glumes haurien de ser mínimes. De fet, als nostres resultats la $\delta^{13}C$ de les glumes i els grans

no van ser significativament diferents (Taula 2, Capítol 2). Per tant, la importància de la fotosíntesi de l'espiga a l'ompliment del gra es podria estar subestimant, ja que les glumes no es van incloure en l'aproximació tot i el seu paper en la re-assimilació del CO_2 que s'allibera durant els processos de respiració.

2.3 Contribució relativa de les arestes i la tija durant l'ompliment del gra en condicions de sequera i deficiència de nitrogen

En els apartats anterior, la contribució relativa de l'espiga a l'ompliment del gra va ser en tots els casos elevada tot i variar en funció de l'estatus hídric de la planta. Resumint els resultats en els capítols anteriors, er una banda es va comparar la contribució relativa del conjunt de l'espiga amb la de la fulla bandera (Capítol 1) sota un ampli rang ambiental (diferents adobats nitrogenats i nivells d'estrès hídric). Per altra banda, es va comparar específicament la contribució de les arestes (com a indicador de la contribució relativa de l'espiga) amb la del peduncle (el qual englobava els assimilats que provenen de les fulles inferiors, beina, i reserves pre-antesi) en bones condicions agronòmiques de creixement (Capítols 2 i 3). Com ja s'ha comentat la contribució de les reserves emmagatzemades a les parts vegetatives de la tija pot augmentar en condicions de sequera (Bidinger et al., 1977). Així, per tenir en compte això, es va aplicar l'aproximació emprada per el blat tendre crescut sota bones condicions hídriques de CIMMYT (Capítol 2), al blat dur sota les condicions de sequera i dèficit de nitrogen (Figura 3), considerant el peduncle i les arestes. Així, utilitzant el peduncle, es tenen en compte les reserves emmagatzemades prèvies a la floració a més de la activitat fotosintètica durant l'ompliment del gra. De fet, aquestes reserves estan constituïdes per carbohidrats no estructurals de baix pes molecular com la fructosa i el midó (Blacklow et al., 1984), que principalment s'emmagatzemen en els entrenusos superiors de la tija (Seide, 1996), convertint al peduncle com el potencial contribuïdor d'aquestes reserves (Hafsi et al., 2001).

Els resultats van mostrar un alta contribució relativa de l'espiga (les arestes) en comparació amb el peduncle (Figura 3 de la discussió). Aquesta tendència va ser més marcada en el tractament no fertilitzat. En aquest cas, al no afegir-hi nitrogen la sequera fa incrementar la contribució relativa de l'espiga a l'ompliment del gra (Tambussi et al., 2007a). Per altra banda, el promig de la contribució relativa de l'espiga en el tractament fetilitzat (mitja dels tres intervals), va ser major en comparació amb el tractament de baix nitrogen. Això concorda amb els resultats que es van obtenir en el Capítol 1, tot i emprar una aproximació metodològica més senzilla (la contribució de l'espiga va ser major en els tractaments amb alt nitrogen, Taula 3, Capítol 1). De fet, s'ha demostrat que al aplicar nitrogen en un cultiu de blat (en condicions de camp), la fotosíntesi de l'espiga pot tindre un paper decisiu (Abbad et al., 2004) alhora d'obtenir un alt rendiment (Olszewski et al., 2014). De fet, s'ha documentat en tractaments amb alt nitrogen una correlació molt més forta entre el rendiment i la fotosíntesi de l'espiga que amb la fotosíntesi de la fulla bandera, des de l'espigat fins a l'ompliment del gra (Olszewski et al., 2014).

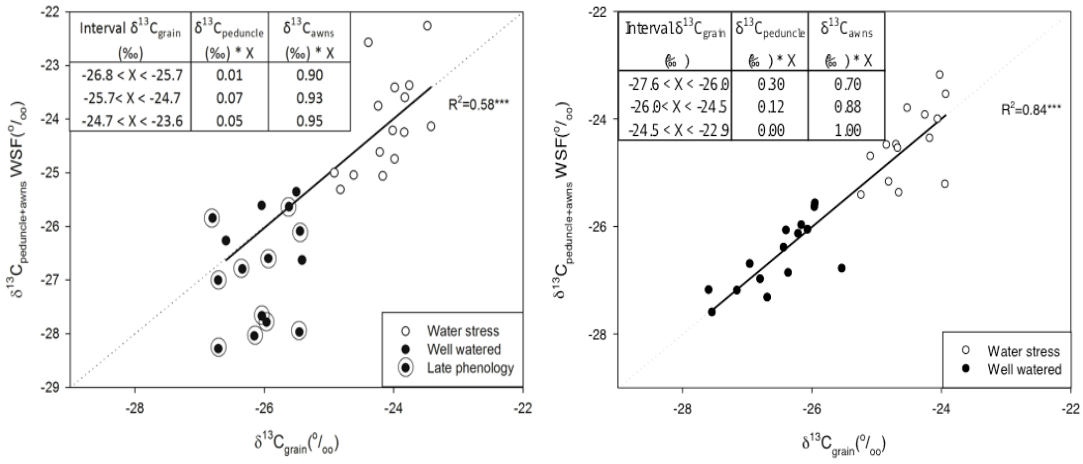

Figura 3. *Regressió lineal de la relació entre la composició isotòpica del carboni en els grans madurs (δ^{13}Cgrains) i la combinació de la δ^{13}C del peduncle i les arestes de les espigues ($\delta^{13}C_{peduncle+awns}$) en la fracció soluble de l'aigua. La figura de l'esquerra presenta els resultats de cinc genotips i tres repeticions per genotip en condicions fertilitzades, mentre que la figura de la dreta considera nou genotips i tres repeticions per genotip en condicions sense nitrogen. Els genotips amb fenologia tardana es van destacar (veure símbols envoltats). Per cada parcel·la, el pes relatiu assignat a la δ^{13}C de cada un dels dos òrgans depen de les condicions hídriques de la parcel·la, la qual s'avalua a través de la composició isotòpica del propi gra ($\delta^{13}C_{grain}$), on es mostra a la taula dins del requadre superior esquerra de les 2 figures. Els nivells de significació: ***, P <0,001. Experiment dut a terme a l'estació de l'INIA de Colmenar de Oreja (Aranjuez, Madrid) durant la temporada 2012.*

2.4. Assumpcions/limitacions de l'aproximació de l'isòtop de carboni

En l'aproximació isotòpica és van assumir uns aspectes: (i) només es van considerar dues fonts de foto-assimilats durant l'ompliment del gra, (ii) els foto-assimilats d'aquestes dues fonts tenien una diferencia significativa en l'abundància natural ^{13}C:^{12}C, (iii) donat que vam mostrejar nomes dues vegades durant l'ompliment del gra, vam considerar que els valors es mantenien constants al llarg del període de l'ompliment del gra i (iv) el fraccionament secundari durant l'emmagatzematge i mobilització als grans era mínim (Cernusak et al., 2009). Aquest últim punt comentat per Raven i Griffiths (2015) podria estar explicant la $\delta^{13}C$ més elevada del peduncle observada en els nostres resultats. Tal enriquiment del ^{13}C al peduncle podria ser causa del fraccionament secundari durant la (re) mobilització i emmagatzematge dels carbohidrats (per exemple, de la fulla bandera). Cernusak et al. (2009), explica en detall una sèrie d'hipòtesis que podrien explicar el potencial fraccionament entre els òrgans 'font' i els orgàns 'destí'. Tot i no haver-se demostrat en el blat un fraccionament durant la remobilització dels carbohidrats al gra (Yoneyama et al., 1997), un dels mecanismes addicionals que podria induir a un fraccionament de l'isòtop de carboni, és el possible desplaçament dels carbohidrats emmagatzemats durant la nit enlloc de durant el dia (Tcherkez et al. 2004). No obstant, estudis en el girasol i el blat no van poder demostrar una variació al llarg del dia de la $\delta^{13}C$ en els carbohidrats (Ghashghaie et al., 2001; Kodama et al., 2011). També, altres processos que podrien estar alterant la $\delta^{13}C$ de les espigues en el blat és la matèria orgànica que arriba a l'espiga a través del xilema. El xilema transporta àcids orgànics i aminoàcids sintetitzats a les arrels (en plantes C_3) els quals mostren una $\delta^{13}C$ molt menor que aquells sintetitzats a les fulles (Yoneyama et al., 1997). Així, el subministrament de C del xilema i el floema durant l'ompliment del gra, des de les arrels o el peduncle, podria estar afegir fonts addicionals de variació en la $\delta^{13}C$ de l'espiga. De totes maneres el subministrament via xilema de C a l'espiga es quantitativament molt petit

(Taiz and Zeiger, 2002). Tot i així, l'aproximació metodològica que aquí es proposa per inferir les particions de la font-destí, s'ha de tenir en compte que no informa sobre la contribució real dels òrgans fotosintètics a l'ompliment del gra, si no que estima les màximes capacitats fotosintètics de cada òrgan de manera comparativa.

3. Comparativa entre metodologies per estimar la contribució relativa de l'espiga i la tija durant l'ompliment del gra

Tot i les limitacions comentades, aquesta aproximació metodològica s'ha provat en diversos experiments de camp sota diferents condicions de creixement ambientals. Així aquesta metodologia basada en l'estudi de la composició isotòpica del carboni en la seva abundància natural, s'ha demostrat que funciona per elucidar la contribució relativa dels diferents òrgans fotosintètics. A més, l'aproximació es pot utilitzar en diferents règims hídrics i de nitrogen, amb l'avantatge afegida de que és una aproximació no intrusiva. Així, s'eviten efectes compensatoris no desitjats entre els òrgans (Chanishvili et al., 2005). Tot i això, l'aproximació es va comparar amb altres dues metodologies de caire més intrusiu sota bones condicions agronòmiques. Les tècniques que es van utilitzar per inhibir la fotosíntesi de l'espiga i de la tija van ser (i) l'aplicació d'un herbicida DCMU i (ii) l'ombrejat (Capítol 4).

En el tractament de DCMU, el pes dels grans per espiga (GW_{ear}) va assignar un rol major a la fotosíntesi de la tija (70%) comparat amb el de l'espiga (40%) (Figura 3, Capítol 4). No obstant, quan el DCMU es va aplicar a la tija, no només va afectar a la fotosíntesi d'aquesta, si no que també es va inhibir parcialment la fotosíntesi de l'espiga. En la mateixa línia, en un estudi realitzat per Nicolas i Turner (1993), les fulles i tiges es van ruixar amb tres dessecants diferents (paraquat, clorat de magnesi i clorat de sodi). En el mencionat estudi, els dessecants van afectar també a les espigues, el que en conseqüència va reduir el pes dels grans en més d'un 70%. Tot

plegat suggereix que els compostos químics es van transportar des de la tija i les fulles (via el floema) a l'espiga i a continuació als grans en desenvolupament (Blum et al., 1983). No obstant això, no tots els compostos químics afecten de la mateixa manera els grans en desenvolupament. De fet, en un estudi de Saeidi et al. (2012) en blat, van ruixar les tiges i les espigues amb el dessecant de iodur de potassi. A continuació, van observar una major contribució de l'espiga a l'ompliment del gra comparat amb la tija. La diferència bàsica entre aquest dessecant i els utilitzats per Nicolas i Turner (1993) va ser que el iodur potàssic es transportava pel xilema (Herrett et al., 1962), i no pel floema, creant una afectació menor a l'espiga i els grans en desenvolupament. Així, per tal d'evitar transports no desitjats dels productes químics dins de la planta, l'ombrejat va ser el sistema que es va utilitzar per inhibir la fotosíntesi en el següent any. En aquest cas el pes dels grans per espiga (GW_{ear}) van ser similar a les espigues i a les tiges ombrejades, indicant que la fotosíntesis de l'espiga i la tija podien ser comparables en termes d'ompliment del gra.

No obstant, els resultats obtinguts per aquestes metodologies de naturalesa intrusiva s'han d'interpretar amb cautela, perquè poden aparèixer mecanismes compensatoris que incrementin la contribució a l'ompliment del gra dels òrgans no tractats o bé de les reserves pre-antesi (Aggarwal et al., 1990; Eyles et al., 2013). De fet, la contribució total de l'espiga sumada a la de la tija durant l'ompliment del gra dels tractaments de DCMU (110%) i d'ombrejat (119%), van ser majors comparades amb el control (100%). En conseqüència, aquests resultats indiquen els possibles efectes compensatoris dels òrgans no tractats (Figura 3, Capítol 3). En aquesta línia, en els estudis realitzats per Aggarwal et al. (1990) i Ahmadi et al. (2009), el pes final dels grans no va disminuir quan es va defoliar la planta, i els autors van indicar que l'ompliment del gra estava governat per l'òrgan 'destí' més que pels òrgans 'font'. Paradoxalment, la fotosíntesi de l'espiga no es va considerar en aquests

treballs (Saeidi et al., 2012), de manera que els seus resultats podrien interpretar-se de dues maneres. Per una banda, els resultats podrien estar esbiaixats degut als mecanismes compensatoris dels òrgans no afectats (com és l'espiga) pel tractament de defoliació (Chanishvili et al., 2005). Per una altra banda, el pes del grans no va disminuir, revelant l'important rol que pot estar exercint la fotosíntesi de l'espiga durant l'ompliment del gra. Comparativament, tot i les limitacions imposades pels tractaments experimentals de naturalesa intrusiva, la inhibició de la fotosíntesi de l'espiga (per acció del DCMU o de l'ombrejat) va reduir de manera similar el pes final dels grans en un 35-40% el que està d'acord amb treballs previs (Maydup et al., 2010).

En definitiva, les diferents metodologies en bones condicions agronòmiques, ja sigui per l'aplicació de DCMU, d'ombrejat o per l'aproximació de la $\delta^{13}C$, van mostrar una contribució de l'espiga a l'ompliment del gra al menys comparable a la de la tija (on s'inclou la fulla bandera, les fulles inferiors, i la beina). En aquest cas, l'aproximació va mostrar una contribució relativa màxima de l'espiga d'un 74% de promig (Figura 3, Capítol 3). En canvi, en condicions de sequera la contribució relativa de l'espiga va augmentar fins al 100% (Figura 1, Capítol 1), el que està d'acord amb la literatura existent (Araus et al., 1992, 1993; Tambussi et al., 2005, 2007a; Maydup et al., 2010, 2012, 2014), En un estudi dut a terme per Merah i Monneveux (2014) també es van comparar els efectes de l'ombrejat i l'excisió amb la remobilització del carboni de diferents òrgans durant l'ompliment del gra. No obstant, en aquest estudi no es va quantificar de manera precisa la contribució relativa de cadascun dels òrgans a l'ompliment del gra. Però així i tot, es van obtenir resultats similars als duts a terme en el Capítol 3, recalcant la importància de la l'espiga i les arestes com a majors proveïdors de foto-assimilats (Jia et al., 2015), i per contra la modesta contribució de la fulla bandera al rendiment sota condicions Mediterrànies.

4. Relació entre la composició isotòpica de l'oxigen i el rendiment

Com s'ha comentat en els punts anteriors d'aquesta tesi, l'isòtop estable de carboni ($\delta^{13}C$) és una eina molt útil per (i) avaluar l'estatus hídric del cultiu sota condicions de creixement diferents,(ii) avaluar les diferencies genotípiques de les plantes exposades a diferents condicions, i de manera més novedosa per (ii) seleccionar genotips amb una fotosíntesi de l'espiga més elevada. De fet, la $\delta^{13}C$ en plantes C_3 s'ha utilitzat àmpliament per seleccionar genotips amb alta eficiència transpiratoria (ET) especialment durant les etapes inicials del conreu (Farquhar i Richards 1984; Richards et al. 2002) i en aquest sentit el CSIRO australià ha emprat un alt $\delta^{13}C$ en la plàntula com a criteri de selecció de diferents varietats de blat adaptades a les condicions mediterrànies australianes (Condon and Richards, 1992; Condon et al., 1993, 2004; Richards et al., 1993). No obstant, la utilització de la $\delta^{13}C$ com una eina de selecció de genotips amb un major rendiment no és senzilla ja que com s'ha vist en les seccions anteriors que depèn de les condicions hídriques del cultiu i també de les condicions de nitrogen. Així, pel ambients de la conca Mediterrània on son esperables pluges durant el creixement del conreu, incloent l'ompliment del gra, la selecció basada en una alta $\delta^{13}C$ en els grans es una possibilitat (Araus et al. 2002a; Araus et al. 2008; Taula 1 en aquesta discussió). L'alta $\delta^{13}C$ estaria indicant que les plantes que exhibeixen menor ET son possiblement aquelles més capaces d'utilitzar aigua disponible al terra i per tant aquelles que tenen un ús efectiu de l'aigua millor (Araus et al., 2008; Blum, 2009). Per altra banda la $\delta^{13}C$ en plantes C_4 com el blat de moro, mostra una variació molt petita i en conseqüència la seva utilitat és sovint limitada per diferenciar entre condicions de creixement (Hubick et al., 1990; Monneveux et al., 2007; Cabrera-Bosquet et al., 2009a). Com a alternativa, s'ha proposat la $\delta^{18}O$ dels teixits de la planta com un tret per seleccionar cultius C_4 millor adaptats a la sequera (Cabrera-Bosquet et al., 2009b). A més, a diferència de la $\delta^{13}C$, no hi ha divergències particulars pel que fa al fraccionament de la $\delta^{18}O$ en cultius C_3 i C_4.

D'acord amb la base teòrica (Barbour and Farquhar, 2000) la variació de la $\delta^{18}O$ prové de les condicions d'evaporació les quals es van conrear les plantes. Així, en aquesta Tesis de manera general i sense tenir en compte les característiques específiques de cada experiment (diferents camps experimentals), les espècies estudiades (és a dir, el blat dur (C_3) i el blat de moro (C_4)), així com la ubicació (és a dir, Aranjuez, Espanya o Tlaltizapán, Mèxic), la $\delta^{18}O$ en els teixits de la planta va augmentar sempre com a resposta a la limitació d'aigua en cada un dels experiments. No obstant això, també es van observar diferències en l'enriquiment de la $\delta^{18}O$ en els diferents òrgans analitzats en blat i blat de moro així com la seva relació amb el rendiment.

4.1 Relació entre la composició isotòpica de l'oxigen i el rendiment en blat

Durant l'experiment del 2011 la $\delta^{18}O$ dels grans madurs va associar-se fortament amb el rendiment combinant tots els règims hídrics i de nitrogen (Taula 4, Capítol 5). Però les correlacions dins de cada condició en particular van ser casi inexistents (Taula 1 Discussió Tesi). De manera similar, a l'experiment del 2010 (amb les mateixes 4 condicions de creixement) les correlacions no van ser significatives ni en sequera ni en el tractament fertilitzat (Taula 3, Capítol 5). Els agents implicats en la major o menor força de les correlacions podrien ser les condicions ambientals favorables del 2011, el que van causar rendiments molt més alts al 2011 que al 2010 i per tant un rang de rendiments més elevant al englobar diferents condicions de creixement. La manca de constància de les correlacions entre els dos anys està d'acord amb altres estudis realitzat en blat (Barbour et al., 2000; Araus et al., 2013). A més, a l'estudi de Barbour et al., (2000), la correlació entre la $\delta^{18}O$ dels grans madurs i el rendiment no va ser constant al llarg dels tres cicles que va durar l'experiment. L'esmentada correlació va ser només significativa l'any on va haver-hi una precipitació més alta i una radiació solar baixa. Això suggereix que la $\delta^{18}O$ en els grans madurs no es preserva sota ambients amb un rang ambiental baix i un estrès moderat. La causa de la disparitat

de les correlacions observades entre la $\delta^{18}O$ dels grans madurs i GY durant els nostres experiments en el blat, podria ser la proporció de foto-assimilats que s'han remobilitzat (Barbour et al., 2000) durant l'ompliment del gra. Sota condicions de dèficit hídric s'han observat alts nivells de remobilització dins la planta, els quals podrien estar interferint en la preservació de les condicions evaporatives impreses en la $\delta^{18}O$ dels grans (Barbour et al., 2000). Aquests foto-assimilats provenen de les trioses fosfat formades a partir de la fotosíntesi durant el dia, i es convertiran a sacarosa per transportar-se així a través del floema (Barbour and Farquhar, 2000). A més, la transmissió de la $\delta^{18}O$ des de l'aigua de les fulles fins a la matèria orgànica engloba molts processos, fet que complica la seva interpretació. De fet, el principal intercanvi de l'aigua amb l'oxigen dels grups carbonil es produeix durant la formació de les molècules trioses fosfat (Sternberg et al., 1986; Barbour et al., 2000), ja que dos dels tres àtoms d'oxigen presents a la molècula provenen de l'aigua i un del CO_2 (Schmidt et al., 2001). No obstant, la $\delta^{18}O$ dels carbohidrats de la fulla bandera van correlacionar molt bé amb el GY en quasi totes les condicions de creixement de reg i de sequera (amb alt i baix nitrogen), i d'alt i baix nitrogen (amb reg i sequera) durant el 2010 (Taula 3 i Taula S2, Capítol 5) i també durant el 2011 unint totes les condicions de creixement (en aquest la correlació es va fer amb la $\delta^{18}O$ de la matèria seca de la fulla bandera). Per contra, no es van trobar correlacions fenotípiques significatives quan es separar les 4 condicions de creixement (Taula 1 de la discussió). No obstant, la força de les correlacions unint varies condicions de creixement indica que les condicions evaporatives ocorregudes durant el cultiu encara es preserven en els assimilats de la fulla bandera (Gessler et al., 2013). L'enriquiment evaporatiu de la $\delta^{18}O$ a l'aigua de la fulla està àmpliament establert pels models mecanístics que caracteritzen els factors ambientals i fisiològics que controlen la transpiració (Dongmann et al., 1974; Cernusak et al., 2005). S'ha de tindre en compte però, que l'enriquiment evaporatiu de l'aigua de la fulla està governat per un intercanvi bidireccional del vapor d'aigua

entre la fulla i l'aire de l'ambient, i que pot estar afectat al mateix temps pel dèficit de vapor d'aigua de l'aire i la composició isotòpica del vapor d'aigua (Gessler et al., 2013).

En canvi, la $\delta^{18}O$ de la matèria orgànica total dels grans, les arrels, o l'espiga, no estava correlacionada de manera constant contra el rendiment dins de cada una de les diferents condicions de creixement. La raó per aquesta manca de correlació podria ser conseqüència de l'alteració de la $\delta^{18}O$ de la matèria orgànica des de els òrgans fotosintetitzadors fins als grans o altres òrgans heterotròfics. Així les reaccions bioquímiques dels diferents processos de la síntesi de la matèria orgànica (Farquhar i Lloyd, 1993) com la càrrega, transport i descàrrega dels assimilats depositats als grans (Offermann et al., 2011a) poden causar aquest fraccionament de la $\delta^{18}O$. Durant el transport de la sacarosa pel floema, s'ha observat un baix intercanvi d'àtoms d'oxigen entre l'aigua del floema i la sacarosa (Gessler, 2011). Però el model de flux de pressió que caracteritza el transport del floema (Van Bel, 2003) implica l'intercanvi continu de sacarosa entre els tubs cribosos del floema i les cèl·lules d'acompanyament. És en aquest moment quan podrien aparèixer noves oportunitats de interconversió metabòlica de la sacarosa amb àtoms d'oxigen de l'aigua del floema (en equilibri amb el xilema), causant una pèrdua parcial de la senyal enriquida que prové de les fulles (Offermann et al., 2011). Per altra banda, l'aigua font també pot estar interferint en la $\delta^{18}O$ de la matèria orgànica (Epstein et al., 1977; Yakir et al., 1990b; Roden et al., 2000; Williams et al., 2005; Barbour, 2007). Paral·lelament, la font d'aigua (aigua de la base de la tija) pot estar sotmesa a un enriquiment degut a la evaporació en la fulla bandera durant la transpiració (Farquhar et al., 1993) i durant la formació del gra (Pande et al., 1994).

A més, en el blat la $\delta^{18}O$ de l'aigua en els grans en creixement està subjugada a un enriquiment bifàsic en comparació amb l'aigua de la tija (Pande et al., 1994). Aquest enriquiment bifàsic té relació per una banda amb el metabolisme del gra en desenvolupament i la subsegüent ràpida pèrdua d'aigua, i per altra banda amb el metabolisme oxidatiu durant les darreres etapes de la maduració (Pande et al., 1995). Per tant, l'enriquiment bifàsic dels grans podria ser una font més de variació de la $\delta^{18}O$ de la matèria orgànica total. De fet, en els nostres resultats l'aigua dels grans en desenvolupament van mostrar una $\delta^{18}O$ major comparada amb l'aigua de la tija (Taula 2, Capítol 5). En conseqüència, l'enriquiment de l'aigua que conté el gra podria estar obstaculitzant la capacitat de la $\delta^{18}O$ de registrar les condicions ambientals a la matèria orgànica total dels grans madurs.

D'altra banda la $\delta^{18}O$ de l'aigua de la fulla bandera està molt més enriquida en comparació a l'aigua dels grans en desenvolupament i la tija (Taula 2, Capítol 5). Aquests resultats concorden amb els processos d'evaporació àmpliament documentats que ocorren a les fulles (Farquhar and Gan, 2003; Barbour et al., 2004). A més la $\delta^{18}O$ de l'aigua de la fulla bandera es va correlacionar fortament amb el GY (Taula 4, Capítol 5), el que estarà principalment reflectint l'enriquiment per evaporació i per tant les condicions ambientals, amb l'avantatge addicional que s'evita el fraccionament associat a les reaccions bioquímiques durant la síntesi de la matèria orgànica (Farquhar and Lloyd, 1993).

Finalment un altra font de variació podria ser també la contribució de les reserves pre-antesi (Bidinger et al., 1977) durant l'ompliment del gra. Això seria especialment evident en el 2010, ja que s'ha documentat que pot ser major en condicions de creixement més limitades (Slafer and Andrade, 1991; Bingham et al., 2007; Dreccer et al., 2009).

4.2 Relació entre la composició isotòpica de l'oxigen i el rendiment en el blat de moro

Per altra banda, el blat de moro va mostrar baixes correlacions entre la $\delta^{18}O$ dels grans madurs i el rendiment, sobretot alhora de detectar diferències genotípiques (Capítol 4). En el cas del blat de moro, els sucres transportats des del floema als grans es produeix via pedicel, on la sacarosa es divideix en glucosa i fructosa, i es torna a re-sintetitzar a sacarosa abans de convertir-se en midó (Felker and Shannon, 1980; Griffith et al., 1987). De fet, el midó és el principal component del gra en el blat de moro (Nelson and Pan, 1995; James et al., 2003). Com a conseqüència de la hidròlisi de la sacarosa i la seva posterior re-síntesi, les possibilitats d'intercanvi amb l'aigua augmenten (Barbour and Farquhar, 2000). Aquestes conversions metabòliques podrien impedir que les condicions evaporatives impreses en els foto-assimilats produïts a les fulles es conservin en la $\delta^{18}O$ dels grans madurs.

A més, no només la re-síntesi de la sacarosa als grans podria estar interferint en la conservació de la "senyal" isotòpica de les condicions evaporatives de les fulles als grans, sinó que també el temps invertit pels assimilats en ser transportats pel floema (Song et al., 2014; Gessler et al., 2014). En els nostres resultats la $\delta^{18}O$ dels assimilats transportats pel floema van donar valors més enriquits en condicions òptimes de reg en comparació a la sequera en blat de moro (Capítol 4). Aquests resultats van presumiblement en contra de la teoria que estableix que l'evaporació de la fulla és més alta en condicions de sequera (Farquhar and Lloyd, 1993; Cernusak et al., 2003). Aquesta aparent contradicció podria ser una conseqüència d'un temps més curt de permanència a la tija en condicions de sequera dels sucres no estructurals. Una curta permanència dels sucres dins del floema pot provocar un intercanvi major amb l'aigua font, el que empobreix més la $\delta^{18}O$ dels assimilats que es transporten pel floema tot i estar sota condicions de sequera. Per contra, un temps de rotació

més lent (és a dir una llarga permanència dels sucres dins del floema) en condicions òptimes, podria haver resultat en que els sucres transportats pel floema no s'hagin intercanviat tant amb l'aigua font i que els seus valors s'assemblin més als de la $\delta^{18}O$ dels sucres que provenen de les fulles (més enriquits) (Song et al., 2014; Gessler et al., 2014). A més, aquest efecte pot haver-se accentuat per una major assignació basipètala dels sucres en sequera per promoure el desenvolupament de les arrels (Palta and Gregory, 1997), incrementant així la quantitat relativa dels sucres a la base de la tija (Gessler et al., 2014). Però, l'enriquiment dels assimilats transportats pel floema s'ha vist que depèn en gran mesura de l'espècie (Gessler et al., 2013). A diferència del blat, en el blat de moro la contribució al ompliment dels grans de les reserves pre-antesis sintetitzades abans de la floració són mínimes ja que l'ompliment del gra en aquesta espècie es manté bàsicament pels assimilats actuals produïts pels òrgans fotosintètics (Cliquet et al., 1990; Prioul et al., 1990). En els nostres resultats, tot i la manca de fortes correlacions entre la $\delta^{18}O$ dels grans madurs contra el GY, si es van observar fortes correlacions entre la $\delta^{18}O$ dels grans madurs amb la g_s. De fet, Barbour et al., (2000), també observà que la $\delta^{18}O$ dels grans madurs va correlacionar dèbilment contra el GY però fortament amb la g_s. En definitiva, tot i els esmentats processos de fraccionament del gra, les condicions transpiratives de la fulla sembla que encara es conserven (al menys en part) en el gra.

Per altra banda, es va trobar que les "sedes" (estigmes) podien reflectir el rendiment millor que els grans madurs i que les fulles (Taula 4, Capítol 4). De fet, factors com l'interval entre la floració masculina (antesis) i femenina (aparició de les "sedes"), que rep el nom d'ASI (de l'acrònim en anglès de Anthesis-to-Silking Interval), l'extrusió de les sedes o la viabilitat del pol·len són paràmetres que determinaran en número de grans per panotxa i la productivitat de la planta (Barker et al., 2005). Així es va trobar que les sedes podrien estar reflectint la demanda evapo-transpirativa durant

la floració femenina. En aquest sentit, les sedes podrien ser un bon indicador de la tolerància a la sequera durant la floració femenina, ja que aquestes contenen un percentatge d'aigua molt major que les fulles i per altra banda estan menys protegides que les fulles en front a l'evaporació. A més, tenint en compte que el blat de moro és particularment sensible a la sequera durant l'estadi reproductiu donat el caràcter al·logam de l'esmentada especie, la $\delta^{18}O$ de les sedes podria estar integrant les condicions ambientals durant la important fase reproductiva, i en conseqüència el rendiment.

En definitiva, i tenint en compte l'absència de correlacions entre la $\delta^{18}O$ dels sucres transportats pel floema i el rendiment (Taula 3, Capítol 4), tot plegat suggereix que encara hi ha una manca de coneixement dels mecanismes que controlen la transferència de la senyal isotòpica de l'oxigen dels òrgans autotròfics als òrgans heterotròfics. No obstant, l'absència de correlacions entre la $\delta^{18}O$ sucres transportats pel floema i l'aigua font (Taula 3, Capítol 4) suggereix que l'aigua font podria estar intercanviant-se després de descarregar els assimilats a l'òrgan 'destí' i no abans (Sternberg et al., 1986). En canvi, si s'hagués trobat una correlació significativa entre $\delta^{18}O$ sucres transportats pel floema i l'aigua font hauria indicat que l'aigua de l'oxigen s'ha intercanviat en el floema, abans d'entrar a l'òrgan heterotròfic.

En definitiva, en el blat de moro la $\delta^{18}O$ de les "sedes" van poder reflectir millor el rendiment que la $\delta^{18}O$ d'altres òrgans, mentre que en el blat van ser les fulles.

4.3 Comparativa entre la composició isotòpica de l'oxigen en el blat i el blat de moro

El blat de moro té la $\delta^{18}O$ dels grans més empobrida que la resta d'òrgans ("sedes" i fulles), en comparació al blat que en general té la $\delta^{18}O$ dels grans més enriquida que la resta d'òrgans (ex. l'espiga, fulles i arrels). La diferència es podria explicar en part per les diferències morfo-fisiològiques del blat i el blat de moro (Barbour et al., 2000). Per exemple la posició apical de l'espiga en el blat fa que aquesta estigui sotmesa a condicions de més irradiació i en conseqüència de més calor, el que podria estar provocant un enriquiment de $\delta^{18}O$ dels grans comparat amb les fulles (Taula 1, Capítol 5). Per contra, la posició a mitja alçada de la panotxa en la planta de blat de moro, li proporciona més ombra (i temperatures més baixes) el que segurament estarà afavorint una $\delta^{18}O$ dels grans més empobrida comparat amb de les fulles (Taula 1, Capítol 4). També per la quantitat d'aigua font intercanviada amb els foto-assimilats que s'envien als grans, o la formació de midó en el blat de moro, o l'enriquiment bifàsic en els grans del blat podrien ser factors que també estiguessin alterant la $\delta^{18}O$ dels grans madurs.

5. Relació de la composició isotòpica de l'hidrogen amb el rendiment

Com s'ha vist als apartats anteriors la $\delta^{13}C$ es un bon indicador del rendiment, encara que s'han de tenir en compte algunes consideracions en quant a la seva utilització i interpretació com a eines de millora. En el cas del $\delta^{18}O$, encara que el valor assolit pels assimilats reflecteix les condicions d'evaporació i la signatura isotòpica no depèn directament del metabolisme fotosintètic, el seu ús està limitat per processos de fraccionament. En aquest sentit es important estudiar les possibilitats de les signatures isotòpiques d'altres elements com ara l'hidrogen. A l'igual que la $\delta^{18}O$, la $\delta^{2}H$ també reflecteix condicions evaporatives, però a diferència de l'autonomia de la $\delta^{18}O$ de la fotosíntesi (Barbour and Farquhar, 2000; Barbour et al., 2000), la $\delta^{2}H$ si es veu afectada

per aquesta. Els nostres resultats evidencien que la δ^2H dels assimilats està sotmesa a grans fraccionaments post-fotosintètics. Les diferencies en la δ^2H dels diferents òrgans de la planta eren molt elevades comparades amb els valors relativament propers de $\delta^{13}C$ i $\delta^{18}O$ entre les diferent parts de la planta (Figura 3, Capítol 5). Ara bé, com es mostra a la Taula 2 (Discussió de la Tesi), la δ^2H, tant a la matèria orgànica total com dels carbohidrats solubles, són capaces de captar les diferències entre règims hídrics, metabolismes fotosintètics i inclús l'efecte de la salinitat. A més, la δ^2H dels carbohidrats de l'espiga van mostrar correlacions fenotípiques amb el rendiment en comparació als grans i les fulles (excepte en WW-LN), (Taula 1 de la discussió de la tesi). Curiosament en l'únic ambient de creixement (tractament fertilitzat i reg suplementari) on aquesta correlació va ser positiva va ser el mateixa on es va trobar l'única correlació positiva entre $\delta^{13}C$ (en aquest cas de la fracció soluble de la fulla banderal) i GY.

No obstant les grans diferències observades entre els òrgans, la δ^2H dels grans, es va correlacionar molt bé amb el rendiment, inclús millor que la $\delta^{13}C$ i la $\delta^{18}O$ en el tractament de sequera (combinant els dos nivells de N). A més, en els tractament fertilitzats i sense fertilitzar (incloent els dos règims hídrics) on les correlacions entre la $\delta^{13}C$ i el GY (apartat 1) van ser més dèbils, la δ^2H va correlacionar més fortament contra el GY (Taula 3, Capítol 5).

Així mentre que en el cas de la $\delta^{18}O$, l'enriquiment bifàsic podria estar creant una pèrdua de la senyal isotòpica original en els grans (o altre òrgan heterotròfic), en el cas del δ^2H aquesta signatura isotòpica no sembla estar afectada de la mateixa manera. De fet, l'absència de correlacions entre la $\delta^{18}O$ i la δ^2H dels grans madurs, suggereix que els dos isòtops no estan sotmesos als mateixos processos de fraccionament. Inclús les baixes correlacions observades entre la $\delta^{18}O$ dels grans madurs i el rendiment, especialment en els tractaments combinant dos nivells de nitrogen sota

un únic nivell hídric, suggereix que l'isòtop ^{18}O és més sensible als processos de fraccionament bioquímics durant la formació del gra (Farquhar and Lloyd, 1993) o amb l'intercanvi amb l'aigua font (Barbour, 2007) que no pas l'isòtop ^{2}H. A més, els carbohidrats de la fulla bandera van correlacionar millor entre la $\delta^{2}H$ i la $\delta^{13}C$ que no pas entre la $\delta^{2}H$ i la $\delta^{18}O$ (Figura 2, Capítol 5). Això, estaria d'acord amb el fet de que la $\delta^{2}H$ dels carbohidrats de la fulla no només està afectada per la transpiració i la g_s (com es el cas de $\delta^{18}O$) sinó també per les reaccions fotosintètiques (Yakir et al., 1990b). A més, tant la $\delta^{13}C$ com la $\delta^{2}H$ van mostrar comportaments similars en els tractament fertilitzats (amb dos règims hídrics) i de sequera (amb dos nivells de N), així com la seva relació amb el nitrogen total de la fulla bandera i la g_s. El comportament similar entre els dos isòtops indica que podrien estar exposats a fonts de variació anàlogues, amb la diferència que la $\delta^{2}H$ podria estar donant informació de l'estat autotròfic del teixit de la planta (Yakir and Deniro, 1990). Així, a diferencia del $\delta^{13}C$, la $\delta^{2}H$ dels carbohidrats reflexa l'efecte net de dots efectes antagònics (Yakir, 1992). Com s'ha comentat a la introducció, comparat amb l'aigua del medi, l'efecte de la fotosíntesi produeix carbohidrats amb l'hidrogen unit al carboni empobrit en ^{2}H (Yakir et al., 1990a), mentre que el metabolisme post-fotosintètic mostra un efecte oposat enriquit en ^{2}H (Ziegler, 1989). Així, la $\delta^{2}H$ dels carbohidrats serà un balanç dels dos processos oposats (Yakir et al., 1990b). Així, si s'observen valors empobrits de la $\delta^{2}H$ estaria indicant l'existència de processos fotosintètics, és a dir l'autotròfia d'una òrgan. Per contra, si els valors de la $\delta^{2}H$ són enriquits, estaria indicant l'existència de processos post-fotosintètics i en conseqüència l'heterotròfia d'una òrgan (Yakir, 1992).

5.1 Mecanisme autotròfic de l'espiga

Els valors empobrits de la $\delta^{2}H$ dels carbohidrats de la fulla comparats amb els grans (Taula 1, Capítol), recolzen la hipòtesi de que l'activitat fotosintètica (autotròfica) de

les fulles provoca un fraccionament negatiu de l'isòtop d'hidrogen. En canvi, els valors enriquits de la δ^2H dels grans madurs, segons la hipòtesi formulada per Ziegler (1989), estarien representant el fraccionament degut al metabolisme heterotròfic dels carbohidrats enviats al gra (Yakir and Deniro, 1990) i també (al menys en part) el fraccionament bifàsic que experimenta el gra en el procés de maduració (Pande et al., 1994, 1995). Si seguim el mateix raonament pel que fa a l'espiga, la δ^2H dels carbohidrats dona uns valors més empobrits que els grans madurs però més enriquits que la fulla bandera. Tot i ser merament especulatiu, ja que aquest és el primer experiment publicat on s'analitza la δ^2H de l'espiga, aquests resultats ens estarien indicant l'activitat autotròfica (Yakir and Deniro, 1990) de l'espiga (pels empobrits valors obtinguts en la δ^2H en comparació amb els grans). Això recolzaria la importància constatada en els capítols 1, 2 i 3 de la capacitat intrínseca fotosintètica de l'espiga durant l'ompliment del gra.

Una altra hipòtesi per explicar les diferencies de δ^2H entre la fulla i l'espiga, podria ser que la δ^2H de l'espiga estigués més enriquida que la fulla degut al fraccionament per transpiració associat a la posició apical que l'espiga ocupa en la planta. Ara bé, si els valors enriquits s'haguessin donat pel fenomen de la transpiració (Wershaw et al., 1966) i d'evaporació (Gonfiantini et al., 1965; Deniro and Epstein, 1979), caldria esperar correlacions entre la δ^2H i la $\delta^{18}O$ dels carbohidrats (Sternberg and Deniro, 1983) de l'espiga (el que indicaria una mateixa font de variació) però aquestes correlacions no es van observar. Per contra, les majors correlacions en els carbohidrats de l'espiga es van mostrar entre la δ^2H i la $\delta^{13}C$ (Figura 2, Capítol 5). Això indica, que els valors més enriquits de la δ^2H de l'espiga comparat amb la fulla bandera no sembla que siguin únicament el resultat del fraccionament físic degut a la transpiració (Ziegler et al., 1976). El fet de que la discriminació de l'isòtop d'hidrogen vagi en la mateixa direcció que l'isòtop de carboni i no que l'isòtop d'oxigen, estaria

*T**aula 2.** Composició isotòpica (‰) de l'hidrogen (δ^2H) en diferents experiments i ubicacions: estació experimental d'Aranjuez (Araus et al., 2013), assaig Fontagro (Buenos Aires, Argentina) sota condicions controlades, l'hivernacle de Barcelona i un camp experimental al CIMMYT, Ciudad de Obregón (Mèxic) (). La composició isotòpica d'hidrogen es va analitzar en diferents òrgans (fulles, espigues i grans madurs) en les plantes amb diferents metabolismes fotosintètics (blat, C3; blat de moro, C_4 i Corpobrotus edulis, CAM) i sota diferents condicions de creixement, incloent salinitat moderada i severa (Yousfi et al., 2013), en condicions òptimes (WW) i de sequera (WS), en la matèria seca (DM) i la fracció soluble en aigua (WSF).*

Espècie	Òrgan	Ubicació	Any	Tractament	Metabolisme	N	δ^2H
Zea mays L.	fulla	Font Agro Argentina (Flint, Pissigallom, TxT)	2010	WW	C4	4	-11.2
Corpobrotus edulis L.	fulla	Barcelona	2012	WW	CAM	8	-3.3
Triticum turgidum L. var. *durum*	gra	Aranujez	2006	WS	C3	2	-35.5
Triticum turgidum L. var. *durum*	gra	Aranujez	2006	WW	C3	2	-32.0
Triticum turgidum L. var. *durum*	gra	Aranujez	2007	WS	C3	2	-14.3
Triticum turgidum L. var. *durum*	gra	Aranujez	2007	WW	C3	2	-27.0
Triticum turgidum L. var. *durum*	gra	Aranujez	2008	WS	C3	2	-28.4
Triticum turgidum L. var. *durum*	gra	Aranujez	2008	WW	C3	2	-33.7
Zea mays L.	gra	Fontagro Argentina (control Flint, Pissigallom TxT)	2010	WW	C4	6	-20.6
Zea mays L.	gra	Fontagro Argentina Antietilènic 1-MCP	2010	WW	C4	4	-22.3
Zea mays L.	gra	Mèxic (llaura convencional vs sembra directa)	2010	WW	C4	4	-19.2
Triticum turgidum L. var. *durum*	fulla DM	Experiment hivernacle	2009	WW	C3	4	-88.8
Triticum turgidum L. var. *durum*	fulla DM	Experiment hivernacle	2009	WS	C3	4	-86.1
Triticum turgidum L. var. *durum*	fulla DM	Experiment hivernacle	2009	Salinitat moderada	C3	4	-83.7
Triticum turgidum L. var. *durum*	fulla DM	Experiment hivernacle	2009	Salinitat severa	C3	4	-86.2
Triticum turgidum L. var. *durum*	fulla WSF	Experiment hivernacle	2009	WW	C3	6	-41.9
Triticum turgidum L. var. *durum*	fulla WSF	Experiment hivernacle	2009	WS	C3	4	-45.9
Triticum turgidum L. var. *durum*	fulla WSF	Experiment hivernacle	2009	Salinitat moderada	C3	8	-40.7
Triticum turgidum L. var. *durum*	fulla WSF	Experiment hivernacle	2009	Severe salinity	C3	13	-31.4

d'acord amb la dependència de la δ^2H de les reaccions bioquímiques associades a la fotosíntesis (Sternberg and Deniro, 1983) tals com el procés fotosintètic de la reducció del $NADP^+$ (Luo and Sternberg, 1991).

5.2 Metabolisme fotosintètic de l'espiga

Per altra banda, Ziegler et al. (1976) observà en un experiment a l'hivernacle que el quocient de $^2H/^1H$ en la matèria orgànica total de plantes amb el metabolisme CAM era molt més alt que a les plantes amb metabolisme C_3 i C_4. Els autors van postular que les plantes CAM estan enriquides en δ^2H en comparació a les plantes C_3 i C_4 degut a la seva habilitat de mantenir l'activitat metabòlica sota condicions d'estrès. De fet, en plantes CAM, com *Kalanchoe daigremontiana*, Ziegler et al. (1976) van observar correlacions significatives entre la δ^2H i la $\delta^{13}C$, tant en plantes crescudes en el laboratori com en condicions de camp, però que al privar-les d'aigua, la δ^2H de la fracció tant soluble com de la insoluble de fulles va esdevenir menys negativa mentre que el $\delta^{13}C$ augmentava (Ziegler et al., 1976). Així, van postular que aquests canvis podrien estar indicant l'enriquiment del deuteri en l'aigua dels teixits durant la transpiració (Wershaw et al., 1966). Però, més tard, es demostrà que els processos responsables del fraccionament isotòpic de la δ^2H en les plantes CAM, eren conseqüència de les reaccions bioquímiques, més que de l'evaporació, ja que la δ^2H va correlacionar dèbilment amb la $\delta^{18}O$ de la cel·lulosa (Sternberg and Deniro, 1983).

Així, per comparar els diferents isòtops analitzats en aquesta Tesi, i els seus valors en funció del metabolisme fotosintètic, es va elaborar la Figura 4. Així mateix, al comparar la $\delta^{13}C$ de fulles de les plantes amb metabolisme "CAM-cycling" amb la $\delta^{13}C$ de les fulles C_3, els valors que es van obtenir van ser similars (Figura 4). En canvi, els valors de la δ^2H de les plantes C_3 mostraren uns valors molt més negatius que les CAM-cycling. Si extrapolem aquests resultats amb l'enriquiment en ^2H de

l'espiga en comparació a la fulla bandera tot i se purament especulatiu, ens podria estar indicant una possible interferència del metabolisme CAM, especialment en condicions d'estrès hídric a l'espiga. De fet, les diferències entre la δ^2H de la fulla bandera versus l'espiga van ser majors en condicions d'estrès hídric, principalment perquè la δ^2H de l'espiga es va enriquir més que la fulla bandera (Taula 1, Capítol 5). Aquest major enriquiment podria estar en part recolzant l'esmentada interferència del metabolisme CAM a l'espiga especialment en condicions de sequera. De fet, s'ha demostrat que en fulles madures de plantes C_3 tals com *Jatropha curcas* L. el metabolisme CAM pot ser que s'expressi dèbilment quan apareixen severes condicions però essent igualment la fotosíntesi C_3 la principal proveïdora de C a la planta (Winter i Holtum, 2015).

Així, ja sigui per processos transpiratoris o bioquímics, l'enriquiment de la δ^2H dels carbohidrats de l'espiga podria estar relacionat amb l'existència de cert metabolisme CAM (Figura 3). El metabolisme fotosintètic de l'espiga ha sigut un tema controvertit durant les últimes tres dècades (Singal et al., 1986; Bort et al., 1995). Estudis més recents demostren que a l'espiga del blat, l'activitat de l'enzim RuBP carboxilasa decreix significativament sota condicions de dèficit hídric mentre que la de l'enzim de la PEP carboxilasa augmenta junt amb el NADP-malat especialment en les glumes i les lemnes (Jia et al., 2015). De fet, depenent de les espècies, hi ha varies modalitats de plantes amb metabolisme CAM (Nobel, 2003; Silvera et al., 2005, 2014). Però, la modalitat que s'ha observat en certes espècies i que potser existeix en l'espiga i altres òrgans com les tijes, en la utilització del metabolisme CAM d'una manera opcional facultativa, en el que s'utilitza la via C_3 sota condicions òptimes de creixement, o s'utilitza la via CAM en resposta a la sequera a través la capacitat de canviar de manera reversible a la via CAM e(Winter et al., 2008; Winter and Holtum, 2014).

En definitiva, l'existència d'un cert metabolisme CAM facultatiu per un estrès hídric recolzaria l'important rol de la fotosíntesi de l'espiga durant l'ompliment del gra especialment en condicions de sequera (Capítol 1), però també pot ser important en condicions òptimes de creixement degut a la seva plasticitat fotosintètica (Capítols 2 i 3).

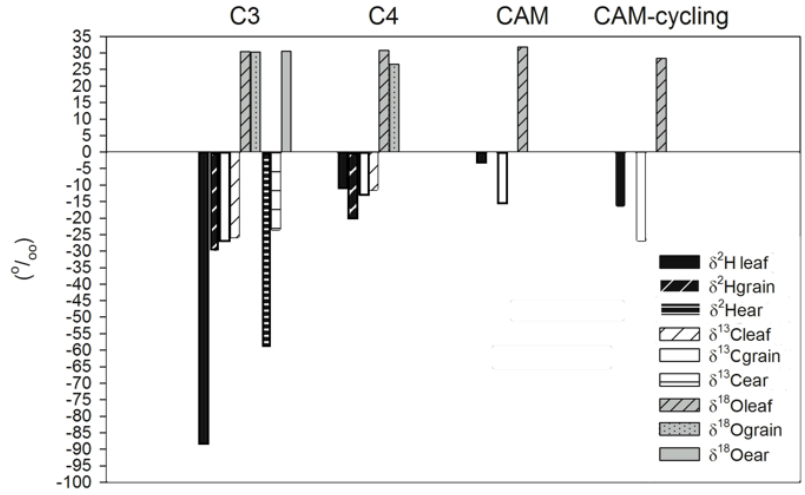

***Figura 4**. Composició isotòpica (‰) de l'hidrogen (δ^2H), l'oxigen ($\delta^{18}O$) i el carboni ($\delta^{13}C$) en la fracció soluble de fulles i espiga i el grans madurs en el metabolisme C_3 (Titicum turgidum L. var. Durum), metabolisme C_4 (Zea mays L.) i el metabolisme CAM (Corpobrotus edulis L.) i la mitja de plantes CAM-cycling cultivades en condicions òptimes d'aigua. Les dades de plantes C_3, C_4 i CAM són resultats propis, mentre que les dades de δ^2H, $\delta^{13}C$ i $\delta^{18}O$ de l'especie CAM-cycling així com la $\delta^{13}C$ i la $\delta^{18}O$ de la planta del metabolisme CAM es van completar amb la bibliografia (Sternberg et al., 1984). Les plantes CAM-cycling actuen com una C_3 quan estan en bones condicions hídriques, però canvien a CAM quan estan estressades, mostrant una fixació del CO_2 nocturna però fluctuacions al llarg del dia dels àcids orgànics (Sternberg and Deniro, 1983).*

Conclusions

CONCLUSIONS

1. $\delta^{13}C$, particularly from mature grains in wheat reflected growing conditions. However, phenotypic correlations between $\delta^{13}C$ in the mature kernels with GY, increased with better growing conditions.
2. $\delta^{13}C$, when analized in different plant parts was also a good indicator for selecting genotypes with enhanced ear photosynthesis during grain filling. The $\delta^{13}C$ approach assigned a higher role to the ear compared to the flag leaf, supporting new evidences on the important role of the ear providing assimilates to the grain.
3. However, contribution of the ear decreased in modern cultivars compared to landraces, and this is probably associated with the appearance, to some extent, of source limitation driven by the increase in HI.
4. Moreover, it was not only the genotypic variability but also the improving growing conditions decreased the relative contribution of the ear and the flag leaf to grain filling
5. Even under good agronomical conditions, the contribution of the ear was more important than the flag leaf during grain filling. Such a conclusion is also supported by similar photosynthetic contribution per whole organ recorded during the reproductive period by the ears and flag leaf blades.
6. Ear contribution to grain filling may still be underestimated because the glumes were not included in the approach using $\delta^{13}C$. Whereas awns may be the organ of the ear that is pre-eminent in fixing atmospheric CO_2, the glumes may also play a major photosynthetic role in re-assimilating CO_2 respired by the ear.
7. Experimental approaches of intrusive nature such as shading treatments indicate similar contribution of the ear and the culm. Conversely, the DCMU approach assigned a higher role to culm photosynthesis, but herbicide application in the culm affected the ear, biasing the final grain weight. Nevertheless, the results

from any intrusive treatment should be interpreted with caution, as unwanted compensatory mechanisms in the remaining unaffected organs could affect final grain weight.

8. In general without taking into account the specific characteristics of each experiment (field conditions or plots), the species studied (i.e. durum wheat (C_3) and corn (C_4)), and location (i.e, Aranjuez, Spain or Tlaltizapán, Mexico), the $\delta^{18}O$ in plant tissues always increased in response to water stress.
9. However $\delta^{18}O$ of kernels in durum wheat performed very poorly (lost all its capacity) for predicting GY even combining years and growing conditions. Only when combining water regimes, the $\delta^{18}O$ of the flag leaf water was strongly correlated with GY, indicating that $\delta^{18}O$ flag leaf water may be reflecting leaf evaporative enrichment and therefore the environmental conditions, with the additional advantage of avoiding fractionation associated with biochemical reactions in the synthesis of organic.
10. In the case of maize, although GY was only marginally correlated through genotypes with $\delta^{18}O$ of different tissues in maize, the silks were the tissue best related with GY.
11. Our results for both maize and durum wheat provide indirect evidence that exchange with un-enriched source water is likely to occur within the sink tissues, rather than during phloem transport to the reproductive organs. This finding will eventually help to discard plant tissues, which are more susceptible to post-photosynthetic fractionation processes, and may help to understand the use of δ18O as a genotype selection tool for the adaptation of maize and other crops to drought.
12. $\delta^{2}H$ performed better than the other two isotopes predicting GY and N content in durum wheat under water stress but combining contrasting N regimes. $\delta^{2}H$, similarly than $\delta^{13}C$, correlated negatively with g_s and grain yield.

13. The absence of correlations between $\delta^{18}O$ and $\delta^{2}H$ in mature kernels suggest that the two isotopes are not subjected to the same isotope fractionation processes. In addition, when analyzed the water soluble fraction of the leaf flag, $\delta^{2}H$ correlated better with $\delta^{13}C$ than with $\delta^{18}O$, suggesting that $\delta^{2}H$ of carbohydrates in the leaf was affected not only by the transpiration and g_s (as the case $\delta^{18}O$) but also by photosynthetic reactions.
14. $\delta^{2}H$ in the water soluble fraction of the ear was lower compared to the grains, but much higher than the flag leaf. This observation further support the photosynthetic nature of the capacity of the ear (autotrophic activity)

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