Grain carbon isotope composition is a marker for allocation and harvest index in wheat

Jean-Baptiste Domergue[1], Cyril Abadie[1], Julie Lalande[1], Jean-Charles Deswarte[2], Eric Ober[3], Valérie Laurent[4], Céline Zimmerli[5], Philippe Lerebour[6], Laure Duchalais[7], Camille Bédard[8], Jérémy Derory[9], Thierry Moittie[10], Marlène Lamothe-Sibold[11], Katia Beauchêne[12], Anis M. Limami[1] & Guillaume Tcherkez[1,13]*

1. Institut de Recherche en Horticulture et Semences, Université d'Angers, INRAe, 42 rue Georges Morel, 49071 Beaucouzé, France.
2. Arvalis Institut du Végétal, Pôle valorisation de l'écophysiologie, ZA des Graviers, 91190 Villiers le Bâcle, France.
3. National Institute of Agricultural Botany, 93 Lawrence Weaver Road, Cambridge, CB3 0LE, United Kingdom.
4. Florimond Desprez Veuve et fils, BP 41, 59242 Cappelle-en-Pévèle, France.
5. BASF France S.A.S., La Ferme du Paly, 91490 Milly-la-Forêt, France.
6. Unisigma, GIE Recherche et Création Variétale, 2 rue Petit-Sorri, 60480 Froissy, France.
7. RAGT 2n, Route Epincy, 28150 Louville-la-Chenard, France.
8. Secobra Recherches, Centre de Bois-Henry, 78580 Maule, France.
9. Limagrain Europe, Centre de recherche, CS 50005, Saint Beauzire, 63360 Gerzat, France.
10. ASUR Plant Breeding, 163 ter avenue de Flandre, 60190 Estrées-Saint-Denis, France.
11. SPOmics plant métabolisme métabolome platform, Institute of Plant Sciences Paris-Saclay IPS2, CNRS, INRAe, Universities Paris-Saclay, Evry and Paris, Batiment 630, 91405 Orsay, France.
12. Arvalis Institut du Végétal, Pôle PhenoHD3, 45 voie Romaine, Ouzouer-le-Marché, 41240 Beauce-La-Romaine, France.
13. Research School of Biology, ANU College of Science, Australian National University, 2601 Canberra ACT, Australia.

*Contact author to whom correspondence should be addressed

Email: guillaume.tcherkez@anu.edu.au

Word count (w/o references): 4,966

Keywords: wheat, isotope, carbon 13, harvest index, respiration use efficiency

Abstract (222 words)

The natural ^{13}C abundance ($\delta^{13}C$) in plant leaves has been used for decades with great success in agronomy to monitor water use efficiency and select modern cultivars adapted to dry conditions. However, in wheat, breeding also implies looking for genotypes with high carbon allocation to spikes and grains, and thus with a high harvest index and/or low carbon losses via respiration. Finding isotope-based markers of optimal carbon partitioning to grains would be extremely useful since isotope analyses are inexpensive and can be performed routinely at high throughput. Here, we took advantage of a set of field trials made of more than 600 plots with several wheat cultivars and measured agronomic parameters as well as $\delta^{13}C$ values in leaves and grains. We find a linear relationship between the apparent isotope discrimination between leaves and grain (denoted as $\Delta\delta_{corr}$), and the respiration use efficiency-to-harvest index ratio. It means that overall, efficient carbon allocation to grains is associated with a small isotopic difference between leaves and grains. This effect is explained by post-photosynthetic isotope fractionations, and we show that this can be rationalised by equations describing the carbon isotope composition in grains along the wheat growth cycle. Our results thus show that ^{13}C natural abundance in grains has some potential to help finding genotypes with better carbon allocation properties and assisting current wheat breeding technologies.

Introduction

The quantitation of stable carbon isotopes (^{12}C, ^{13}C) is currently a major technology for crop cultivar selection and authentication of food products, representing a huge market in fraud detection and quality assessment (Kelly *et al.*, 2005). In particular, the natural ^{13}C abundance (or isotope composition, $\delta^{13}C$) is used routinely to probe photosynthetic pathways (C_3, C_4, CAM) and thereby certify the origin of many food products consumed worldwide, such as flavours (Remaud & Akoka, 2017) (e.g., vanillin) and beverages (Santesteban *et al.*, 2015) (e.g., wine). In C_3 plants like wheat, the natural isotope composition in plant organic matter is ^{13}C-depleted by *c*. 20‰ compared to atmospheric CO_2 while C_4 plants are ^{13}C-depleted by *c*. 4‰ only.

In C_3 crops, $\delta^{13}C$ is crucial to screen water-efficient cultivars by taking advantage of the linear relationship between the isotope discrimination during photosynthesis (Δ) and water use efficiency (WUE) (Condon *et al.*, 1987; Condon *et al.*, 1990; Condon *et al.*, 2004). Photosynthetic isotope discrimination essentially comes from two main steps: First, the strong isotope effect (29‰) associated with CO_2 fixation catalysed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco); Second, the small isotope effect (4.4‰) by CO_2 diffusion from the atmosphere to leaf mesophyll cells (Farquhar *et al.*, 1989). It has also been demonstrated recently that internal conductance of CO_2 (from intercellular spaces to chloroplasts) also affects significantly the estimation of intrinsic WUE (iWUE) from carbon isotopes (Ma *et al.*, 2020). In water-efficient cultivars, the generally lower stomatal conductance and/or higher photosynthetic capacity are so that CO_2 diffusion is relatively more limiting and thus the isotope discrimination Δ is low, causing a general ^{13}C-enrichment in plant matter. As a result, the $\delta^{13}C$ value in vegetative material (typically flag leaf in wheat) directly reflects leaf photosynthetic properties and can thus be used as a biomarker to find cultivars with high WUE values (Condon *et al.*, 2004). This technique has been widely and successfully implemented in wheat and other species since the eighties because $^{13}C/^{12}C$ analyses are rapid and inexpensive using current elemental analysis/mass spectrometry devices (Sanchez-Bragado *et al.*, 2020).

Nevertheless, although leaf $\delta^{13}C$ reflects photosynthetic performance and associated water usage, it does not provide information on other parameters that are essential for yield (Y) such as the harvest index (HI). In wheat, high-performing cultivars are generally associated with higher tiller number, a high grain number per spike, and relatively high HI (Sinclair, 1998; Reynolds *et al.*, 2012; Quintero *et al.*, 2018). In other words, grain properties and carbon allocation to grains is a fundamental aspect of wheat breeding that cannot be accounted for with current $\delta^{13}C$ analyses in vegetative material. The question thus arises as to whether the isotope signature of grains, which are readily amenable for biochemical analyses and cultivar ranking unlike fastidious and expensive flag leaf sampling at anthesis, can be used to gain direct information on Y or HI. About 20 years ago, relationship was found between Y or HI and grain $\delta^{13}C$ ($\delta^{13}C_{grain}$) but this

was mostly driven by changes in iWUE, i.e., stomatal conductance and photosynthetic capacity (Merah *et al.*, 2001a; Merah *et al.*, 2001b; Merah *et al.*, 2002). Whether $\delta^{13}C_{grain}$ can be exploited further to give access to agronomic parameters other than photosynthesis or WUE has never been addressed.

In particular, variations in $\delta^{13}C_{grain}$ can be anticipated because of metabolic isotope discriminations in grain biomass synthesis and/or respiratory CO_2 loss (so-called "post-photosynthetic" isotope fractionations). Post-photosynthetic fractionations are generally so that heterotrophic organs are ^{13}C-depleted compared to leaves (Badeck *et al.*, 2005; Cernusak *et al.*, 2009). In addition, there is an isotope discrimination during metabolic decarboxylation and respired CO_2 is often naturally ^{13}C-enriched in leaves and ^{13}C-depleted in heterotrophic organs (Bathellier *et al.*, 2017). Conversely, vegetative plant organic matter is mostly made of cellulose, which is generally ^{13}C-enriched compared to photosynthetically fixed carbon (Gleixner *et al.*, 1993; Schmidt & Gleixner, 1998; Kodina, 2010). At the metabolic scale, it has been shown that the intramolecular ^{13}C distribution ($\delta^{13}C$ values at the different C-atom positions) in wheat grain starch is dictated by isotope effects in metabolism and partitioning between starch production and glycolysis (Gilbert *et al.*, 2012). Similarly, at the plant scale, variations in carbon partitioning to grains at the expense of respiratory CO_2 loss or vegetative organic matter likely cause changes in $\delta^{13}C_{grain}$ due to isotopic mass-balance.

Here, we exploited a dataset of $\delta^{13}C$ values and agronomic variables (such as biomass, N content, yield, grain weight, consumed water, etc.) obtained in winter wheat grown in the field, across different sites (Table 1 and Fig. S1), representing a total of 644 field plots and 70 cultivars. We also developed new equations describing the isotope composition in grains as a function of carbon allocation parameters, HI or Y. We find that the leaf-to-grain carbon isotope discrimination relates to the respiration use efficiency-to-HI ratio. The observed relationship depends on neither the cultivation site nor irrigation and thus offers good potential for allocation-based cultivar selection using $\delta^{13}C_{grain}$ values.

Material and methods

Cultivation

Three series of randomized block field experiments (Fig. S1 and Table 1) were undertaken at different locations, with different cultivars and varying water availability, irrigated to maximal evapotranspiration, and non-irrigated (rainfed). Experiments followed cultivation of wheat or other crops (sunflower, pea, rapeseed) as mentioned in figures, on calcareous or clay substratum depending on the site considered. Crop development was typical of French climatic regions with sowing in October-November, seedling establishment in November, visible stem extension (growth stage Z30) at the end of March or beginning of April, anthesis at the end of May and harvest maturity in July.

Measurement of agronomic parameters

Experimental plots measured between 6 and 12 m² across experimental sites. Destructive measurements (above-ground biomass and nitrogen content at Z30, Z65 and maturity, leaf sampling at anthesis) were performed on a dedicated additional plot in each block, with 2-3 biological replicates per plot. Non-destructive measurements (plant and spike density at heading (Z55) and anthesis (Z65)) were carried out using the plot dedicated to final harvest. Final yield was measured at the plot level using an experimental combine. Grain parameters were measured as in (Touzy *et al.*, 2019). Grain samples from the combine harvester was used to measure plot grain weight, grain humidity at harvest, grain protein content, specific weight. Grain yield, grain number per m², grain number per spike and grain weight per spike were calculated. At Saint-Pierre d'Amilly (VAR site), actual soil water content was measured at Z30, Z65 and harvest using a soil neutron probe (at 1.8-m depth) and water content variations between measurement dates were used to monitor crop water consumption. At other sites (BPA, BVG), water balance was followed using soil properties, meteorological data and actual irrigation when applicable.

Isotopic analyses

Isotopic analyses were done using oven-dry material ground in fine power and weighted in tin (^{13}C) or silver (^{18}O) capsules. Samples were analysed using an isotope ratio mass spectrometer Isoprime (Elementar, France) coupled to an elemental analyser (Carlo-Erba, Italy) run in combustion (^{13}C) or pyrolysis (^{18}O). Delta value accuracy was checked using IEAE standards USGS-40 (glutamic acid) and IAEA-CH7 (polyethylene) and IAEA-602 (benzoic acid) (^{13}C) and ANU sucrose (home standard) (^{18}O) every 10 samples. Isotopic analyses were carried out by facilities Plateforme Métabolisme Métabolome (France) and ANU Stable Isotope Laboratory (Australia). Delta values are expressed with respect to V-PDB (^{13}C) and V-SMOW (^{18}O), in ‰. Climatic parameters used for computations

(temperature) was from Meteo France stations. $\delta^{18}O$ values in precipitation in the regions considered are from the database Nucleus (IAEA).

Theory and calculations

Notations and assumptions. The isotope composition ($\delta^{13}C$ value) is denoted as δ. Second order terms are neglected and thus, if the isotope fractionation of a given biochemical process is denoted as Δ then the isotope composition of the product $\delta_{product}$ is equal to $\delta_{substrate} - \Delta$. Symbols associated with carbon amounts are illustrated in Fig. S2. The decomposition in carbon flow and three different stages (pre-anthesis, early grain filling, late grain filling) in Fig. S2 is from model III in (Gent, 1994). Symbols associated with isotope compositions and fractionations are listed in Table S1. The term "isotope fractionation" refers to the change in delta value related to the observed isotope effect IE ($^{12}C/^{13}C$ ratio of velocity) as follows: fractionation = IE – 1. That way, the fractionation is positive when against the heavy (^{13}C) isotope. Here, for simplicity, we assumed that fractionations associated with reserve establishment (p_r), respiration (e_g, e_m) and grain biomass synthesis (β) did not vary between stages. We also neglect denominators ($\delta + 1$) which are assumed to be equal to unity (that is, we neglect second-order terms, see above).

Flux mass balance. Carbon balance is applied to C amounts as follows:

Stage 1: $A_1 = V_{r1} + V_s + R_{g1}$ and $V_{r1} = B_1 + R_{m1}$ (1)

Stage 2: $A_2 = V_{r2} + G + R_{g2}$ and $V_{r2} = B_2 + g_2 + R_{m2}$ (2)

Stage 3: $A_3 = V_{r3} + R_{g3}$ and $V_{r3} + B_3 = g_3 + R_{m3}$ (3)

Harvest index. The harvest index (HI) is defined as the ratio of grain biomass to total net biomass, which gives, using amounts of carbon:

$$HI = \frac{\text{grain carbon}}{\text{net total carbon}} = \frac{\Sigma g}{\Sigma A - \Sigma R_g - \Sigma R_m} = \frac{\Sigma g}{V_s + \Delta B + \Sigma g} \quad (4)$$

where $\Sigma g = G + g_2 + g_3$, $\Sigma A = A_1 + A_2 + A_3$, $\Sigma R_g = R_{g1} + R_{g2} + R_{g3}$, $\Sigma R_m = R_{m1} + R_{m2} + R_{m3}$, and $\Delta B = B_1 + B_2 - B_3$. In principle, carbon remobilization is so that $\Delta B \approx 0$, that is, $B_1 + B_2 = B_3$. This assumption will be used here. Also, V_s and Σg are given by straw and grain carbon amounts, respectively.

Isotopic mass balance. The isotopic composition of photosynthetically fixed carbon (δ_{fix}) must be equal to the weighted average of isotopic compositions of sink compartments (which account for fractionations) and thus, at stage 1:

$$A_1\delta_{fix1} = V_{r1}(\delta_{fix1} - p_r) + V_s(\delta_{fix1} - p_s) + R_{g1}(\delta_{fix1} - e_g) \quad (5)$$

where p_r, p_s and e_g is the isotope fractionation associated with reserves establishment, structural biomass construction and growth respiration, respectively. Similarly, isotopic mass balance on metabolism leads to:

$$V_1(\delta_{fix1} - p_r) = B_1\delta_{B1} + R_{m1}(\delta_{B1} - e_m) \quad (6)$$

By substitution, the isotope composition of the metabolic reserve pool, δ_{B1}, can be calculated as:

$$\delta_{B1} = \frac{V_1(\delta_{fix1} - p_r) + R_{m1}e_m}{B_1 + R_{m1}} \quad (7)$$

The same procedure was applied to stages 2 and 3. For stage 2, it leads to:

$$\delta_{B2} = \frac{V_2(\delta_{fix2} - p_r) + g_2\beta + R_{m2}e_m}{B_2 + g_2 + R_{m2}} \quad (8)$$

In stage 3, we have:

$$\delta_{B3} = \frac{V_3(\delta_{fix3} - p_r) + B_3\delta_{B2} + g_3\beta + R_{m3}e_m}{g_3 + R_{m3}} \quad (9)$$

where β is the isotope fractionation during grain biomass synthesis. While there is some variations depending on the organ considered, CO_2 respired by heterotrophic organs is generally ^{13}C-depleted (Ghashghaie & Badeck, 2014; Bathellier *et al.*, 2017) and thus across the full wheat life cycle, overall respiratory fractionation (e_{mg} used thereafter) is likely positive and different scenarios (from 2.5 to 4‰) are shown in Fig. 4 in main text. In our study, the fact that $e_g > 0$ is further supported by the fact that grains are ^{13}C-enriched, in agreement with the possible difference in sign between β and e_g when V_{r2}/V_{r3} is small (equation 13 below). The isotope fractionation in structural biomass synthesis is negative (favours ^{13}C) due to the fact that straw is cellulose-rich and cellulose is naturally ^{13}C-enriched. In wheat, it has been shown that straw and straw cellulose is ^{13}C-enriched by up to 3‰ compared to flag leaf at anthesis (Merah *et al.*, 2002; Kodina, 2010). In numerical applications, we thus used $p_s = -3‰$.

By definition, the isotope composition in grains is the weighted average of contributions of the different sources, and therefore:

$$\delta_{grain} = \frac{g_2\delta_{B2} + g_3\delta_{B3} + G\delta_{fix2}}{g_3 + g_2 + G} - \beta \quad (10)$$

Substituting (7), (8) and (9) in (10) gives:

$$\delta_{grain} = \frac{g_2 + g_3\varphi}{\Sigma g} \cdot \frac{B_1}{B_1 + V_{r2}} \cdot \delta_{fix1} + \left(\frac{g_2 + g_3\varphi}{\Sigma g} \cdot \frac{V_{r2}}{B_1 + V_{r2}} + \frac{G}{\Sigma g} \right) \cdot \delta_{fix2} + \frac{g_3(1-\varphi)}{\Sigma g} \cdot \delta_{fix3}$$
$$- p_r \cdot \left(\frac{g_2 + g_3\varphi}{\Sigma g} + \frac{g_3(1-\varphi)}{\Sigma g} \right) + \beta \cdot \left(\frac{g_2 + g_3\varphi}{\Sigma g} \cdot \frac{g_2}{B_1 + V_{r2}} + \frac{g_3}{\Sigma g} \cdot \frac{g_3}{B_3 + V_{r3}} - 1 \right)$$
$$+ e_m \cdot \left(\frac{g_2 + g_3\varphi}{\Sigma g} \cdot \frac{\frac{B_1 R_{m1}}{B_1 + R_{m1}} + R_{m2}}{B_1 + V_{r2}} + \frac{g_3}{\Sigma g} \cdot \frac{R_{m3}}{B_3 + V_{r3}} \right) \quad (11)$$

where $\varphi = B_3/(B_3 + V_{r3})$. (11) can be simplified using relationships obtained from mass balance on fixed carbon (with amounts A_2 and A_3) that are such that:

$$p_r = -R_{g3} e_g / V_{r3} \quad (12)$$

$$\beta = e_g \cdot (-R_{g2} + V_{r2} R_{g3} / V_{r3}) / G \quad (13)$$

Also, the sum of coefficients in front of δ_{fix1}, δ_{fix2} and δ_{fix3} in (11) equals 1 and thus we can simplify notations using the weighted average isotope composition of fixed carbon $\langle \delta_{fix} \rangle$. Then we obtain:

$$\delta_{grain} = \langle \delta_{fix} \rangle + \frac{e_g}{\Sigma g} \cdot \left(R_{g3} \cdot \frac{g_2 + g_3}{\Sigma g} + \left(R_{g3} \frac{V_{r2}}{V_{r3}} - R_{g2} \right) \cdot \frac{(g_2 + g_3\varphi) \frac{g_2}{B_1 + V_{r2}} + \frac{g_3^2 \varphi}{B_3} - \Sigma g}{G} \right)$$
$$+ \frac{e_m}{\Sigma g} \cdot \left(\left(R_{m1} \cdot \frac{B_1}{B_1 + R_{m1}} + R_{m2} \right) \cdot \frac{g_2 + g_3\varphi}{B_1 + V_{r2}} + R_{m3} \cdot \frac{g_3 \varphi}{B_3} \right) \quad (14)$$

Equation (14) can be abbreviated as follows:

$$\delta_{grain} = \langle \delta_{fix} \rangle + e_g \cdot \frac{\langle R_g \rangle}{\Sigma g} + e_m \cdot \frac{\langle R_m \rangle}{\Sigma g} \quad (15)$$

where $\langle R_g \rangle$ and $\langle R_m \rangle$ represent growth and respiratory sums, corrected for carbon allocation to grains as described by parentheses in (14).

Of course, $\langle \delta_{fix} \rangle$ is not very convenient since it is a weighted average and furthermore, it cannot be measured routinely by isotopic online gas exchange in the field. Therefore, it is more useful to use the isotope composition of leaves (vegetative material). Here, we used the isotope composition of leaves at anthesis, where tissues are made of structural material and metabolites (reserves) and are generated up to the end of stage 1/onset of stage 2 (Fig. S2). The weighted average of reserves and structural material produced during stage 1 (full vegetative phase up to anthesis) is given by:

$$\delta_{leaf} = \frac{\delta_{B1} B_1 + (\delta_{fix1} - p_s) V_s}{B_1 + V_s} \qquad (16)$$

which gives, using (7):

$$\delta_{leaf} = \delta_{fix1} + e_m \cdot \frac{R_{m1}}{B_1 + R_{m1}} \theta - p_s \cdot (1 - \theta) \qquad (17)$$

Where θ is allocation to reserves at stage 1, equal to $B_1/(B_1 + V_s)$. If we assume that $\delta_{fix1} \approx \langle \delta_{fix} \rangle$, we have:

$$\delta_{grain} - \delta_{leaf} \approx \frac{\langle R_m \rangle}{\Sigma g} \cdot e_m + \frac{\langle R_g \rangle}{\Sigma g} \cdot e_g - \frac{R_{m1}}{B_1 + R_{m1}} \theta \cdot e_m + p_s \cdot (1 - \theta) \qquad (18)$$

If the respiration use efficiency calculated with respect to net fixed carbon (not gross assimilation) is denoted as RUE^*, then (18) can be abbreviated using (4) to:

$$\delta_{grain} - \delta_{leaf} \approx \frac{RUE^*}{HI} \cdot e_{mg} - \frac{R_{m1}}{B_1 + R_{m1}} \theta \cdot e_m + p_s \cdot (1 - \theta) \qquad (19)$$

Where e_{mg} stands for the average fractionation in respiration (growth, maintenance).

(19) simply says that the isotope composition in grains is the result of carbon partitioning and as such, can be considered (due to mass balance) as reflecting the carbon material left behind by the action of fractionations in respiration and structural vegetative biomass. If the leaf-to-grain difference is denoted as $\Delta\delta$, (19) can be rewritten as:

$$\Delta\delta_{corr} = \Delta\delta - \ell(\theta, p) \approx \frac{RUE^*}{HI} \cdot e_{mg} \qquad (20)$$

where

$$\ell(\theta, p) = -\frac{R_{m1}}{B_1 + R_{m1}} \theta \cdot e_m + p_s \cdot (1 - \theta) \text{ and } \Delta\delta = \delta_{grain} - \delta_{leaf}$$

Also in (19), it should be noted that the order of magnitude of the different terms differs. Fractionations e_{mg}, e_m and p_s are of a few per mil. The middle term multiplies two quantities smaller than 1, θ and $R_{m1}/(B_1 + R_{m1})$, and thus it is of minor importance. Since RUE^* is of the order of 0.6-0.8 and HI of about 0.5 in wheat, the first and third terms predominate numerically in (19). Further information is provided in the Supplementary Text on estimating RUE^*, θ and $R_{m1}/(B_1 + R_{m1})$. RUE^* was estimated from biomass increment and %N using two methods, which gave essentially the same results (Fig. S3).

Estimation of $\delta^{13}C$ values in leaves. While measuring the carbon isotope composition in grains is easy because it just requires sampling upon final harvesting, having $\delta^{13}C$ values of leaves at anthesis is much more demanding, both financially and in personnel. Therefore, we explored the possibility to reconstruct the average $\delta^{13}C$ value in leaves from (*i*) observed agronomic variables via machine learning conducted by multivariate

analysis OPLS (Eriksson *et al.*, 2008; Eriksson *et al.*, 2013); (*ii*) the $\delta^{18}O$ value observed in grains. These two methods are presented in the Supplementary Text. Method *(i)* involves multivariate statistics which were performed with Simca® (Umetrics, Sweden).

Statistics

Univariate statistics and linear regressions were done in R and with Sigmaplot®. Tests used and significance levels are indicated in figure-e legends.

Results

Relationship with yield

As expected, there was a negative linear relationship between $\delta^{13}C_{grain}$ and yield (Fig. 1), whereby low-yielding plots were up to 3‰ enriched in ^{13}C compared to high-yielding plots. In fact, grain carbon inherits photosynthetically fixed carbon and thus is ^{13}C-enriched when stomatal closure is more pronounced in non-irrigated field plots and in turn restricts isotope discrimination (blue vs. grey points in Fig. 1). This is further illustrated with isolines that showed that the expected generic relationship between yield and $\delta^{13}C_{grain}$ was positive, while its slope increased considerably as stomatal closure increased (dashed lines). Interestingly, the relationship between $\delta^{13}C_{grain}$ and yield was not influenced by field site showing that wheat cultivars did not differ enormously in their physiological response to environmental conditions.

Prediction of $\delta^{13}C$ in leaves

To extract valuable information on grain properties from $\delta^{13}C_{grain}$, we then used the isotopic difference between grain and leaves ($\Delta\delta$), i.e., the apparent grain-to-leaf discrimination. Based on wheat phenology and carbon allocation pattern (see *Theory and calculations* in Materials and Methods), we anticipate a generic relationship between $\Delta\delta$, respiratory loss (R_t) and yield (Y) as (equations 18-19):

$$\Delta\delta \approx \frac{R_t}{Y} \cdot e_{mg} + \ell(\theta, p) \qquad (21)$$

where e_{mg} is respiratory (growth + maintenance) isotope fractionation and $\ell(\theta,p)$ combines the carbon allocation coefficient to reserves (θ) and post-photosynthetic fractionation in structural biomass (straw) production (p). This equation can be rewritten using HI as follows (equation 20):

$$\Delta\delta_{corr} = \Delta\delta - \ell(\theta, p) \approx \frac{RUE^*}{HI} \cdot e_{mg} \qquad (22)$$

where RUE^* is the net carbon-based respiration use efficiency. Note this equation is written in such as way to make proportionality apparent between $\Delta\delta_{corr}$ and RUE^*/HI. However, applying (21) and (22) implies prior knowledge of the $\delta^{13}C$ value in leaves. This is not something that can be done routinely in the field unlike isotope analysis of grains, which are sampled anyway during harvesting. We thus explored three methods to obtain $\delta^{13}C_{leaf}$ (Table 1). In the first dataset (VAR), leaves were sampled and $\delta^{13}C_{leaf}$ was thus measured. In the second dataset (BPA), $\delta^{13}C_{leaf}$ was reconstructed from agronomic parameters using machine-learning (OPLS multivariate analysis with $\delta^{13}C$ as the quantitative objective variable). In the third dataset (BVG), $\delta^{13}C_{leaf}$ was estimated from the natural ^{18}O abundance in grains (calculations explained in Supplementary Text). This method took advantage of the rather well-conserved $\delta^{18}O$ difference between leaves and grains, of 2.9‰ (Fig. 5a)

Outputs of the multivariate analysis are provided in Fig. 2. The statistical model was trained with dataset 1 (VAR). It was highly significant ($P_{CV\text{-}ANOVA} = 1.7\times10^{-9}$), robust ($R^2 = 0.68$; $Q^2 = 0.51$) and representative (Q^2[intercept] = –0.33). There was a very strong relationship between predicted and observed $\delta^{13}C_{leaf}$ values, with a slope extremely close to unity (0.9998) (Fig. 2a). The role played by the different variables is shown using a volcano plot, representing the variable importance for the projection (VIP) against the loading (pq) (Fig. 2b). In such a representation, the most important drivers have a VIP value above unity and a high loading. $\delta^{13}C_{leaf}$ appeared to be most influenced by the previous crop species and N-related traits (red and turquoise points), suggesting a strong effect of nitrogen availability. Multiple linear models are then carried out and similarly, showed the importance of the previous crop species, N-related traits in addition to yield (Fig. 2c). The statistical model was then implemented to calculate $\delta^{13}C_{leaf}$ at the second site (BPA). The average was found to be –27.9‰, with values up to about –25‰ and 90% of values being between –27‰ and –29‰ (Fig. 2d).

$\delta^{13}C_{leaf}$ values computed from $\delta^{18}O_{grain}$ at the collective site 3 (BVG) are presented in Fig. S5. The applicability of this method was first checked by comparing observed and computed values at site 1 on different cultivars (Fig. S5b). Average observed and computed $\delta^{13}C$ values were found to be consistent, by about 1‰, and calculations were thus implemented at site 3.

Leaf-to-grain isotope difference

The uncorrected leaf-to-grain $\delta^{13}C$ difference is shown in Fig. 3. Grains were always found to be ^{13}C-enriched compared to leaves, showing the occurrence of a post-photosynthetic fractionation. Such a fractionation was comprised within 0 and –4‰ (negative sign since it was in favour of ^{13}C in grains), except at site 3 were it reached 10‰. As expected from equation 21, there was a negative hyperbolic relationship between $\Delta\delta$ and yield, which fell into the region where the respiratory loss represented about 200 dt CO_2 ha^{-1} (isolines in Fig. 3) at both sites 1 and 2 (VAR, BPA). Also, $\Delta\delta$ was affected by irrigation, with smaller post-photosynthetic fractionation (≈1‰) under irrigation and high yield, reflecting the impact of water limitation on isotope allocation to grains. Although in the same graphical region, dataset 3 (BVG) was more scattered due to the much higher imprecision in $\delta^{13}C_{leaf}$ estimated from $\delta^{18}O_{grain}$.

Also, undesirable noise in the relationship between $\Delta\delta$ and yield may have come from reserve remobilisation and variations in biomass allocation. Therefore, we looked at the relationship between corrected $\Delta\delta$ ($\Delta\delta_{corr}$) and the ratio RUE^*/HI (equation 22). At both sites (VAR and BPA), $\Delta\delta_{corr}$ fell within a narrow region delimited by isolines obtained with different respiratory isotope fractionations (from 2.5 to 4‰) (Fig. 4a-b). It formed a significant linear relationship with RUE^*/HI (dash-dotted black line). This relationship was not examined using dataset 3 (BVG) since biomass, %N and growth data

were not available and thus RUE^* and $\Delta\delta_{corr}$ could not be estimated. Interestingly, data points obtained under irrigated and non-irrigated conditions partly overlapped and were in the same region of the graph, with no significant isotope offset, and thus appeared to be on the same relationship.

Possible links to grain properties

Under our conditions, the relationship between $\Delta\delta_{corr}$ and RUE^*/HI was probably not driven by differences in grain specific weight (GSW, mass per grain volume) since no difference was found between conditions in GSW (Fig. 4c and 4f). It suggests that changes in carbon allocation reflected by RUE^* and HI were not associated with different grain size but rather changes in total grain set per plant. There were differences in straw or grain nitrogen (%N) across conditions (Fig. 4d, e, g and h). Since the nitrogen content was also a determinant of $\delta^{13}C_{leaf}$ (see above), N assimilation and remobilisation thus appeared to be an important parameter in $\Delta\delta_{corr}$ typically via both photosynthetic capacity and grain metabolism.

Discussion

Our results show that the apparent isotope fractionation between leaves (at anthesis) and grain (at maturity), $\Delta\delta_{corr}$, correlates to parameters associated with respiration use efficiency (RUE^*) and harvest index (HI) in wheat. In addition to giving information on isotope biochemistry beyond photosynthesis (i.e. on grain production itself), it suggests that $\Delta\delta_{corr}$ is a potentially useful marker to trace carbon partitioning carbon utilisation. In fact, the RUE^*-to-HI ratio reflects C allocation: the larger the ratio, the higher the respiratory loss and/or the lower the grain relative biomass fraction. In what follows, we address this point by discussing causes of the leaf-to-grain isotope fractionation and possible limitations in using $\Delta\delta_{corr}$.

Origin of the relationship between grain isotope fractionation $\Delta\delta_{corr}$ and RUE^*/HI

The relationship between $\Delta\delta_{corr}$ and RUE^*/HI (Fig. 4) is a consequence of respiratory loss and biomass production, which are accompanied by isotope effects (represented by fractionation factors e_{mg} and p in equations). In practice, this suggests that highly performing wheat plants are naturally less ^{13}C-enriched due to a lower relative impact of respiration and higher commitment to biomass synthesis, thus minimizing apparent, post-photosynthetic isotope fractionations.

The proportionally lower respiration in highly performing wheat lines has effectively been observed experimentally (McCullough & Hunt, 1989). Of course, our interpretation of variations in $\Delta\delta_{corr}$ is somewhat simplified since parameters other than e_{mg} and p participate in equations (such as partitioning to reserves, θ) and furthermore, equations (18-22) are a simplification (i.e., require approximations, see also discussion below). This probably explains part of the noise present in Fig. 4, along with variation in e_{mg} itself. Still, it is striking that nearly all of the datapoints are within the graphical region defined by e_{mg} = 2 and 4‰ (Fig. 4).

The fact that carbon partitioning translates into changes in $\Delta\delta_{corr}$ is also likely associated with changes in nitrogen partitioning: First, $\delta^{13}C_{leaf}$ was found to depend on leaf N content and availability (Fig. 2); Second, some changes were observed in grain or straw N content between conditions (Fig. 4); Third, nitrogen availability has a well-known effect on carbon partitioning (including the shoot-root ratio) and respiration in wheat (Pearman *et al.*, 1981; Cox *et al.*, 1986; Hay, 1995; Sinclair, 1998; Kichey *et al.*, 2007; Allard *et al.*, 2013). In our case, wheat plots were fertilised at relatively similar levels (*c.* 200 kg ha^{-1}) which are commonly used under ordinary culture conditions (200 kg ha^{-1} is the average value compiled by the FAO, www.fao.org). In other words, $\Delta\delta_{corr}$ would probably appear to be less strongly related to RUE*/HI if fertilisation regimes had been extremely different between sites or plots (for example, no fertilisation at all and more than 250 kg N ha^{-1}).

Importantly, the relationship between $\Delta\delta_{corr}$, RUE*/HI did not depend much on water relations. In effect, while the response of $\delta^{13}C_{grain}$ to yield was mostly driven by water availability and thus conductance and thus photosynthetic conditions as in (Merah *et al.*, 2001a; Merah *et al.*, 2001b) (Fig. 1), irrigation had a rather small effect on the relationship between $\Delta\delta_{corr}$ and RUE*/HI, all data points being apparently on the same line (Fig. 4). For example, under oceanic climate (site VAR), the average offset between irrigated and non-irrigated conditions was *c*. 0.7‰ only (Fig. 4a) and was statistically insignificant. In other words, although non-irrigated conditions impacted on overall allocation to grains and thus the harvest index, there was little effect on fractionation factors and as a result, datapoints followed the same relationship as that found under irrigated conditions. Accordingly, drought conditions have effectively been shown to have little effect on metabolic discriminations, in particular in respiration in other crops (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001).

Pros and cons of the theoretical background used here

The theoretical background used here to obtain a relationship between $\Delta\delta_{corr}$ and RUE*/HI (equations 20 and 22) implied several approximations. We assumed that reserves accumulated during first stages are remobilised at later stage with minimal left overs of unused carbon reserves ($\Delta B = 0$ in equation 4). This assumption is likely not critical since wheat straw is effectively very poor in non-structural carbohydrates (and protein) [for a recent overview, see (Wang *et al.*, 2020)] showing very efficient remobilisation. Also, isotopic labelling has shown that up to 94% of available protein and water-soluble carbohydrates are effectively used for grain filling (Gebbing *et al.*, 1999). We further assumed that leaves sampled at anthesis were representative of fixed carbon (accounting for biomass and reserves deposition, equation 16). This assumption was driven by technical imperatives because the true value of the weighted average of fixed carbon, $\langle\delta_{fix}\rangle$, is not accessible. It is probably not too critical since reserves accumulated during the vegetative stage have a high contribution to grain carbon, of up to 50% (Schnyder, 1993; Gent, 1994; Gebbing *et al.*, 1999). In addition, if photosynthetic properties of leaves were to vary significantly (with typically lower c_i/c_a during summertime), it would mean that the isotope composition of fixed carbon up to anthesis would be slightly ^{13}C-depleted compared to $\langle\delta_{fix}\rangle$. This isotopic difference would add in the intercept of the relationship in (19) and Fig. 4, not in the slope, and therefore the correlation between $\Delta\delta_{corr}$ and RUE*/HI would remain valid, with some noise due to variations in $\langle\delta_{fix}\rangle$. In other words, our assumption $\langle\delta_{fix}\rangle \approx \delta_{fix1}$ probably explains some of the scattering but cannot explain the linear relationship in Fig. 4. It is also possible that the small offset between irrigated and non-irrigated conditions (blue vs. grey points in Fig. 4) was explained by this effect. That said, the ^{13}C-enriching effect of dry conditions in summer must be compensated for by the ^{13}C-depleting effect of refixation of CO_2

respired by glumes, which has a non-negligible contribution to grain organic matter (Araus *et al.*, 1993; Gebbing & Schnyder, 2001). Taken as a whole, variations in overall photosynthetic isotopic input are probably not huge and cannot alter the relationship described here.

We also recognise that although convenient, equation (22) is an abbreviated formulation of the full expression describing post-photosynthetic processes (compare equations 14 and 15 and compare 15 and 19). Nevertheless, the abbreviated final equation (22) allows application with readily accessible variables in the field. Taken as a whole, a generic respiratory fractionation (here denoted as e_{mg}), which encapsulates weighted maintenance and growth respiration parameters, seems to be sufficient to model $\Delta\delta_{corr}$. In addition, the apparent value of e_{mg} adapted to linear fitting (2.5 to 4‰) (Fig. 4, coloured lines) indicates that at the plant scale (above-ground organs), respiratory metabolism yields ^{13}C-depleted CO_2. The generation of ^{13}C-depleted CO_2 is a general feature in heterotrophic organs of C_3 plants (Klumpp *et al.*, 2005; Bathellier *et al.*, 2009; Ghashghaie & Badeck, 2014; Bathellier *et al.*, 2017).

Potential utilisation of the relationship between $\Delta\delta_{corr}$ and RUE/HI*

The observed robustness of the relationship between $\Delta\delta_{corr}$ and RUE*/HI (with respect to water availability and sites) is essential to envisage potential applications. In fact, having low values of the ratio RUE*/HI reflect minimal respiratory losses (low RUE*) and/or good allocation to grains (high HI), and is thus beneficial. That is, selecting for low $\Delta\delta_{corr}$ values could thus help finding cultivars with better performance in carbon partitioning to grains. This would be of prime interest for current strategies to get higher yield in wheat, which include an increase in HI and minimization of carbon losses by respiration (Reynolds *et al.*, 2012). Also, HI appears to condition responsiveness to high CO_2 (Aranjuelo *et al.*, 2013) and is thus of importance under current conditions of global change. In the recent past, considerable efforts have been devoted to biometrics, QTL or GWAS to identify key markers associated with yield or harvest index (Reynolds *et al.*, 2017; Quintero *et al.*, 2018; Pradhan *et al.*, 2019; Rivera-Amado *et al.*, 2019; Porker *et al.*, 2020). In addition, domesticated high-yielding dwarf varieties with changed carbon (as well as nitrogen and sulphur) allocation, show changes in grain specific weight (Casebow *et al.*, 2016) although the trade-off between grain number and specific weight seems to depend on environmental conditions (Quintero *et al.*, 2018).

Future studies are warranted to determine whether the isotope difference $\Delta\delta_{corr}$ can be used as an advantageous trait for breeding, since *(i)* it encapsulates a complex carbon allocation parameter (RUE*/HI) that is not accessible otherwise, and *(ii)* the $\Delta\delta_{corr}$-RUE*/HI relationship changes minimally with growth conditions. When $\delta^{13}C_{leaf}$ at anthesis is not available to compute $\Delta\delta_{corr}$, our study further suggests it can be estimated with multivariate analysis or $\delta^{18}O$. It is now well-accepted that there is good heritability

459 of $^{12}C/^{13}C$ isotope composition not only in leaves but also in grains (Merah *et al.*, 2001a).
460 Taken as a whole, we suggest that interesting allocation properties can be accessed via
461 $\Delta\delta_{corr}$, which gives direct information on events during grain filling and thus may help
462 isotope-based implementation of genotype selection.

Acknowledgments

This work was supported by the *Région Pays de la Loire* and *Angers Loire Métropole* via the program *Connect Talent* Isoseed and the University of Angers via a PhD grant to JB.D. The authors also thank the financial support of the *Groupement Interprofessionnel des Semences et Plants* via the project 2012K "Identification of drought tolerance traits in winter wheat and constitution of tools adapted to their evaluation" of the scheme *Fonds de Soutien à l'Obtention Végétale* (field trials BPV and VAR). The field trial BPA was carried out jointly by PIA-ANR via the project BreedWheat (ANR-10-BTBR-003). The authors thank Hilary Stuart-Williams (ANU Isotope Facility) for $^{18}O/^{16}O$ isotope analyses.

Author Contributions

JC.D., E.O., V.L., C.Z., L.D., C.B., P.L., J.D., and T.M. conceived the field experiment, organised wheat cultivation field trials and sampling. K.B. performed agronomic measurements. C.A. prepared samples for the isotopic analysis. M. LS. Carried out isotopic $^{12}C/^{13}C$ analyses. JB.D. and G.T. conceived the concept and developed the scenarios associated with $\Delta\delta$ in grains. J.L. and AM.L. checked the validity of isotopic analyses. G.T. wrote the paper. JB.D. managed data storing and availability on the INRAe server. All authors read or contributed to the final version of the paper.

Competing Interest Statement: The authors declare no conflict of interest.

References

Allard V, Martre P, Le Gouis J. 2013. Genetic variability in biomass allocation to roots in wheat is mainly related to crop tillering dynamics and nitrogen status. *European Journal of Agronomy* **46**: 68-76.

Aranjuelo I, Sanz-Sáez Á, Jauregui I, Irigoyen JJ, Araus JL, Sánchez-Díaz M, Erice G. 2013. Harvest index, a parameter conditioning responsiveness of wheat plants to elevated CO_2. *Journal of experimental botany* **64**: 1879-1892.

Araus JL, Brown HR, Febrero A, Bort J, Serret MD. 1993. Ear photosynthesis, carbon isotope discrimination and the contribution of respiratory CO_2 to differences in grain mass in durum wheat. *Plant Cell and Environment* **16**: 383-392.

Badeck FW, Tcherkez G, Nogues S, Piel C, Ghashghaie J. 2005. Post-photosynthetic fractionation of stable carbon isotopes between plant organs—a widespread phenomenon. *Rapid Communications in Mass Spectrometry* **19**: 1381-1391.

Bathellier C, Badeck F-W, Ghashghaie J 2017. Carbon isotope fractionation in plant respiration. In: Tcherkez G, Ghashghaie J eds. *Plant respiration: Metabolic fluxes and carbon balance*. Berlin: Springer, 43-68.

Bathellier C, Tcherkez G, Bligny R, Gout E, Cornic G, Ghashghaie J. 2009. Metabolic origin of the $\delta^{13}C$ of respired CO_2 in roots of *Phaseolus vulgaris*. *New Phytologist* **181**: 387-399.

Casebow R, Hadley C, Uppal R, Addisu M, Loddo S, Kowalski A, Griffiths S, Gooding M. 2016. Reduced height (*Rht*) alleles affect wheat grain quality. *Plos one* **11**: e0156056.

Cernusak LA, Tcherkez G, Keitel C, Cornwell WK, Santiago LS, Knohl A, Barbour MM, Williams DG, Reich PB, Ellsworth DS. 2009. Why are non-photosynthetic tissues generally ^{13}C enriched compared with leaves in C_3 plants? Review and synthesis of current hypotheses. *Functional Plant Biology* **36**: 199-213.

Condon A, Farquhar G, Richards R. 1990. Genotypic Variation in Carbon Isotope Discrimination and Transpiration Efficiency in Wheat. Leaf Gas Exchange and Whole Plant Studies. *Functional Plant Biology* **17**: 9-22.

Condon A, Richards R, Farquhar G. 1987. Carbon Isotope Discrimination is Positively Correlated with Grain Yield and Dry Matter Production in Field-Grown Wheat. *Crop Science* **27**: 996-1001.

Condon AG, Richards R, Rebetzke G, Farquhar G. 2004. Breeding for high water-use efficiency. *Journal of Experimental Botany* **55**: 2447-2460.

Cox MC, Qualset CO, Rains DW. 1986. Genetic variation for nitrogen assimilation and translocation in wheat. III. Nitrogen translocation in relation to grain yield and protein. *Crop Science* **26**: 737-740.

Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G. 1999. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant Cell and Environment* **22**: 515-523.

Eriksson L, Byrne T, Johansson E, Trygg J, Vikström C. 2013. *Multi-and megavariate data analysis basic principles and applications*. Malmö: Umetrics Academy.

Eriksson L, Trygg J, Wold S. 2008. CV-ANOVA for significance testing of PLS and OPLS® models. *Journal of Chemometrics: A Journal of the Chemometrics Society* **22**: 594-600.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual review of plant biology* **40**: 503-537.

Gebbing T, Schnyder H. 2001. ^{13}C labeling kinetics of sucrose in glumes indicates significant refixation of respiratory CO_2 in the wheat ear. *Functional Plant Biology* **28**: 1047-1053.

Gebbing T, Schnyder H, Kühbauch W. 1999. The utilization of pre-anthesis reserves in grain filling of wheat. Assessment by steady-state $^{13}CO_2/^{12}CO_2$ labelling. *Plant Cell and Environment* **22**: 851-858.

Gent M. 1994. Photosynthate reserves during grain filling in winter wheat. *Agronomy Journal* **86**: 159-167.

Ghashghaie J, Badeck FW. 2014. Opposite carbon isotope discrimination during dark respiration in leaves versus roots–a review. *New Phytologist* **201**: 751-769.

Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline MT, Deleens E. 2001. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. *Plant Cell and Environment* **24**: 505-515.

Gilbert A, Robins RJ, Remaud GS, Tcherkez GG. 2012. Intramolecular ^{13}C pattern in hexoses from autotrophic and heterotrophic C_3 plant tissues. *Proceedings of the National Academy of Sciences* **109**: 18204-18209.

Gleixner G, Danier HJ, Werner RA, Schmidt HL. 1993. Correlations between the ^{13}C content of primary and secondary plant products in different cell compartments and that in decomposing basidiomycetes. *Plant physiology* **102**: 1287-1297.

Hay RKM. 1995. Harvest index: a review of its use in plant breeding and crop physiology. *Annals of Applied Biology* **126**: 197-216.

Kelly S, Heaton K, Hoogewerff J. 2005. Tracing the geographical origin of food: The application of multi-element and multi-isotope analysis. *Trends in Food Science Technology* **16**: 555-567.

Kichey T, Hirel B, Heumez E, Dubois F, Le Gouis J. 2007. In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. *Field Crops Research* **102**: 22-32.

Klumpp K, Schäufele R, Lötscher M, Lattanzi FA, Feneis W, Schnyder H. 2005. C-isotope composition of CO_2 respired by shoots and roots: fractionation during dark respiration? *Plant Cell and Environment* **28**: 241-250.

Kodina L. 2010. Carbon isotope fractionation in various forms of biogenic organic matter: I. Partitioning of carbon isotopes between the main polymers of higher plant biomass. *Geochemistry International* **48**: 1157-1165.

Ma WT, Tcherkez G, Wang XM, Schäufele R, Schnyder H, Yang Y, Gong XY. 2020. Accounting for mesophyll conductance substantially improves ^{13}C-based estimates of intrinsic water-use efficiency. *New Phytologist* **229**: 1326-1338.

McCullough DE, Hunt LA. 1989. Respiration and dry matter accumulation around the time of anthesis in field stands of winter wheat (*Triticum aestivum*). *Annals of Botany* **63**: 321-329.

Merah O, Deléens E, Al Hakimi A, Monneveux P. 2001a. Carbon isotope discrimination and grain yield variations among tetraploid wheat species cultivated under contrasting precipitation regimes. *Journal of Agronomy and Crop Science* **186**: 129-134.

Merah O, Deléens E, Monneveux P. 2001b. Relationships between carbon isotope discrimination, dry matter production, and harvest index in durum wheat. *Journal of Plant Physiology* **158**: 723-729.

Merah O, Deléens E, Teulat B, Monneveux P. 2002. Association between yield and carbon isotope discrimination value in different organs of durum wheat under drought. *Journal of Agronomy and Crop Science* **188**: 426-434.

Pearman I, Thomas SM, Thorne GN. 1981. Dark respiration of several varieties of winter wheat given different amounts of nitrogen fertilizer. *Annals of Botany* **47**: 535-546.

Porker K, Straight M, Hunt JR. 2020. Evaluation of G × E × M interactions to increase harvest index and yield of early sown wheat. *Frontiers in Plant Science* **11**: 994.

Pradhan S, Babar MA, Robbins K, Bai G, Mason RE, Khan J, Shahi D, Avci M, Guo J, Maksud Hossain M, et al. 2019. Understanding the genetic basis of spike fertility to improve grain number, harvest index, and grain yield in wheat under high temperature stress environments. *Frontiers in Plant Science* **10**: 1481.

Quintero A, Molero G, Reynolds MP, Calderini DF. 2018. Trade-off between grain weight and grain number in wheat depends on GxE interaction: A case study of an elite CIMMYT panel (CIMCOG). *European Journal of Agronomy* **92**: 17-29.

Remaud GS, Akoka S. 2017. A review of flavors authentication by position-specific isotope analysis by nuclear magnetic resonance spectrometry: the example of vanillin. *Flavour and Fragrance Journal* **32**: 77-84.

Reynolds M, Foulkes J, Furbank R, Griffiths S, King J, Murchie E, Parry M, Slafer G. 2012. Achieving yield gains in wheat. *Plant, Cell and Environment* **35**: 1799-1823.

Reynolds MP, Pask AJD, Hoppitt WJE, Sonder K, Sukumaran S, Molero G, Pierre CS, Payne T, Singh RP, Braun HJ, et al. 2017. Strategic crossing of biomass and harvest index—source and sink—achieves genetic gains in wheat. *Euphytica* **213**: 257.

Rivera-Amado C, Trujillo-Negrellos E, Molero G, Reynolds MP, Sylvester-Bradley R, Foulkes MJ. 2019. Optimizing dry-matter partitioning for increased spike growth, grain number and harvest index in spring wheat. *Field Crops Research* **240**: 154-167.

Sanchez-Bragado R, Newcomb M, Chairi F, Condorelli GE, Ward RW, White JW, Maccaferri M, Tuberosa R, Araus JL, Serret Molins MD. 2020. Carbon Isotope Composition and the NDVI as Phenotyping Approaches for Drought Adaptation in Durum Wheat: Beyond Trait Selection. *Journal of Agronomy* **10**: 1679.

Santesteban L, Miranda C, Barbarin I, Royo J. 2015. Application of the measurement of the natural abundance of stable isotopes in viticulture: a review. *Australian journal of grape wine research* **21**: 157-167.

Schmidt H, Gleixner G 1998. Carbon isotope effects on key reactions in plant metabolism and ^{13}C-patterns in natural compounds. In: Griffiths H ed. *Stable Isotopes: Integration of biological, ecological geochemical processes*. Oxon, UK: Garland Science (Taylor and Francis), 13-25.

Schnyder H. 1993. The role of carbohydrate storage and redistribution in the source-sink relations of wheat and barley during grain filling - a review. *New Phytologist* **123**: 233-245.

Sinclair TR. 1998. Historical changes in harvest index and crop nitrogen accumulation. *Crop Science* **38**: 638-643.

Touzy G, Rincent R, Bogard M, Lafarge S, Dubreuil P, Mini A, Deswarte J-C, Beauchêne K, Le Gouis J, Praud S. 2019. Using environmental clustering to identify specific drought tolerance QTLs in bread wheat (*T. aestivum* L.). *Theoretical and Applied Genetics* **132**: 2859-2880.

Wang X, Yang Z, Liu X, Huang G, Xiao W, Han L. 2020. The composition characteristics of different crop straw types and their multivariate analysis and comparison. *Waste Management* **110**: 87-97.

Table 1. Summary of agronomic properties of cultivation sites. Average temperature and precipitations are associated with the growing and maturation season (March-June). Plant density was within 200-300 m^{-2} in all parcels. Sowing date was Nov 1st (VAR), 17th (BPA) and at various dates in the second half of October (BVG).

Abbr.	Name	Climate	Av. temp. (°C)	Prec. (mm)	Previous crop	Fert. (kgN/ha)	No. of parcels	No. of cultivars	Isotopes
Field site 1									
VAR	Saint Pierre d'Amilly (France)	Oceanic	13.4	283	Rapeseed or pea	200	87	8	$\delta^{13}C$, $\delta^{18}O$ measured in both grains and leaves
Field site 2									
BPA	Gréoux les Bains (France)	Supramediterranean	14.4	156	Sunflower	210	379	70	$\delta^{13}C$ measured in grains, $\delta^{13}C$ in leaves simulated by machine learning
Collective field site 3									
BVG	Various (France, Hungary)	Various (oceanic to mediterranean)	11.4-13.9	180-250	Sunflower, wheat or pea	180-220	178	38	$\delta^{13}C$ and $\delta^{18}O$ in grains; $\delta^{13}C$ in leaves reconstructed from $\delta^{18}O$ in grains

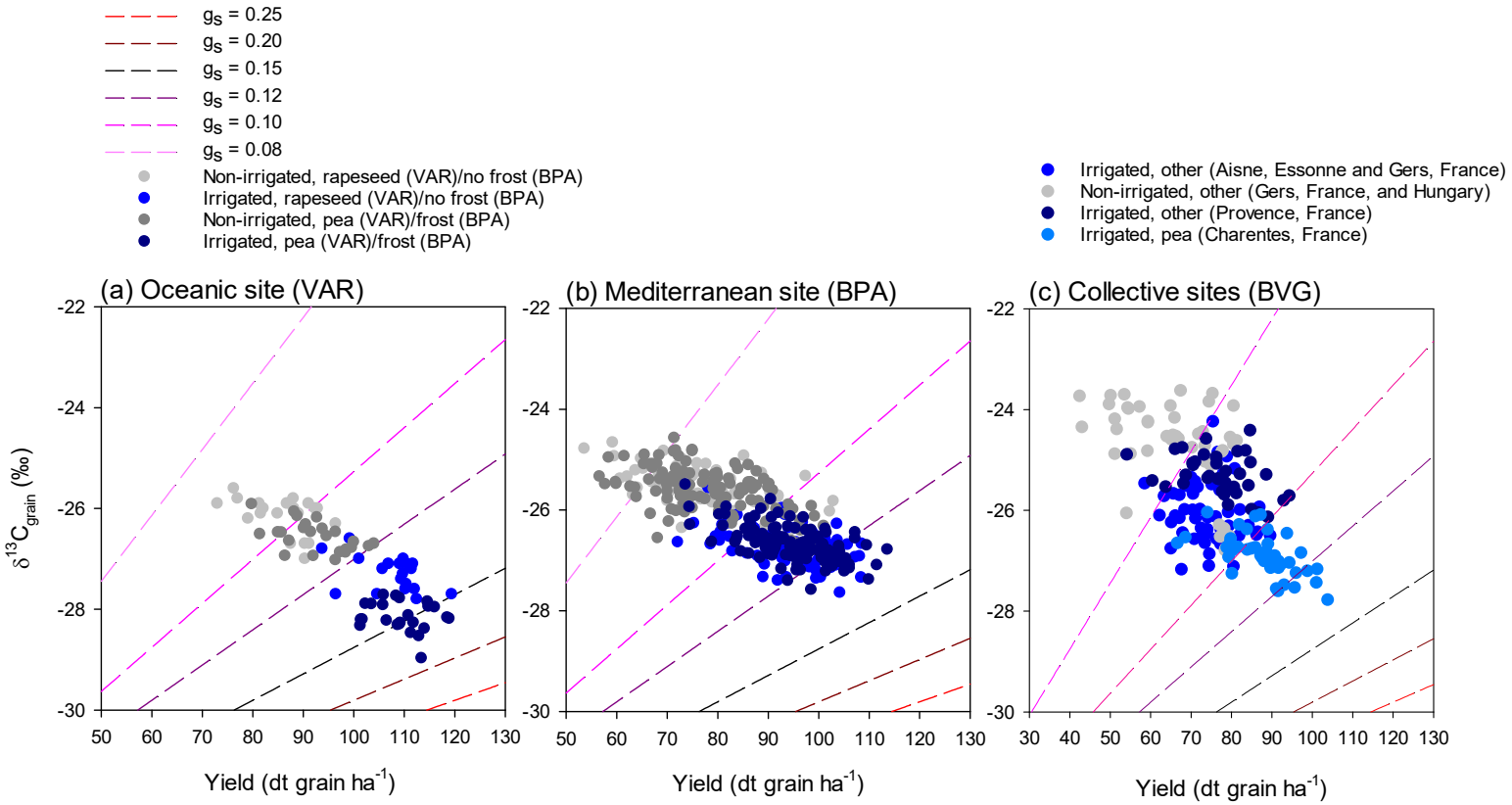

Fig. 1. Carbon isotope composition in wheat grains, expressed in absolute V-PDB scale as a $\delta^{13}C$. Wheat was cultivated under oceanic (VAR, a), Mediterranean (BPA, b) climatic conditions or across different sites (BVG, c). Wheat was grown under irrigated or non-irrigated plots, used previously to grow pea or other plants, and experiencing occasional frost or not (BPA). $\delta^{13}C$ values are plotted against yield, in decitons grain (at standard 15% humidity) per hectare. Isolines stand for expected linear relationship using the simplified photosynthetic model of isotope fractionation, with different values of average stomatal conductance g_s (in mol m^{-2} s^{-1}) (equation S11 in Supplementary Material). Note that all data points are not on the same isoline due to variations in average stomatal conductance across growth conditions, however, the relationship between $\delta^{13}C$ and yield is similar across sites.

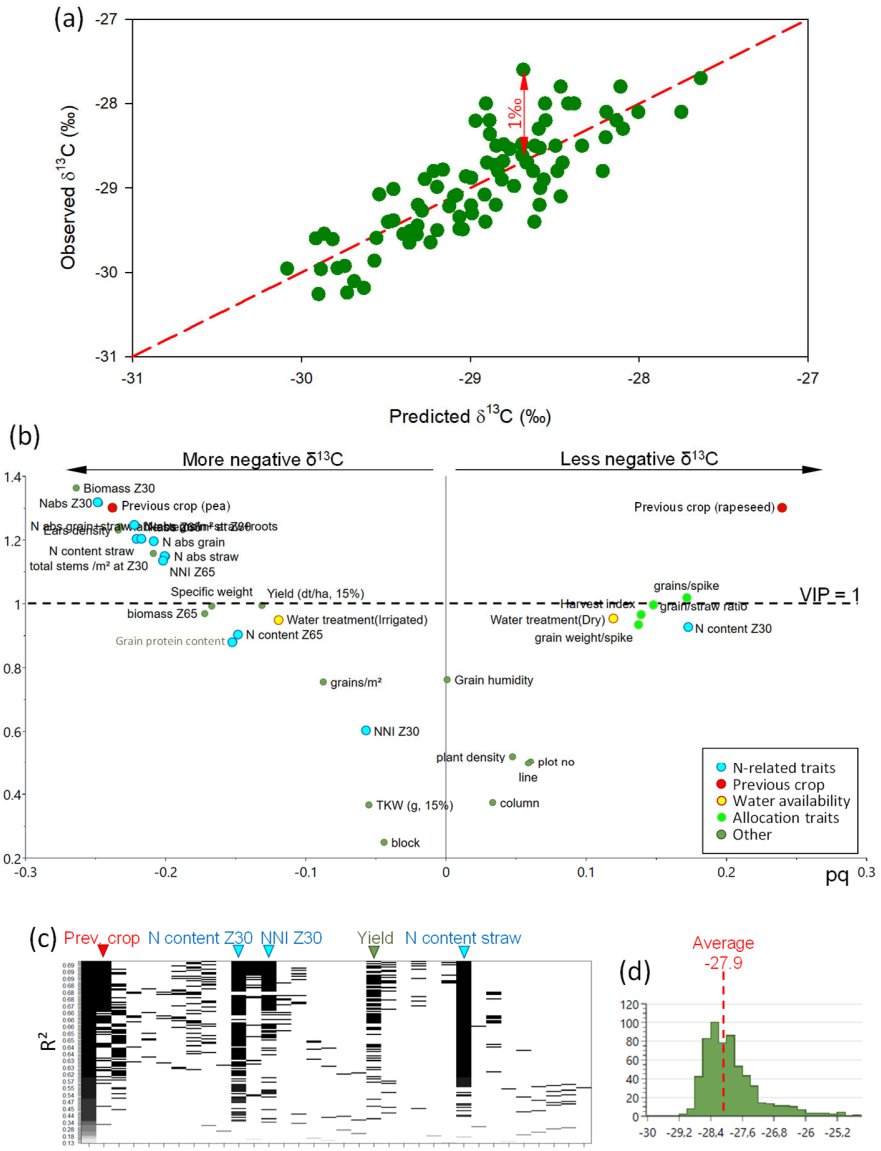

Fig. 2. Statistical analysis of $\delta^{13}C$ in leaves using dataset 1 (VAR) (a-c) **and application of the multivariate model to dataset 2 (BPA) to predict $\delta^{13}C$ values** (d). (a) Output of the OPLS model showing the correlation between predicted and observed $\delta^{13}C$ values. The maximum error made by the model is 1‰ (red arrow). Dashed line, regression line (y = 0.9998 x – 0.00518; P<0.001; R^2 = 0.68). (b) Volcano plot showing the most important variables using the loading value (pq, x-axis) against the variable importance for the projection (VIP, y-axis). Different colours are used to distinguish the types of agronomic variables (see legend). The dashed horizontal line stands for the usual threshold value used in multivariate analyses (VIP = 1). (c) Output of multiple linear models (sampling of 7 variables amongst 33 variables) showing the distribution of R^2 values. The four best variables are the previous crop, the N content in straw and in leaves at stage Z30 and the nitrogen nutrition index (NNI) at Z30 (arrowheads). The left lane corresponds to the grey scale. (d) Spectrum of predicted $\delta^{13}C$ values in leaves for the Mediterranean site (BPA) using a frequency graph, where the average value was found to be –27.9‰ (red).

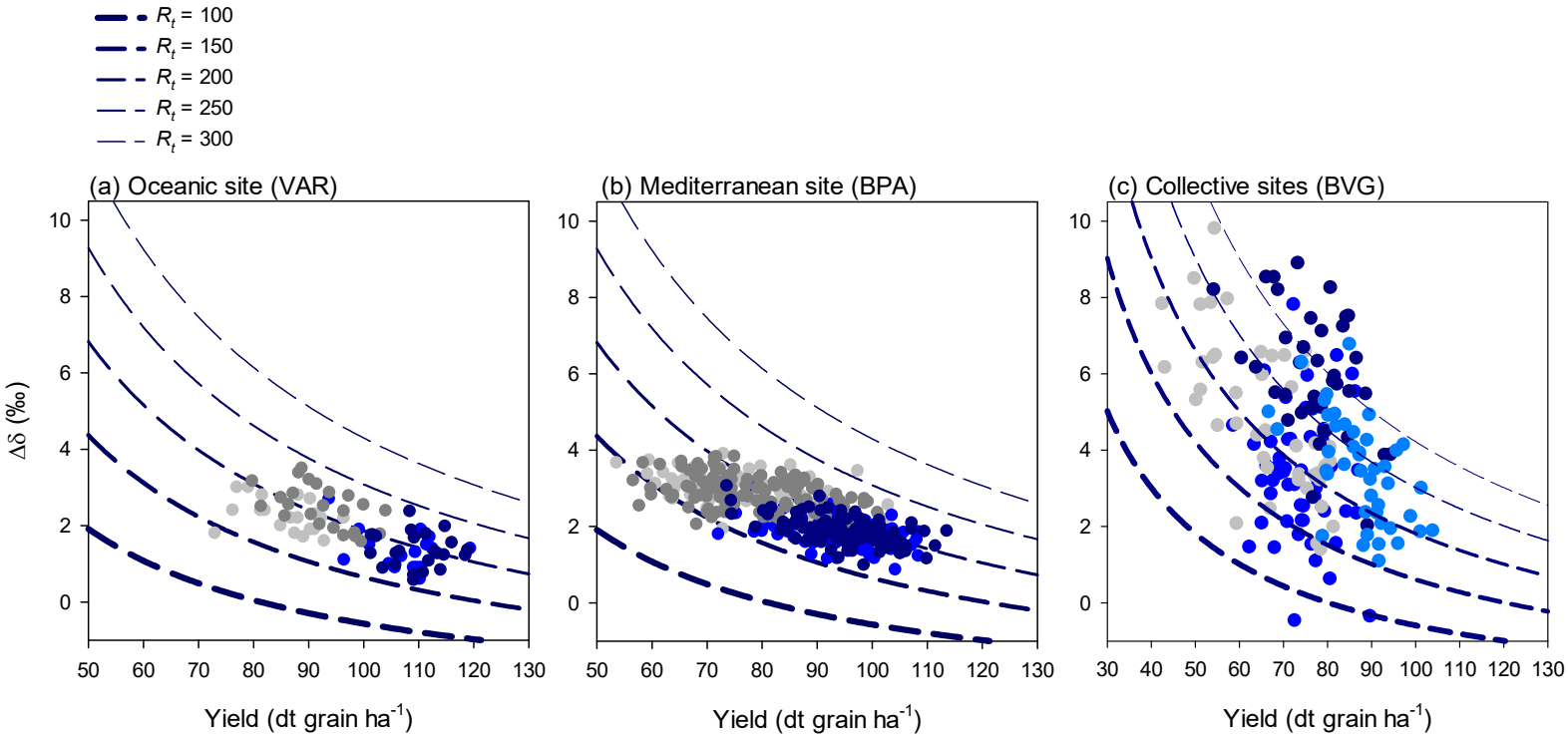

Fig. 3. Uncorrected $\delta^{13}C$ difference between grains and leaves, $\Delta\delta$. Same legend as in Fig. 1. $\delta^{13}C$ value in grains was measured and the $\delta^{13}C$ value in leaves was either measured or estimated from multivariate modelling or observed $\delta^{18}O_{grain}$ (Table 1). Isolines represent the reciprocal relationship with yield ($Y = \Sigma g$) and total respiration (R_t) (equation 21), with p = –3‰, e_{mg} = 3‰ and different values of total respiration R_t (in decitons CO_2 per hectare).

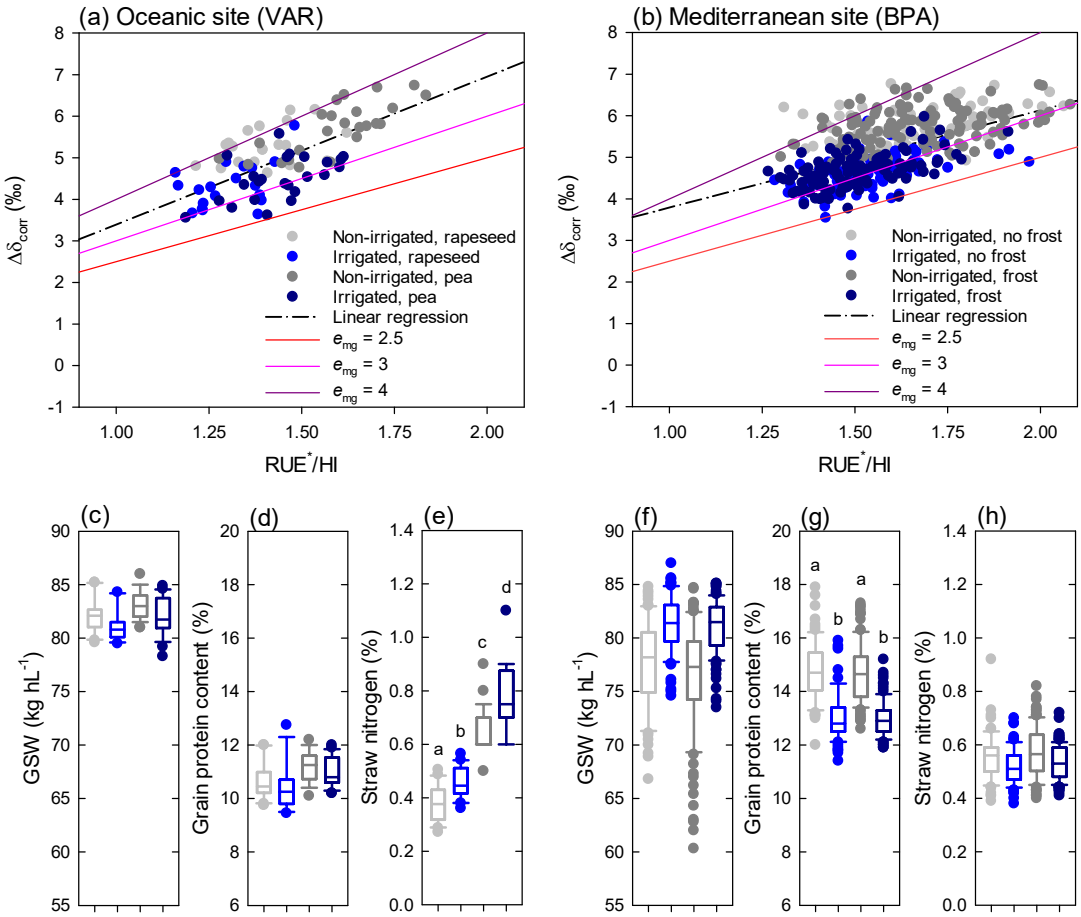

Fig. 4. Corrected isotope leaf-grain difference $\Delta\delta_{corr}$ and physiological properties of grain and straw in wheat cultivated at sites 1 (VAR) and 2 (BPA) (same symbols as in Fig. 1). (a,b) $\Delta\delta_{corr}$ plotted against estimated respiration use efficiency-to-harvest index ratio, RUE*/HI. Colored lines stand for isolines (expected proportionality relationships) with respiratory fractionation e_{mg} equal to to 2.5, 3 or 4‰. Dashed-dotted line, linear regression, which is significant (P<0.0001) with R^2 = 0.48 (a) and 0.61 (b). (c,f) Grain specific weight, in kilograms per hectoliter. (d,g) Grain protein content, in % weight. (e,h) Nitrogen elemental content in straw. Letters stand for statistical classes (ANOVA, P<0.01).