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► To cite this version:

Jean-Baptiste Domergue, Cyril Abadie, Julie Lalande, Jean-Charles Deswarte, Eric Ober, et al.. Grain carbon isotope composition is a marker for allocation and harvest index in wheat. *Plant, Cell and Environment*, 2022, pce.14339. 10.1111/pce.14339 . hal-03687116

HAL Id: hal-03687116

<https://hal.inrae.fr/hal-03687116v1>

Submitted on 3 Jun 2022


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

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Funding information

Conseil Régional des Pays de la Loire, Grant/Award Number: Connect Talent Isoseed

Abstract

The natural ¹³C abundance ($\delta^{13}\text{C}$) 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, it is also important to find genotypes with high carbon allocation to spikes and grains, and thus with a high harvest index (HI) and/or low carbon losses via respiration. Finding isotope-based markers of carbon partitioning to grains would be extremely useful since isotope analyses are inexpensive and can be performed routinely at high throughput. Here, we took the advantage of a set of field trials made of more than 600 plots with several wheat cultivars and measured agronomic parameters as well as $\delta^{13}\text{C}$ values in leaves and grains. We find a linear relationship between the apparent isotope discrimination between leaves and grain (denoted as $\Delta\delta_{\text{corr}}$), and the respiration use efficiency-to-HI ratio. It means that overall, efficient carbon allocation to grains is

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associated with a small isotopic difference between leaves and grains. This effect is explained by postphotosynthetic isotope fractionations, and we show that this can be modelled by equations describing the carbon isotope composition in grains along the wheat growth cycle. Our results show that ^{13}C natural abundance in grains could be useful to find genotypes with better carbon allocation properties and assist current wheat breeding technologies.

KEYWORDS

carbon 13, partitioning, post-photosynthetic fractionation, respiration use efficiency

1 | INTRODUCTION

The quantitation of stable carbon isotopes (^{12}C and ^{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, $\delta^{13}\text{C}$) is used routinely to probe photosynthetic pathways (C_3 , C_4 and 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 with atmospheric CO_2 while C_4 plants are ^{13}C -depleted by c. 4‰ only.

In C_3 crops, $\delta^{13}\text{C}$ 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) (A. Condon et al., 1987, 1990; A. G. Condon et al., 2004). Photosynthetic isotope discrimination essentially comes from two main steps: Firstly, the isotope effect (29‰) associated with CO_2 fixation catalysed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco); Secondly, the 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 $\delta^{13}\text{C}$ 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 1980s because $^{13}\text{C}/^{12}\text{C}$ analyses are rapid and inexpensive using current elemental analysis/mass spectrometry devices (Sanchez-Bragado et al., 2020).

Nevertheless, although leaf $\delta^{13}\text{C}$ 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 (Quintero et al., 2018; M. Reynolds et al., 2012; Sinclair, 1998). In other words, grain properties and carbon allocation to grains is a fundamental aspect of wheat breeding that cannot be accounted for with current $\delta^{13}\text{C}$ 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, a relationship was found between Y or HI and grain $\delta^{13}\text{C}$ ($\delta^{13}\text{C}_{\text{grain}}$) but this was mostly driven by changes in iWUE, that is, stomatal conductance and photosynthetic capacity (Merah, Deléens, Al Hakimi et al., 2001a; Merah, Deléens, & Monneveux, 2001b; Merah et al., 2002). Whether $\delta^{13}\text{C}_{\text{grain}}$ can be exploited further to give access to agronomic parameters other than photosynthesis or WUE has never been addressed.

In particular, variations in $\delta^{13}\text{C}_{\text{grain}}$ can be anticipated because of metabolic isotope discriminations in grain biomass synthesis and/or respiratory CO_2 loss (the so-called 'postphotosynthetic' isotope fractionations). Postphotosynthetic fractionations generally cause heterotrophic organs like roots to be ^{13}C -enriched compared with leaves (Badeck et al., 2005; Cernusak et al., 2009). That is, isotope fractionations in metabolism (biosynthesis and respiration) impact on the final isotope composition of sink organs because of isotopic mass balance. In mathematical terms, the isotope mass balance is such that the $\delta^{13}\text{C}$ of available source carbon (δ_{source}) equals the weighted average of $\delta^{13}\text{C}$ in products, accounting for fractionations. This translates into equations of the form $A \times \delta_{\text{source}} = V \times (\delta_{\text{source}} - p) + R \times (\delta_{\text{source}} - e)$, where A is the influx of source carbon, V is biomass synthesis and R is respiration (with $A = V + R$), p and e are fractionation associated with biomass production and respiratory CO_2 release, respectively (isotope fractionations are positive when against ^{13}C and negative when against ^{12}C). There is an isotope discrimination during metabolic decarboxylation and thus respired CO_2 is generally ^{13}C -depleted in heterotrophic organs (i.e., $e > 0$) (Bathellier et al., 2017). Vegetative plant organic matter is mostly made of cellulose, which is ^{13}C -enriched compared with photosynthetically fixed carbon (Gleixner et al., 1993; Kodina, 2010; Schmidt & Gleixner, 1998), therefore $p < 0$. Using intramolecular ^{13}C distribution analysis ($\delta^{13}\text{C}$ values at the different C-atom positions), it has been shown that isotope effects in metabolism (such as glucose isomerase and glycolysis)

cause a ^{13}C -enrichment in specific C-atom positions of starch in wheat grains (Gilbert et al., 2012). Taken as whole, grains are ^{13}C -enriched compared with leaves, but the extent of such a ^{13}C -enrichment likely depends on carbon partitioning to grain matter and respiration (respiratory CO_2 loss) because of isotopic mass balance. It means that $\delta^{13}\text{C}_{\text{grain}}$ is likely related to carbon allocation at the plant scale, but to our knowledge, this has never been studied.

To answer this question, we aim to examine possible relationships between $\delta^{13}\text{C}_{\text{grain}}$ and agronomic parameters that reflect carbon allocation (HI and respiration use efficiency). To do so, we exploited a data set of $\delta^{13}\text{C}$ 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 Supporting Information: Figure S1), representing a total of 644 field plots and 199 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 $\delta^{13}\text{C}_{\text{grain}}$ values.

2 | MATERIALS AND METHODS

2.1 | Cultivation and trial management

Three series of randomized block field experiments (Supporting Information: Figure S1 and Table 1) were undertaken at different locations in 2012–2015, with different cultivars (Supporting Information: Table S1) and varying water availability, irrigated to maximal evapotranspiration, and nonirrigated (rainfed). Experiments followed cultivation of wheat or other crops (sunflower, pea and rapeseed) as mentioned in figures, on calcareous or clay substratum depending on the site considered. Crop development was typical of French/European 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. Sowing density was within 200 and 350 seeds m^{-2} , depending on soil conditions and sowing date. Irrigation, when applied, was based on soil sensors or water balance information to ensure actual differentiation between treatments (irrigated and nonirrigated) while avoiding water-logging. Each irrigation sequence provided 25–35 L m^{-2} and was repeated as frequently as necessary (i.e., one to six applications from stage Z30 to stage Z75, depending on weather conditions). Crop protection (pests, diseases and weeds) was applied to avoid biotic stress. Nitrogen fertilisation consisted of two to four applications from mid-tilling (Stage Z25, in February) till expended flag leaf (Z39, in April–May), according to the local recommendation. The nitrogen status was monitored during stem growth using either nondestructive measurements (SPAD reading on the last fully expended leaf) (Prost & Jeuffroy, 2007), or destructive measurements (nitrogen nutrition index, NNI) (Justes et al., 1994). P and K deficiencies were avoided by applying P and K fertilisation when soil P and K content before sowing was insufficient.

TABLE 1 Summary of agronomic properties of cultivation sites

Abbr.	Name	Climate	Av. temp. (°C)	Prec. (mm)	Previous crop	N fert. (kg/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	88	9	$\delta^{13}\text{C}$, $\delta^{18}\text{O}$ measured in both grains and leaves
Field site 2									
BPA	Gréoux les Bains (France)	Supramediterranean	14.4	156	Sunflower	210	378	193	$\delta^{13}\text{C}$ measured in grains, $\delta^{13}\text{C}$ 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	48	$\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in grains; $\delta^{13}\text{C}$ in leaves reconstructed from $\delta^{18}\text{O}$ in grains

Note: Average temperature and precipitations are associated with the growing and maturation season (March–June 2015). Plant density was within 200–300 m^{-2} in all parcels. Sowing date was 1 November 2014 (VAR), 17 November 2012 (BPA) and at various dates in the second half of October 2014 (BVG).

2.1.1 | Measurement of agronomic parameters

The surface area of experimental plots was between 6 and 12 m² across sites of the study. 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 two to three biological replicates per plot. Nondestructive measurements (plant density at emergence Z11, heading and flowering [Z55 and Z65], and spike density during grain filling [Z75]) were carried out using the plot dedicated to final harvest. The final yield was measured at the plot level using a combine harvester. Grain parameters were measured as in Touzy et al. (2019). Grain samples from the combine harvester were used to measure plot grain weight, grain humidity at harvest, grain protein content and 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 and BVG), water balance was followed using soil properties, meteorological data and actual irrigation when applicable.

2.2 | Isotopic analyses

Isotopic analyses were done on leaves collected at anthesis (Z65) and grains at maturity (Z91). Analyses were carried out using oven-dried material ground in fine power and weighted in tin (¹³C) or silver (¹⁸O) capsules. Samples were analysed using an isotope ratio mass spectrometer Isoprime (Elementar) coupled to an elemental analyser (Carlo-Erba) run in combustion (¹³C) or pyrolysis (¹⁸O). Delta value accuracy was checked using IEAE standards USGS-40 (glutamic acid) and IAEA-CH7 (polyethylene) and IAEA-602 (benzoic acid) (¹³C) and ANU sucrose (home standard) (¹⁸O) 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 (¹³C) and V-SMOW (¹⁸O), in ‰. Climatic parameters used for computations (temperature) were from Meteo France stations. $\delta^{18}\text{O}$ values in precipitation in the regions considered are from the database Nucleus (IAEA).

2.3 | Theory and calculations

2.3.1 | Notations and assumptions

The isotope composition ($\delta^{13}\text{C}$ 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 δ_{product} is equal to $\delta_{\text{substrate}} - \Delta$. Symbols associated with carbon amounts are illustrated in Supporting Information: Figure S2. The decomposition in carbon flow and three different stages

(pre-anthesis, early grain filling and late grain filling) in Supporting Information: Figure S2 is from model III in Gent (1994). Symbols associated with isotope compositions and fractionations are listed in Supporting Information: Table S2 and assumptions used in ¹²C/¹³C calculations are summarized in Supporting Information: Table S3. The term 'isotope fractionation' refers to the change in delta value related to the observed isotope effect IE (¹²C/¹³C ratio of velocity) as follows: fractionation = IE - 1. That way, the fractionation is positive when against the heavy (¹³C) 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 (i.e., we neglect second-order terms, see above).

2.3.2 | Flux mass balance

Carbon balance is applied to C amounts as follows:

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

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

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

2.3.3 | Harvest index

The HI is defined as the ratio of grain biomass to total net biomass, which gives, using amounts of carbon:

$$\text{HI} = \frac{\text{graincarbon}}{\text{nettotalcarbon}} = \frac{\Sigma g}{\Sigma A - \Sigma R_g - \Sigma R_m} = \frac{\Sigma g}{V_s + \Delta B + \Sigma g}, \quad (4)$$

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 $\Delta B = 0$, 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.

2.3.4 | 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:

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

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:

$$V_1(\delta_{\text{fix}1} - p_r) = B_1\delta_{B1} + R_{m1}(\delta_{B1} - e_m). \quad (6)$$

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

$$\delta_{B1} = \frac{V_1(\delta_{\text{fix}1} - p_r) + R_{m1}e_m}{B_1 + R_{m1}}. \quad (7)$$

The same procedure was applied to Stages 2 and 3. For Stage 2, it leads to:

$$\delta_{B2} = \frac{V_2(\delta_{\text{fix}2} - p_r) + g_2\beta + R_{m2}e_m}{B_2 + g_2 + R_{m2}}. \quad (8)$$

In Stage 3, we have:

$$\delta_{B3} = \frac{V_3(\delta_{\text{fix}3} - p_r) + B_3\delta_{B2} + g_3\beta + R_{m3}e_m}{g_3 + R_{m3}}, \quad (9)$$

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 (Bathellier et al., 2017; Ghashghaie & Badeck, 2014). Thus, across the full wheat life cycle, overall respiratory fractionation (e_{mg} used thereafter) is likely to be positive ($e_{\text{mg}} > 0$). Different scenarios (from 2.5‰ to 4‰) are shown in Figure 4 in the 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 with flag leaf at anthesis (Kodina, 2010; Merah et al., 2002). 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:

$$\delta_{\text{grain}} = \frac{g_2\delta_{B2} + g_3\delta_{B3} + G\delta_{\text{fix}2}}{g_3 + g_2 + G} - \beta. \quad (10)$$

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

$$\begin{aligned} \delta_{\text{grain}} = & \frac{g_2 + g_3\varphi}{\Sigma g} \cdot \frac{B_1}{B_1 + V_{r2}} \cdot \delta_{\text{fix}1} + \left(\frac{g_2 + g_3\varphi}{\Sigma g} \cdot \frac{V_{r2}}{B_1 + V_{r2}} \right. \\ & \left. + \frac{G}{\Sigma g} \right) \cdot \delta_{\text{fix}2} + \frac{g_3(1 - \varphi)}{\Sigma g} \cdot \delta_{\text{fix}3} \\ & - p_r \cdot \left(\frac{g_2 + g_3\varphi}{\Sigma g} + \frac{g_3(1 - \varphi)}{\Sigma g} \right) + \beta \cdot \left(\frac{g_2 + g_3\varphi}{\Sigma g} \cdot \frac{g_2}{B_1 + V_{r2}} \right. \\ & \left. + \frac{g_3}{\Sigma g} \cdot \frac{g_3}{B_3 + V_{r3}} - 1 \right) \\ & + e_m \cdot \left(\frac{g_2 + g_3\varphi}{\Sigma g} \cdot \frac{B_1 R_{m1}}{B_1 + R_{m1}} + \frac{R_{m2}}{B_1 + V_{r2}} + \frac{g_3}{\Sigma g} \cdot \frac{R_{m3}}{B_3 + V_{r3}} \right), \end{aligned} \quad (11)$$

where $\varphi = B_3/(B_3 + V_{r3})$. Equation (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 $\delta_{\text{fix}1}$, $\delta_{\text{fix}2}$ and $\delta_{\text{fix}3}$ in (11) equals 1 and thus we can simplify notations using the weighted average isotope composition of fixed carbon ($\langle\delta_{\text{fix}}\rangle$). Then we obtain:

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

Equation (14) can be abbreviated as follows:

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

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).

$\langle\delta_{\text{fix}}\rangle$ 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 (Supporting Information: Figure S2). The weighted average of reserves and structural material produced during Stage 1 (full vegetative phase up to anthesis) is given by:

$$\delta_{\text{leaf}} = \frac{\delta_{B1}B_1 + (\delta_{\text{fix}1} - p_s)V_s}{B_1 + V_s}, \quad (16)$$

which gives, using (7):

$$\delta_{\text{leaf}} = \delta_{\text{fix}1} + e_m \cdot \frac{R_{m1}}{B_1 + R_{m1}}\theta - p_s \cdot (1 - \theta), \quad (17)$$

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

$$\begin{aligned} \delta_{\text{grain}} - \delta_{\text{leaf}} \approx & \frac{\langle R_m \rangle}{\Sigma g} \cdot e_m + \frac{\langle R_g \rangle}{\Sigma g} \cdot e_g - \frac{R_{m1}}{B_1 + R_{m1}}\theta \cdot e_m + p_s \\ & \cdot (1 - \theta). \end{aligned} \quad (18)$$

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:

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

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

Equation (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:

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

where

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

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 permil. 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 Supporting Information: 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 (Supporting Information: Figure S3).

2.3.5 | Estimation of $\delta^{13}\text{C}$ in leaves

While measuring the carbon isotope composition in grains is easy because it just requires sampling upon final harvesting, having $\delta^{13}\text{C}$ values of leaves at anthesis is much more demanding, both financially and in personnel. Therefore, we explored the possibility to reconstruct the average $\delta^{13}\text{C}$ value in leaves from (i) observed agronomic variables via machine learning conducted by multivariate analysis conducted by orthogonal projection on latent structure (OPLS) (Eriksson et al., 2008, 2013); (ii) the $\delta^{18}\text{O}$ value observed in grains. These two methods are presented in the Supporting Information: Text. Method (i) involves multivariate statistics which were performed with Simca[®] (Umetrics).

2.4 | Statistics

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

3 | RESULTS

3.1 | Relationship with yield

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

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

To extract valuable information on grain properties from $\delta^{13}\text{C}_{\text{grain}}$, we then used the isotopic difference between grain and leaves ($\Delta\delta$), that is, the apparent grain-to-leaf discrimination. Based on wheat phenology and carbon allocation pattern (see *Theory and calculations* in Section 2), we anticipate a generic relationship between $\Delta\delta$, respiratory loss (R_t) and yield (Y) as (Equations 18–19):

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

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

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

where RUE^* is the net carbon-based respiration use efficiency. Note this equation is written in such a way to make proportionality apparent between $\Delta\delta_{\text{corr}}$ and RUE^*/HI . However, applying (21) and (22) implies prior knowledge of the $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}_{\text{leaf}}$ (Table 1). In the first data set (VAR), leaves were sampled and $\delta^{13}\text{C}_{\text{leaf}}$ was thus measured. In the second data set (BPA), $\delta^{13}\text{C}_{\text{leaf}}$ was reconstructed from agronomic parameters using machine-learning (multivariate analysis with $\delta^{13}\text{C}$ as the quantitative objective variable). In the third data set (BVG), $\delta^{13}\text{C}_{\text{leaf}}$ was estimated from the natural ^{18}O abundance in grains (calculations explained in Supporting Information: Text). This method took advantage of the rather well-conserved $\delta^{18}\text{O}$ difference between leaves and grains, of 2.9‰ (Figure S5a).

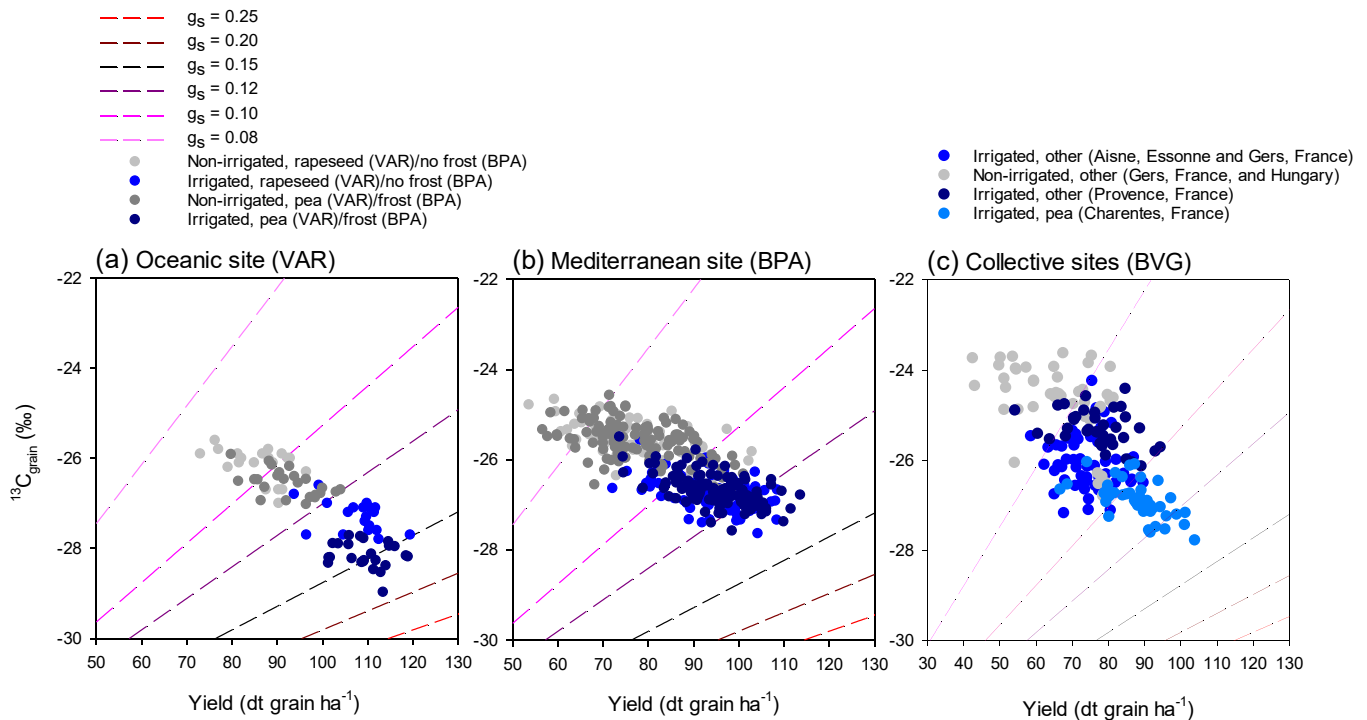


FIGURE 1 Carbon isotope composition in wheat grains expressed in absolute V-PDB scale as a $\delta^{13}\text{C}$. Wheat was cultivated under oceanic (VAR, a), Mediterranean (BPA, b) climatic conditions, or across different sites (BVG, c). Wheat was grown under irrigated or nonirrigated plots, used previously to grow pea or other plants, and experiencing occasional frost or not (BPA). $\delta^{13}\text{C}$ 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 $\text{mol m}^{-2} \text{s}^{-1}$) (Supporting Information: equation S11 in Supporting Information: Material). Note that all data points are not on the same isoline due to variations on average stomatal conductance across growth conditions, however, the relationship between $\delta^{13}\text{C}$ and yield is similar across sites.

Outputs of the multivariate analysis are provided in Figure 2. The statistical model was trained with data set 1 (VAR). It was highly significant ($P_{\text{CV-ANOVA}} = 1.7 \times 10^{-9}$), 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 $\delta^{13}\text{C}_{\text{leaf}}$ values, with a slope extremely close to unity (0.9998) (Figure 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) (Figure 2b). In such a representation, the most important drivers have a VIP value above unity and a high loading. $\delta^{13}\text{C}_{\text{leaf}}$ 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 (Figure 2c). The statistical model was then implemented to calculate $\delta^{13}\text{C}_{\text{leaf}}$ 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‰ (Figure 2d).

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

3.3 | Leaf-to-grain isotope difference

The uncorrected leaf-to-grain $\delta^{13}\text{C}$ difference is shown in Figure 3. Grains were always found to be ^{13}C -enriched compared with leaves, showing the occurrence of a postphotosynthetic 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 where it reached -10‰. As expected from Equation 21, there was a negative hyperbolic relationship between the isotopic difference ($\Delta\delta$) and yield, which fell into the region where the respiratory loss represented about 200 dt $\text{CO}_2 \text{ha}^{-1}$ (isolines in Figure 3) at both Sites 1 and 2 (VAR, BPA). Also, $\Delta\delta$ was affected by irrigation, with smaller postphotosynthetic fractionation ($\approx 1\%$) under irrigation and high yield, reflecting the impact of water limitation on isotope allocation to grains. Although in the same graphical region, data set 3 (BVG) was more scattered due to the much higher imprecision in $\delta^{13}\text{C}_{\text{leaf}}$ estimated from $\delta^{18}\text{O}_{\text{grain}}$.

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 $\Delta\delta$ ($\Delta\delta_{\text{corr}}$) and the ratio $\text{RUE}^{\text{r}}/\text{HI}$ (Equation 22). At both sites (VAR and BPA), $\Delta\delta_{\text{corr}}$ fell within a narrow region delimited by isolines obtained with different respiratory isotope fractionations (from 2.5‰ to 4‰) (Figure 4a,b). It formed a significant linear

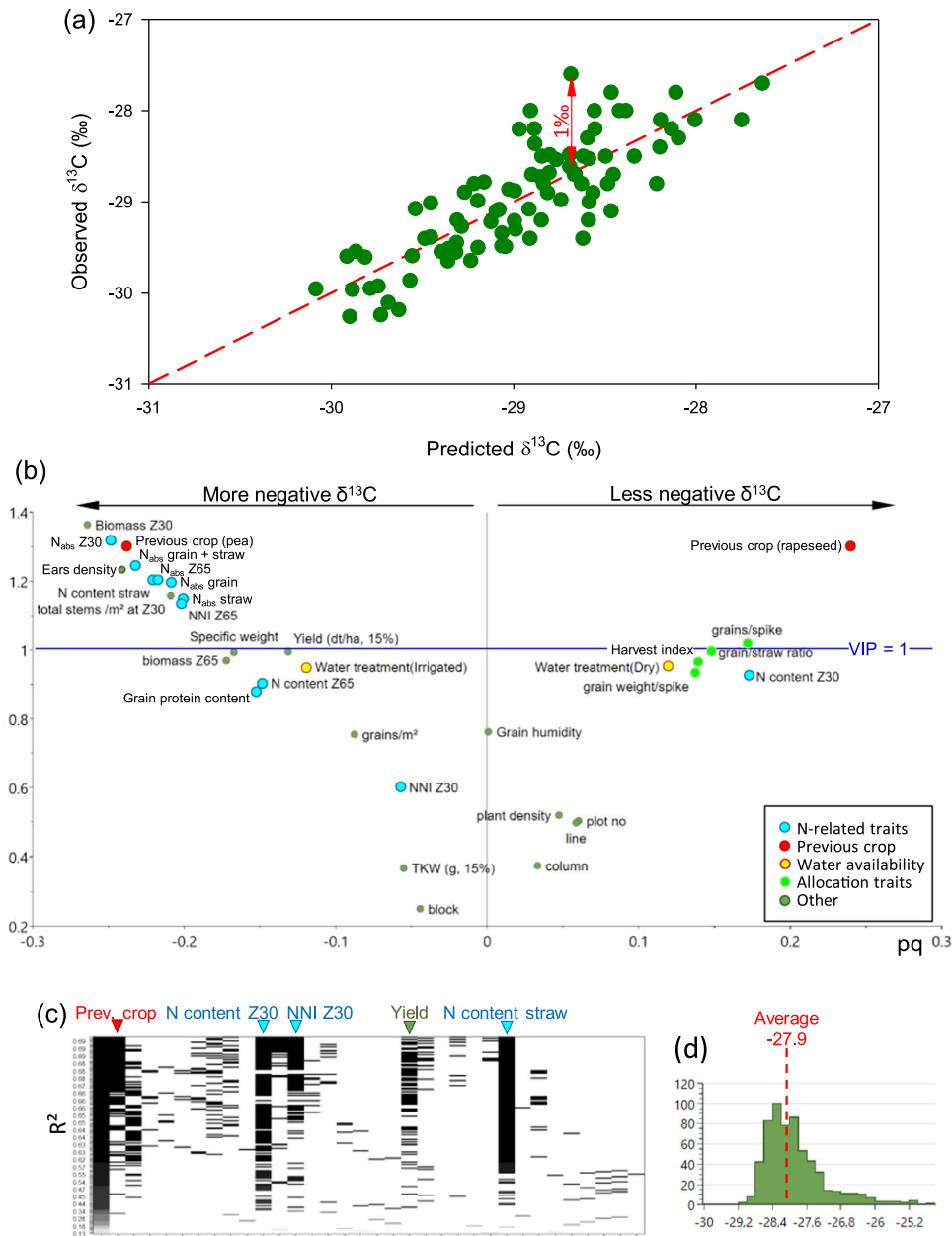


FIGURE 2 Statistical analysis of $\delta^{13}\text{C}$ in leaves using data set 1 (VAR) (a–c) and application of the multivariate model to data set 2 (BPA) to predict $\delta^{13}\text{C}$ values (d). (a) Output of the OPLS model showing the correlation between predicted and observed $\delta^{13}\text{C}$ values. The maximum error made by the model is 1‰ (red arrow). Dashed line, regression line ($y = 0.9998x - 0.00518$; $p < 0.001$; $R^2 = 0.68$). (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 blue horizontal line stands for the usual threshold value used in multivariate analyses (VIP = 1). (c) Output of multiple linear models (sampling of 7 variables among 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 $\delta^{13}\text{C}$ values in leaves for the Mediterranean site (BPA) using a frequency graph, where the average value was found to be -27.9‰ (red).

relationship with $\text{RUE}^{\dagger}/\text{HI}$ (dash-dotted black line). This relationship was not examined using data set 3 (BVG) since biomass, %N and growth data were not available and thus RUE^{\dagger} and $\Delta\delta_{\text{corr}}$ could not be estimated. Interestingly, data points obtained under irrigated and nonirrigated 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.

3.4 | Possible links to grain properties

Under our conditions, the relationship between $\Delta\delta_{\text{corr}}$ and $\text{RUE}^{\dagger}/\text{HI}$ was probably not driven by differences in grain-specific weight (GSW, mass per grain volume) since no difference was found between conditions (Figure 4c,f). It suggests that changes in carbon allocation reflected by RUE^{\dagger} and HI were not associated with different grain size but rather

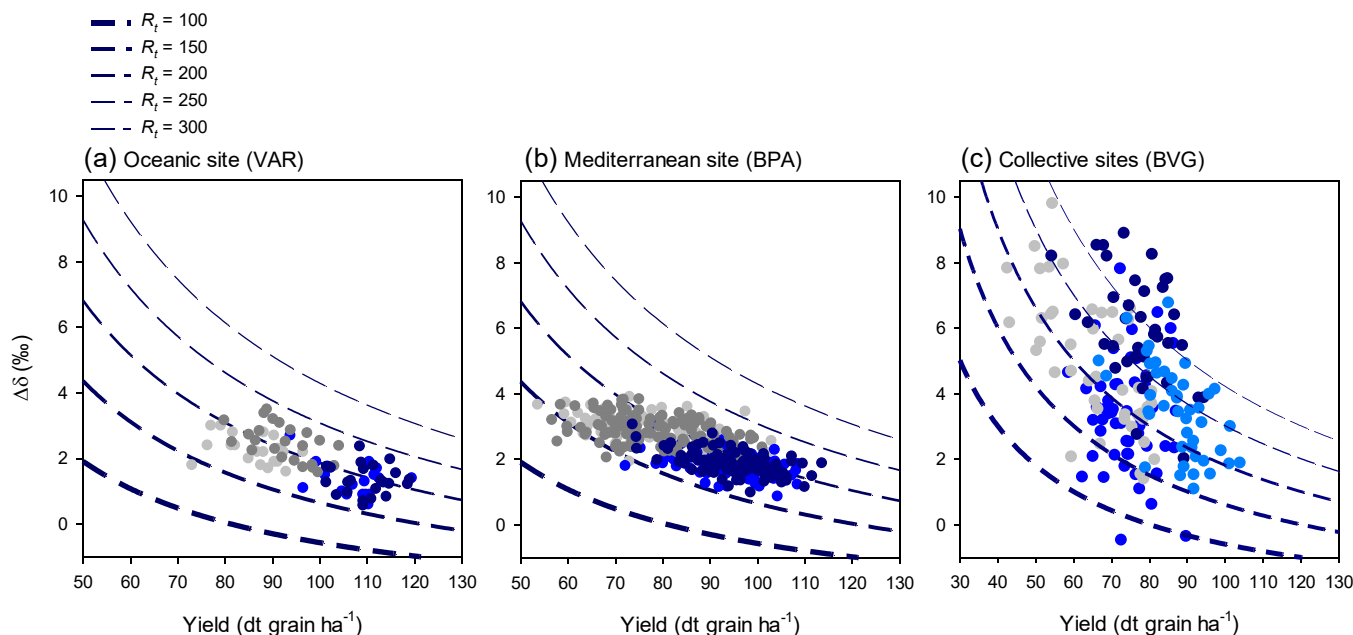


FIGURE 3 Uncorrected $\delta^{13}\text{C}$ difference between grains and leaves, $\Delta\delta$. Same legend as in Figure 1. $\delta^{13}\text{C}$ value in grains was measured and the $\delta^{13}\text{C}$ value in leaves was either measured or estimated from multivariate modelling or observed $\delta^{18}\text{O}_{\text{grain}}$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

changes in total grain set per plant. There were differences in straw or grain nitrogen (%N) across conditions (Figure 4d,e, g,h). Since the nitrogen content was also a determinant of $\delta^{13}\text{C}_{\text{leaf}}$ (see above), N assimilation and remobilisation thus appeared to be an important parameter in $\Delta\delta_{\text{corr}}$ typically via both photosynthetic capacity and grain metabolism.

4 | DISCUSSION

Our results show that the apparent isotope fractionation between leaves (at anthesis) and grain (at maturity), $\Delta\delta_{\text{corr}}$, correlates to parameters associated with respiration use efficiency (RUE^*) and HI in wheat. In addition to giving information on isotope biochemistry beyond photosynthesis (i.e., on grain production itself), it suggests that $\Delta\delta_{\text{corr}}$ 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 the causes of the leaf-to-grain isotope fractionation and possible limitations in using $\Delta\delta_{\text{corr}}$.

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

The relationship between $\Delta\delta_{\text{corr}}$ and RUE^*/HI (Figure 4) is simply due to isotopic mass-balance, that is, consequences of respiratory loss and biomass production, which are accompanied by isotope effects (represented by fractionation factors e_{mg} and p , respectively). In

practice, this means that wheat lines associated with low respiratory loss and high investment into grain biomass should be naturally less ^{13}C -enriched due to a lower relative impact of postphotosynthetic isotope fractionations.

The proportionally lower respiration in highly performing (i.e., associated with high yield) wheat lines has effectively been observed experimentally (McCullough & Hunt, 1989). We recognise that our interpretation of variations in $\Delta\delta_{\text{corr}}$ 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 Figure 4, along with variation in e_{mg} itself. Still, it is striking that nearly all of the data points are within the graphical region defined by $e_{mg} = 2\text{‰}$ and 4‰ (Figure 4).

The fact that carbon partitioning translates into changes in $\Delta\delta_{\text{corr}}$ is also likely associated with changes in nitrogen partitioning: Firstly, $\delta^{13}\text{C}_{\text{leaf}}$ was found to depend on leaf N content and availability (Figure 2); Secondly, some changes were observed in grain or straw N content between conditions (Figure 4); Third, nitrogen availability has a well-known effect on carbon partitioning (including the shoot-root ratio) and respiration in wheat (Allard et al., 2013; Cox et al., 1986; Hay, 1995; Kichey et al., 2007; Pearman et al., 1981; Sinclair, 1998). 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, $\Delta\delta_{\text{corr}}$ would probably appear to be less strongly related to RUE^*/HI if fertilisation regimes had been extremely different between sites or plots (e.g., no fertilisation at all and more than 250 kg N ha^{-1}).

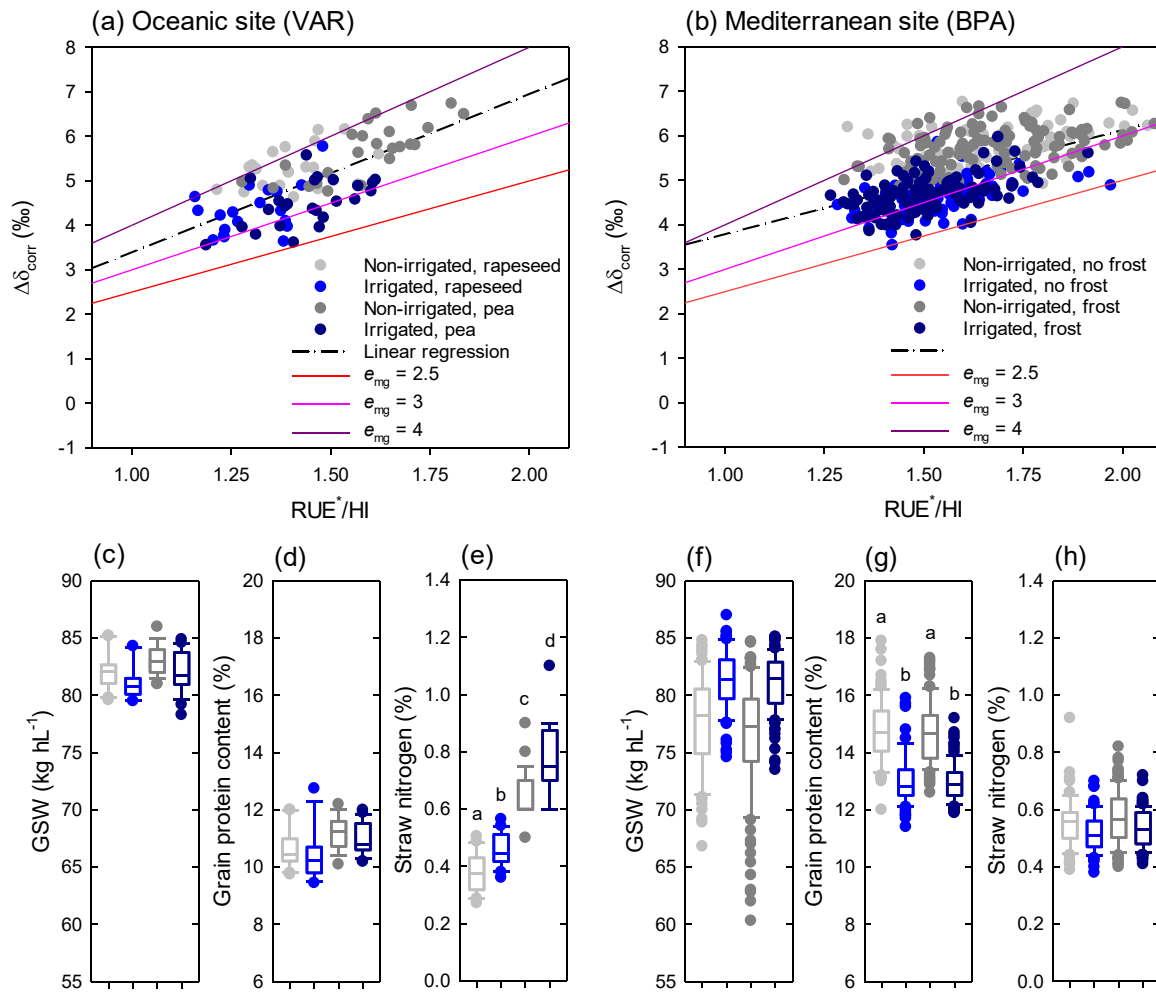


FIGURE 4 Corrected isotope leaf-grain difference $\Delta\delta_{\text{corr}}$ and physiological properties of grain and straw in wheat cultivated at Sites 1 (VAR) and 2 (BPA) (same symbols as in Figure 1). (a,b) $\Delta\delta_{\text{corr}}$ plotted against estimated respiration use efficiency-to-harvest index ratio, RUE^*/HI . Coloured lines stand for isolines (expected proportionality relationships) with respiratory fractionation e_{mg} equal 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 hectolitre. (d,g) Grain protein content, in % weight. (e,h) Nitrogen elemental content in straw. Letters stand for statistical classes (ANOVA, $p < 0.01$).

Importantly, the relationship between $\Delta\delta_{\text{corr}}$, RUE^*/HI did not depend much on water relations. In effect, while the response of $\delta^{13}\text{C}_{\text{grain}}$ to yield was mostly driven by water availability and thus conductance and thus photosynthetic conditions as in Merah, Deléens, Al Hakimi, et al. (2001a) and Merah, Deléens, & Monneveux, (2001b) (Figure 1), irrigation had a rather small effect on the relationship between $\Delta\delta_{\text{corr}}$ and RUE^*/HI , all data points being apparently on the same line (Figure 4). For example, under oceanic climate (site VAR), the average offset between irrigated and nonirrigated conditions was c. 0.7‰ only (Figure 4a) and was statistically insignificant. Although nonirrigated conditions impacted on overall allocation to grains and thus the HI, there was little effect on fractionation factors and as a result, data points 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).

4.2 | Advantages and disadvantages of the present theoretical background

The theoretical background used here to obtain a relationship between $\Delta\delta_{\text{corr}}$ 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 (i.e., $\Delta B = 0$ in Equation 4). This assumption is likely not critical since wheat straw is effectively very poor in nonstructural 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, $\langle \delta_{\text{fix}} \rangle$, 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% (Gebbing et al., 1999; Gent, 1994; Schnyder, 1993). In addition, if photosynthetic properties of leaves were to vary significantly (with typically lower intercellular-to-atmospheric CO_2 mole fraction ratio, 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 with $\langle \delta_{\text{fix}} \rangle$. This isotopic difference would add in the intercept of the relationship in (19) and Figure 4, not in the slope, and therefore the correlation between $\Delta\delta_{\text{corr}}$ and RUE^*/HI would remain valid, with some noise due to variations in $\langle \delta_{\text{fix}} \rangle$. In other words, our assumption $\langle \delta_{\text{fix}} \rangle \approx \delta_{\text{fix}1}$ probably explains some of the scattering but cannot explain the linear relationship in Figure 4. It is also possible that the small offset between irrigated and nonirrigated conditions (blue vs. grey points in Figure 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 nonnegligible contribution to grain organic matter (Araus et al., 1993; Gebbing & Schnyder, 2001). Taken as a whole, variations in overall photosynthetic isotopic input are probably modest and cannot alter the relationship described here.

We also recognise that although convenient, Equation (22) is an abbreviated formulation of the full expression describing postphotosynthetic 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. Also, a generic respiratory fractionation (here denoted as e_{mg}), which encapsulates weighted maintenance and growth respiration parameters, seems to be sufficient to model $\Delta\delta_{\text{corr}}$. In addition, the apparent value of e_{mg} adapted to linear fitting (between 2.5‰ and 4‰) (Figure 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 (Bathellier et al., 2009, 2017; Ghashghaie & Badeck, 2014; Klumpp et al., 2005).

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

The observed robustness of the relationship between $\Delta\delta_{\text{corr}}$ 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 $\Delta\delta_{\text{corr}}$ 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, quantitative trait loci (QTL) discovery or genome-wide association studies (GWAS) to identify key markers associated with yield or HI (Porker et al., 2020; Pradhan et al., 2019; Quintero et al., 2018; M. P. Reynolds et al., 2017; Rivera-Amado et al., 2019). Domesticated high-yielding dwarf varieties with changed carbon (as well as nitrogen and sulphur) allocation, show changes in GSW (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 $\Delta\delta_{\text{corr}}$ 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 $\Delta\delta_{\text{corr}}$ - RUE^*/HI relationship changes minimally with growth conditions. When $\delta^{13}\text{C}_{\text{leaf}}$ at anthesis is not available to compute $\Delta\delta_{\text{corr}}$, our study further suggests it can be estimated with multivariate analysis or $\delta^{18}\text{O}$. It is now well-accepted that there is good heritability of $^{12}\text{C}/^{13}\text{C}$ isotope composition not only in leaves but also in grains (Merah, Deléens, Al Hakimi, et al., 2001a). Taken as a whole, we suggest that interesting allocation properties can be accessed via $\Delta\delta_{\text{corr}}$, which gives direct information on events during grain filling and thus may help isotope-based implementation of genotype selection.

ACKNOWLEDGMENTS

The authors thank Hilary Stuart-Williams (ANU Isotope Facility) for $^{18}\text{O}/^{16}\text{O}$ isotope analyses. This study was supported by the *Région Pays de la Loire* and *Angers Loire Métropole* via the program *Connect Talent* Ioseed and the University of Angers via a PhD grant to Jean-Baptiste Domergue. 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). Open access publishing facilitated by Australian National University, as part of the Wiley - Australian National University agreement via the Council of Australian University Librarians.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Original numerical data are available on request. Metadata are provided in Supporting Information Material.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Domergue, J.-B., Abadie, C., Lalande, J., Deswarte, J.-C., Ober, E., Laurent, V. et al. (2022) Grain carbon isotope composition is a marker for allocation and harvest index in wheat. *Plant, Cell & Environment*, 1–13.
<https://doi.org/10.1111/pce.14339>