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Carbon Isotope Discrimination: A Gateway to Smarter Water Use and Drought-resilient Cereals

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Abstract

Carbon isotope discrimination ($\Delta^{13}C$) is widely used as an integrative physiological trait for assessing intrinsic water use efficiency (iWUE) in cereals under water-limited conditions. This review synthesizes the physiological basis linking $\Delta^{13}C$ to iWUE through intercellular-to-atmospheric CO_2 ratios (c_i/c_a), emphasizing stomatal conductance, photosynthetic capacity, and genotype × environment interactions. Environmental drivers, including drought, temperature, vapor pressure deficit, and atmospheric CO_2, are evaluated alongside differences between C_3 and C_4 cereals. Analytical approaches such as isotope ratio mass spectrometry (IRMS), near-infrared spectroscopy (NIRS), and laser-based methods are compared in terms of precision and scalability. Evidence shows a strong inverse relationship between $\Delta^{13}C$ and iWUE in C_3 species, while C_4 responses are weaker due to CO_2-concentrating mechanisms. Despite limitations related to environmental sensitivity and inconsistent trait-yield relationships, integrating $\Delta^{13}C$ with complementary traits, multi-environment testing, and genomic tools enhances its application in breeding. $\Delta^{13}C$ remains a valuable tool for developing drought-resilient cereal ideotypes.

Keywords

Wheat; drought tolerance; C_3 photosynthesis; crop improvement; climate resilience; carbon isotopic signatures

1. Introduction

Cereal production faces increasing pressure from water scarcity, drought recurrence, and climate variability, which threaten crop stability and global food security [1, 2]. Cereals, including wheat, rice, and maize, constitute the primary staple crops for a large proportion of the world's population [3], highlighting the urgent need to enhance their productivity, especially in drought-prone areas. Crop improvement for drought resilience increasingly depends on physiological traits that can reliably link plant function to yield under field conditions. However, the limited availability of high-throughput physiological traits continues to constrain progress in breeding drought-resilient cereals.

Enhancing water use efficiency (WUE), defined as the ratio of biomass or yield produced per unit of water consumed [4, 5], has become a central objective in crop physiology and breeding programs targeting drought-prone environments. Water use efficiency represents the integrated outcome of stomatal regulation, photosynthetic carbon assimilation, and canopy water loss. This complexity makes direct selection for WUE challenging in breeding populations. Therefore, reliable physiological indicators that quantify and predict WUE are essential for accelerating the development of drought-resilient cultivars.

Among these indicators, carbon isotope-based traits have gained prominence due to their ability to integrate plant physiological processes over time. Carbon occurs naturally as stable isotopes, primarily ^{12}C (≈98.9%) and ^{13}C

(≈1.1%) [6], which differ slightly in atomic mass and bonding behaviour, enabling isotopic fractionation during diffusion and enzymatic reactions. These isotopes originate from atmospheric CO_2, which serves as the primary carbon source for photosynthesis, and their differential behaviour during physical and biochemical processes forms the basis for their use as tracers of plant physiological function. During photosynthesis, enzymatic reactions preferentially fix ^{12}C over ^{13}C, resulting in measurable variation in plant isotopic composition [7]. Carbon isotope discrimination ($\Delta^{13}C$) exploits this fractionation and is widely used as a physiological indicator of plant water use efficiency, as it reflects the balance between stomatal conductance and photosynthetic carbon assimilation. Carbon isotopic signatures ($\delta^{13}C$) represent the relative deviation in the $^{13}C/^{12}C$ ratio of plant material compared to an international standard, expressed in per mil (‰) [8, 9], and provide a time-integrated measure of plant physiological behaviour over the period of carbon assimilation. Unlike instantaneous gas-exchange measurements, $\delta^{13}C$ captures cumulative physiological responses to environmental conditions.

The relationship between $\Delta^{13}C$ and iWUE is grounded in the Farquhar model of photosynthetic fractionation, which links isotopic discrimination to internal CO_2 dynamics [10-12]. This model expresses $\Delta^{13}C$ as a function of the ratio between intercellular and atmospheric CO_2 concentrations (c_i/c_a), which reflects the balance between CO_2 supply through stomata and biochemical demand during photosynthesis. The $\Delta^{13}C$ increases with c_i/c_a, reflecting greater stomatal openness and lower iWUE. The iWUE is defined as the ratio of net photosynthesis (A) to stomatal conductance (g_s), which directly links carbon gain to water loss [13]. This framework positions $\Delta^{13}C$ as an indirect proxy for A/g_s, particularly when stomatal conductance represents the primary limitation to internal CO_2 diffusion. However, the strength of this proxy relationship depends on species, environment, and photosynthetic pathway [14].

C_3 cereals such as wheat, rice, and barley fix atmospheric CO_2 directly through Rubisco-mediated carboxylation, which results in strong isotopic fractionation and a generally inverse relationship between $\Delta^{13}C$ and iWUE [15-17]. This relationship supports the use of $\Delta^{13}C$ as a selection trait in C_3 breeding programs, especially under water-limited conditions. However, physiological complexity within C_3 systems introduces variability, as mesophyll conductance, sink limitation, and stress-induced metabolic constraints can decouple $\Delta^{13}C$ from stomatal behaviour. C_4 cereals such as maize and sorghum exhibit fundamentally different carbon assimilation pathways. Carbon dioxide is initially fixed by phosphoenolpyruvate carboxylase in mesophyll cells and subsequently concentrated in bundle sheath cells, where Rubisco operates. This mechanism modifies isotopic responses by introducing additional biochemical control over internal CO_2 concentration. Bundle sheath leakiness (Φ), internal recycling of CO_2, and compartmentalized metabolism further weaken the direct linkage between $\Delta^{13}C$ and c_i/c_a, resulting in weaker and more genotype-dependent relationships with iWUE.

Environmental conditions exert strong control over isotopic discrimination through their effects on stomatal conductance and photosynthetic activity [18, 19]. Drought stress reduces soil water availability, leading to stomatal closure, reduced internal CO_2 concentration, and lower $\Delta^{13}C$ values, which typically correspond to higher iWUE. Temperature and humidity influence vapor pressure deficit, thereby modifying transpiration demand and stomatal behaviour. Nutrient availability, particularly nitrogen, alters photosynthetic capacity and shifts the balance between CO_2 supply and demand. In addition to environmental factors, the plant developmental stage also influences isotopic composition. Leaf tissues reflect short-term physiological conditions, stem tissues integrate intermediate growth periods, and grain tissues represent cumulative assimilation during reproductive development [5, 14, 20]. Such temporal integration improves stability of the trait but reduces sensitivity to short-term environmental fluctuations.

Genetic variation in $\Delta^{13}C$ arises from differences in stomatal density, leaf anatomy, photosynthetic capacity, and carbon allocation patterns [21]. Moderate to high heritability across cereal species suggests strong genetic control and potential for selection [22]. This genetic basis has enabled the use of $\Delta^{13}C$ as an indirect selection trait for drought adaptation, particularly in wheat breeding programs where grain isotopic composition has been associated with yield under water-limited conditions. However, genotype-by-environment interactions frequently modify trait expression, resulting in inconsistent relationships between $\Delta^{13}C$, iWUE, and yield across environments [5]. Such instability limits the predictive power of $\Delta^{13}C$ unless combined with complementary physiological and agronomic traits.

Accurate measurement of $\Delta^{13}C$ depends on analytical precision and appropriate calibration. Isotope ratio mass spectrometry (IRMS) remains the reference method due to its high accuracy and reproducibility. Near-infrared spectroscopy (NIRS) and laser-based spectroscopy provide higher throughput alternatives but require calibration against IRMS datasets [23]. The trade-off between analytical precision and scalability represents a key constraint for deploying $\Delta^{13}C$ in large breeding populations. Despite significant advances, research on $\Delta^{13}C$ in cereals remains fragmented across physiological, ecological, and breeding disciplines, limiting integration across scales. Major unresolved challenges include the lack of standardized phenotyping protocols, incomplete mechanistic understanding

of $\Delta^{13}C$ regulation in C_4 cereals, and inconsistent translation of isotopic signals into stable agronomic outcomes across environments. This review critically synthesizes current knowledge on carbon isotope discrimination in cereals, focusing on physiological mechanisms, methodological advances, and breeding applications.

2. Principles of Carbon Isotope Discrimination

Carbon isotope discrimination is defined as the preferential fixation of the lighter carbon isotope (^{12}C) over ^{13}C during photosynthesis [10, 24, 25], resulting in measurable variation in plant isotopic composition that reflects internal CO_2 dynamics and stomatal regulation. Isotopic fractionation occurs during two main physiological steps: diffusion of CO_2 from the atmosphere into the leaf through stomata and enzymatic carboxylation during photosynthesis [26]. Diffusion through stomata favours the lighter isotope because ^{12}C diffuses slightly faster than ^{13}C [27]. Additional fractionation occurs during carboxylation, where photosynthetic enzymes preferentially fix ^{12}C over ^{13}C [28]. These processes form the basis of the Farquhar, von Caemmerer, and Berry model, which provides a quantitative framework linking isotopic discrimination to internal CO_2 dynamics [12].

$$\Delta^{13}C = a + (b - a)\left(\frac{c_i}{c_a}\right)$$

In this model, $\Delta^{13}C$ is expressed as a function of the ratio of intercellular to atmospheric CO_2 concentration (c_i/c_a), where a (~4.4‰) represents fractionation during diffusion and b (~27‰) represents fractionation during carboxylation. This formulation links isotopic discrimination to the balance between CO_2 diffusion into the leaf and biochemical fixation by Rubisco. Higher c_i/c_a values reflect greater stomatal openness relative to assimilation capacity, reinforcing the sensitivity of $\Delta^{13}C$ to internal CO_2 dynamics. Lower c_i/c_a values arise when stomatal conductance is restricted, or carboxylation demand is high, resulting in reduced $\Delta^{13}C$ and increased iWUE.

$$iWUE = \frac{A}{g_s}$$

where iWUE = Intrinsic water use efficiency, A = net photosynthesis, and g_s = stomatal conductance. This ratio represents the efficiency with which a plant assimilates carbon relative to water lost through stomatal conductance at the leaf level. Importantly, iWUE differs from whole-plant or agronomic WUE, which is defined as biomass or yield produced per unit of water consumed [29]. While WUE integrates canopy processes, soil evaporation, and plant growth over time [30], iWUE isolates leaf-level physiological processes regulating gas exchange. The theoretical relationship between $\Delta^{13}C$ and iWUE arises through their common dependence on c_i/c_a. $\Delta^{13}C$ is positively related to c_i/c_a, while iWUE is inversely related to c_i/c_a through stomatal control of gas exchange. Therefore, $\Delta^{13}C$ serves as an integrated proxy for iWUE, especially when stomatal conductance is the dominant limitation to photosynthesis and internal CO_2 diffusion.

Variation within C_3 cereals is largely associated with differences in physiological traits that regulate internal CO_2 concentration during assimilation. C_3 species often show a strong inverse relationship between $\Delta^{13}C$ and iWUE (Table 2), which has supported its application in physiological studies and breeding programs. However, this relationship is less consistent in C_4 cereals due to additional biochemical and anatomical controls over CO_2 concentration [7, 31]. In these species, initial CO_2 fixation by phosphoenolpyruvate carboxylase and subsequent concentration in bundle sheath cells modifies isotopic responses [32]. Processes such as bundle sheath leakiness and variable internal carbon cycling decouple c_i/c_a from $\Delta^{13}C$, weakening its reliability as a proxy for iWUE.

Therefore, $\Delta^{13}C$ may become unreliable as a proxy for iWUE when the coupling between stomatal conductance and photosynthesis is disrupted, especially under severe stress or metabolic limitations to photosynthesis. Such conditions include extreme environmental stress, metabolic limitations to photosynthesis, or species-specific carbon concentrating mechanisms, particularly in C_4 crops. Environmental and physiological factors further modify $\Delta^{13}C$ by influencing both CO_2 diffusion and fixation processes [33]. Water availability plays a particularly important role, as reduced soil moisture limits stomatal conductance and restricts CO_2 diffusion into the leaf. Temperature and light intensity affect enzymatic activity and photosynthetic rates [34], while nutrient availability, particularly soil nitrogen, influences photosynthetic capacity through effects on RuBisCO concentration and leaf metabolism [35]. These interacting processes demonstrate that $\Delta^{13}C$ represents an integrated physiological signal over time rather than a direct measurement of instantaneous gas exchange. It specifically reflects iWUE, not whole-plant WUE, which incorporates total transpiration, canopy architecture, and biomass accumulation [30, 36]. Therefore, complementary measurements and multi-environment evaluations are required to ensure reliable physiological interpretation.

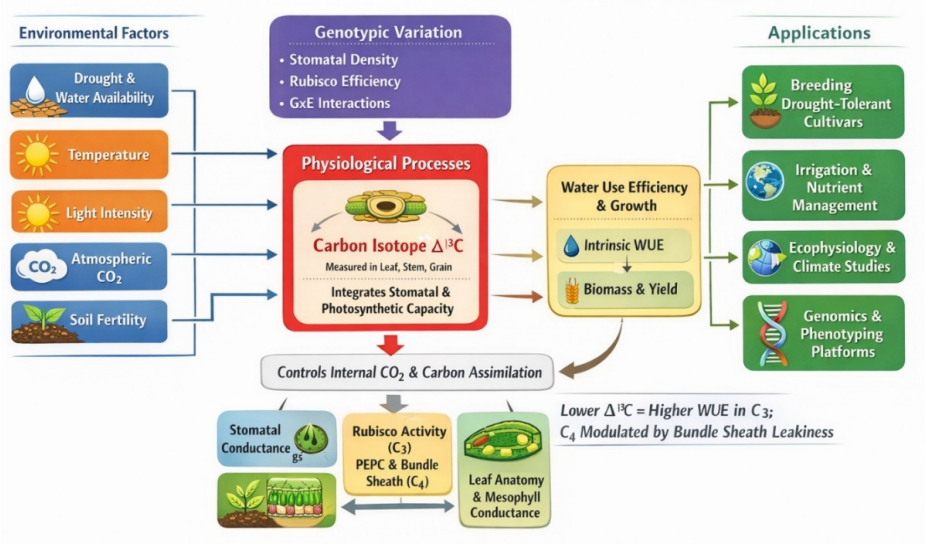

Figure 1. The schematic diagram demonstrates the relationships among environmental factors, physiological processes, genotypic variation, and applied outcomes in cereals, with carbon isotope discrimination ($\Delta^{13}C$) as a central integrative trait. PEPC = phosphoenolpyruvate carboxylase activity; G x E = genotype × environment interactions. (Source: [10-12]).

3. Measurement Techniques

Accurate determination of carbon isotope discrimination ($\Delta^{13}C$) requires appropriate sampling strategies and reliable analytical methods. The selection of plant tissue strongly influences interpretation because isotopic composition varies across plant organs and developmental stages [20]. Leaf tissues are commonly used because they reflect recent photosynthetic activity and short-term environmental conditions [37]. However, leaf $\delta^{13}C$ exhibits higher temporal variability compared to reproductive tissues [20], which may reduce its reliability for estimating season-long iWUE.

Stem and grain tissues integrate carbon assimilation over longer developmental periods [38, 39], providing a more stable representation of physiological behaviour. Grain tissue is particularly important in cereal studies because it integrates carbon assimilation during grain filling and shows strong associations with final yield performance [20]. Empirical studies in wheat have demonstrated that grain $\delta^{13}C$ shows a stronger correlation with yield than leaf $\delta^{13}C$ measurements [40-42, 20], highlighting its value in breeding applications. Therefore, the choice of tissue must align with the research objective, as different tissues capture distinct temporal windows of plant physiological behaviour.

Furthermore, the developmental stage affects isotopic composition because physiological activity varies between vegetative growth and grain filling [43]. Environmental variability during the growing season generates variation in isotopic signatures even within the same field [44]. Replicated sampling across genotypes, environments, and seasons is required to capture genotype-by-environment interactions influencing $\Delta^{13}C$ expression. Sample preparation typically involves drying, grinding, and controlled storage to prevent alteration of isotopic composition. Standardization of sampling protocols is particularly critical for ensuring reproducibility and enabling comparisons across breeding trials and environments.

Several analytical techniques are available for determining $\Delta^{13}C$, including isotope ratio mass spectrometry, near-infrared spectroscopy, and laser-based spectroscopy[45, 46]. Among these methods, IRMS provides the highest analytical precision and serves as the benchmark for calibration of alternative techniques [47] (Table 1). In IRMS, plant material is combusted to produce CO_2, and isotopic ratios are measured relative to international standards with very high analytical precision. High analytical precision makes IRMS the gold standard for physiological studies and calibration of alternative methods. However, high cost, low throughput, and reliance on specialized infrastructure limit its use in large breeding populations where thousands of samples require analysis (Table 1).

Alternative analytical approaches have been developed to improve measurement efficiency and accessibility. Laser-based spectroscopy enables rapid analysis of isotopic composition by measuring CO_2 derived from plant material or gas exchange systems [48]. Near-infrared spectroscopy (NIRS) enables high-throughput estimation once calibrated against IRMS datasets, allowing rapid screening of large breeding populations. Studies have reported that NIRS can predict $\delta^{13}C$ with moderate to high accuracy (R^2 often ranging from ~0.6 to 0.8) when calibration datasets

are used [49-52], making it particularly suitable for large-scale phenotyping. Prediction accuracy in NIRS depends on the quality and representativeness of calibration datasets [53, 54], which require continuous updating to maintain reliability across environments and genotypes. Comparative studies indicate that while IRMS provides the highest precision, NIRS offers the greatest throughput, and laser-based methods provide a balance between speed and analytical resolution (Table 1). These differences influence their suitability for estimating iWUE, with IRMS preferred for mechanistic studies and NIRS for screening applications.

The selection of analytical techniques should be guided by the study scale, objective, and available resources. IRMS is most appropriate for detailed physiological investigations, calibration of alternative methods, and studies requiring high precision. However, NIRS is better suited for high-throughput phenotyping where rapid screening is required despite reduced precision. Laser-based methods provide an intermediate solution, offering improved speed and reduced preparation requirements, although their application remains limited by instrument availability and standardization challenges. The trade-off between analytical precision and throughput represents a major constraint in integrating $\Delta^{13}C$ into breeding programmes. While IRMS provides reliable discrimination among genotypes, its cost and processing time limit scalability, making it impractical for early-generation selection. Conversely, NIRS enables rapid and cost-effective screening [55], but may fail to detect subtle genotypic differences if calibration is inadequate. Therefore, a tiered analytical strategy has been proposed, where IRMS is used to generate high-quality reference datasets for calibration, followed by NIRS-based screening of large populations. Such integration improves the feasibility of using $\Delta^{13}C$ as a proxy for iWUE in breeding pipelines.

Table 1. Comparison of analytical methods for measuring carbon isotope discrimination in plants

Feature	Isotope Ratio Mass Spectrometry	Laser-Based Spectroscopy	Near-Infrared Spectroscopy
Cost	Very high (instrumentation, maintenance, and consumables)	High (specialized optics and calibration systems)	Moderate (relatively low operational cost after calibration)
Operating principle	Measurement of $^{13}C/^{12}C$ isotope ratios in CO_2 after combustion and ion separation based on mass-to-charge ratio	Optical absorption of CO_2 isotopologues using tunable laser or cavity ring-down spectroscopy to determine isotopic composition in gas phase	Indirect estimation of isotopic composition based on absorption of near-infrared radiation (700–2500 nm) associated with chemical bonds in plant tissue
Measurement type	Destructive (combustion of plant material to CO_2 gas)	Non-destructive to sample; requires gas-phase CO_2 extraction or coupling to gas exchange systems	Non-destructive (intact or minimally processed plant tissue)
Precision and accuracy	Extremely high (reference standard method for $\delta^{13}C$ and $\Delta^{13}C$ determination)	High (comparable to IRMS under controlled conditions, but method-dependent)	Moderate to low (indirect estimation; dependent on calibration robustness and dataset quality)
Sample preparation	Intensive (drying, grinding, weighing, encapsulation)	Moderate (gas sampling, system calibration, controlled airflow conditions)	Minimal (drying optional; often requires grinding for improved prediction accuracy)
Speed/throughput	Low to moderate throughput (laboratory-based batch analysis)	Rapid to real-time measurements (especially in gas exchange systems)	Very high throughput (suitable for large breeding populations)
Primary application	High-precision physiological and ecological research; calibration of other methods	Field-based physiological studies, real-time gas exchange and isotope flux analysis	High-throughput phenotyping and screening in breeding programs
Strengths	Highest analytical precision and standardization	Combines isotopic and physiological measurement under dynamic conditions	Scalable, cost-effective for large datasets
Limitations	Expensive, slow, requires specialized infrastructure	Sensitive to calibration and environmental interference	Requires strong calibration against IRMS reference datasets

4. Relationship with Water Use Efficiency

4.1 Theoretical Link between $\Delta^{13}C$ and Intrinsic Water Use Efficiency

Carbon isotope discrimination ($\Delta^{13}C$) integrates the interaction between carbon assimilation and stomatal conductance, providing a quantitative link to iWUE [56]. This relationship is governed by the intercellular-to-atmospheric CO_2 ratio (c_i/c_a), which captures the coordination between stomatal CO_2 supply and photosynthetic demand. The Farquhar model provides a quantitative description linking $\Delta^{13}C$ to c_i/c_a [56], thereby connecting isotopic

discrimination to internal carbon dynamics.

Water-limited conditions promote partial stomatal closure, which reduces c_i and decreases discrimination against ^{13}C, resulting in lower $\Delta^{13}C$ values. This shift corresponds to increased iWUE because carbon assimilation is maintained while stomatal water loss is reduced. C_3 cereals show strong sensitivity of RuBisCO-mediated fractionation to internal CO_2 concentration [57], supporting the use of $\Delta^{13}C$ as a proxy for iWUE. However, C_4 cereals exhibit partial decoupling of $\Delta^{13}C$ from c_i/c_a due to the presence of a CO_2-concentrating mechanism. CO_2 is initially fixed by phosphoenolpyruvate carboxylase in mesophyll cells and subsequently concentrated in bundle sheath cells around RuBisCO [59, 60]. Isotopic discrimination is therefore influenced by both stomatal conductance and internal carbon cycling. Bundle sheath leakiness (Φ), defined as the proportion of CO_2 that diffuses back to the mesophyll, introduces additional variability and weakens the relationship between $\Delta^{13}C$ and iWUE. Importantly, $\Delta^{13}C$ provides a time-integrated signal that enables estimation of seasonal iWUE across plant tissues [5]. Environmental drivers such as vapor pressure deficit, temperature, and soil water availability alter both stomatal behaviour and photosynthetic capacity, modifying the strength of the relationship between $\Delta^{13}C$ and iWUE. Therefore, predictive value depends on the stability of the relationship between c_i/c_a and carbon assimilation across environments and genotypes.

4.2 Empirical Evidence from Crops and Natural Systems

Empirical studies consistently report a negative relationship between $\Delta^{13}C$ and WUE [20, 61]. However, the strength and stability of this relationship vary across environments, genotypes, and measurement scales [20]. The grain $\Delta^{13}C$ has been widely used to identify wheat genotypes with improved drought performance [61], where lower $\Delta^{13}C$ values generally indicate higher iWUE. Reported correlations range from moderate to strong in C_3 cereals [61, 63, 64], depending on environmental conditions and experimental design**.** Rice studies show similar trends, although flooded versus aerobic systems can weaken associations [64]. Multi-environment studies demonstrate that $\Delta^{13}C$ captures cumulative effects of soil moisture, temperature, and evaporative demand [65]. However, cross-environment comparisons reveal that the $\Delta^{13}C$ and iWUE relationship is not universally stable. This highlights reduced predictive power under non-water-limited conditions or when biochemical limitations constrain photosynthesis.

Studies in natural ecosystems confirm that $\Delta^{13}C$ reflects species-specific water-use strategies and adaptation to rainfall variability [66-69]. Comparisons between irrigated and rainfed systems show consistent shifts in $\Delta^{13}C$, although the magnitude varies with environmental intensity and crop type [20]. Studies in mixed cropping systems further indicate that $\Delta^{13}C$ can reflect interspecific competition for water resources [70, 71]. Integration of $\Delta^{13}C$ with complementary measurements, such as gas exchange and canopy-level traits, improves interpretation and strengthens its predictive value for iWUE.

4.3 Relationship between $\Delta^{13}C$ and Transpiration Coefficient

Transpiration coefficient (TC) is the amount of water transpired per unit biomass produced over a specific period [71]. It provides a whole-plant measure of WUE that complements iWUE. While iWUE reflects leaf-level physiological processes, TC integrates transpiration and biomass accumulation over the crop growth period [72]. The reduced rate of transpiration is associated with higher iWUE and lower $\Delta^{13}C$ values. However, this relationship partly reflects shared regulation by stomatal conductance, carbon assimilation, and canopy-level processes. Therefore, $\Delta^{13}C$ is more suitable for assessing physiological efficiency, whereas TC provides a broader measure of WUE at the whole-plant level.

The relationship between $\Delta^{13}C$ and TC is grounded in their shared dependence on stomatal conductance and internal CO_2 concentration (c_i/c_a). Reduced stomatal conductance under water limitation decreases c_i, resulting in lower $\Delta^{13}C$ values and increased iWUE. Concurrently, reduced stomatal aperture limits transpirational water loss, which may reduce TC when biomass production is relatively maintained. This establishes a mechanistic linkage whereby genotypes with lower $\Delta^{13}C$ often exhibit improved transpiration efficiency at the whole-plant level. However, the $\Delta^{13}C$ and TC relationship is indirect and modulated by multiple physiological and structural factors. Canopy architecture influences radiation interception and transpiration demand, while soil evaporation contributes to total water loss but is not captured by $\Delta^{13}C$. Growth duration and phenological variation further affect TC through cumulative transpiration differences independent of leaf-level gas exchange. In addition, carbon allocation patterns influence biomass accumulation, thereby altering TC without necessarily changing isotopic discrimination.

4.4 Factors Modulating the $\Delta^{13}C$ and WUE Relationship

The reliability of $\Delta^{13}C$ as a proxy for iWUE is conditional and strongly influenced by environmental, physiological,

and genetic factors [20]. For instance, vapor pressure deficit modifies stomatal behaviour by increasing atmospheric demand for water, which influences transpiration and carbon assimilation dynamics [73]. Elevated vapor pressure deficit may also indirectly affect photosynthetic rates, thereby altering internal CO_2 concentration and discrimination patterns [74]. The additional physiological traits, including mesophyll conductance, leaf anatomy, and root system architecture, further regulate internal CO_2 diffusion and water availability, potentially decoupling $\Delta^{13}C$ from stomatal conductance alone. In C_4 cereals, the CO_2-concentrating mechanism and variation in bundle sheath leakiness further weaken the $\Delta^{13}C$–iWUE relationship, reinforcing its limited applicability as a universal proxy.

Additional sources of variability include carbon remobilization during grain filling, microclimatic heterogeneity within canopies, and physiological acclimation under prolonged stress, all of which can alter isotopic signatures independently of instantaneous water-use efficiency. These factors suggest that $\Delta^{13}C$ may be more reliable when stomatal regulation is the dominant limitation to photosynthesis but may be less informative when biochemical or structural constraints prevail. Therefore, combining $\Delta^{13}C$ with additional physiological measurements improves interpretation and strengthens its application in breeding programs.

5. Environmental and Genetic Influences

5.1 Impact of Drought, Atmospheric CO_2, and Temperature

Environmental drivers modify $\Delta^{13}C$ by altering stomatal behaviour and photosynthetic activity, thereby influencing internal CO_2 dynamics. Under drought, restricted stomatal conductance reduces internal CO_2 availability [75], resulting in lower $\Delta^{13}C$ values and higher iWUE. This reduction in c_i limits CO_2 availability for carboxylation, resulting in lower $\Delta^{13}C$ and increased iWUE. In C_3 cereals, reductions in $\Delta^{13}C$ under drought reflect tighter stomatal regulation, whereas in C_4 cereals, the CO_2-concentrating mechanism buffers changes in c_i, resulting in weaker and more genotype-dependent responses. Elevated atmospheric CO_2 concentrations further influence $\Delta^{13}C$ [76], though responses vary by photosynthetic pathway. Elevated CO_2 increases c_i in C_3 cereals, potentially enhancing isotopic discrimination; however, the magnitude of this response depends on the relative limitation imposed by photosynthesis versus stomatal conductance. However, the effect is often limited in C_4 cereals, as CO_2 is already concentrated in bundle sheath cells, leading to near saturation of the photosynthetic machinery.

Temperature exerts both direct and indirect effects by influencing enzymatic activity and vapor pressure deficit (VPD). The elevated temperatures increase VPD, which enhances transpirational demand and promotes stomatal closure, thereby reducing c_i and $\Delta^{13}C$ [20]. Simultaneously, temperature affects enzymatic kinetics, potentially constraining photosynthesis and further modifying isotopic discrimination. Combined environmental drivers such as drought × temperature and drought × CO_2 interactions generate non-linear and sometimes opposing responses in $\Delta^{13}C$. For example, high temperature under drought can amplify stomatal limitation, whereas elevated CO_2 may partially offset drought effects by maintaining c_i. These combined effects highlight the need for multi-environment evaluation to capture realistic field variability.

Table 2. Relationship between carbon isotope discrimination and water use efficiency across cereal crops

Crop species	Photosynthetic type	Application of $\Delta^{13}C$	Key findings	Major implications for breeding	Reference
Wheat (*Triticum aestivum*)	C_3	Grain and leaf $\Delta^{13}C$ used to estimate WUE under field drought conditions	Consistently negative relationship between $\Delta^{13}C$ and iWUE; lower $\Delta^{13}C$ genotypes showed higher grain yield under water-limited environments	Strong candidate trait for indirect selection of drought-tolerant, high-yielding lines	[17, 78]
Rice (Oryza sativa)	C_3	Leaf and grain $\Delta^{13}C$ used across flooded and aerobic systems	Moderate correlation between $\Delta^{13}C$ and iWUE; relationship weakened under flooded conditions due to altered stomatal regulation	Useful trait under aerobic and upland rice systems but environment-dependent	[77]
Barley (*Hordeum vulgare*)	C_3	$\Delta^{13}C$ used in drought screening and physiological studies	Variable relationships with iWUE and yield depending on genotype × environment interaction; sometimes weak or inconsistent correlations	Requires multi-environment validation before selection use	[80, 81]

Table 2 Continued

Crop species	Photosynthetic type	Application of $\Delta^{13}C$	Key findings	Major implications for breeding	Reference
Maize (*Zea mays*)	C_4	Leaf $\Delta^{13}C$ used to assess physiological variation under stress	Weak and variable $\Delta^{13}C$–iWUE relationship due to CO_2 concentrating mechanism and bundle sheath leakiness	Limited use as a direct selection trait; better for physiological characterization	[82, 83]
Sorghum (*Sorghum bicolor*)	C_4	$\Delta^{13}C$ used in drought physiology and genotypic screening	Moderate but inconsistent association with WUE; influenced by environmental stress intensity and metabolic regulation	Partial decoupling between $\Delta^{13}C$ and WUE due to C_4 pathway variation and environmental modulation of leakiness	[80]
Proso millet (*Panicum miliaceum*)	C_4	$\Delta^{13}C$ used in stress tolerance screening	Detectable but weak association with iWUE; strongly environment-dependent response	Weak but detectable association; influenced by environmental stress intensity and internal CO_2 recycling	[82]

5.2 Genotypic Variation and Breeding for Improved Water Use Efficiency

Genetic diversity among cereal genotypes drives substantial variation in $\Delta^{13}C$, serving as a key resource for breeding improved water use efficiency. Moderate to high heritability of $\Delta^{13}C$ has been reported across cereal species [83], highlighting strong genetic control. This genetic variability enables breeders to identify germplasm with favourable isotopic signatures associated with improved drought adaptation and WUE. In both C_3 and C_4 cereals, population-level variation in $\Delta^{13}C$ often reflects adaptation to contrasting moisture regimes, although the strength of this signal is more consistent in C_3 species. The $\Delta^{13}C$ offers a practical surrogate trait for indirect selection of iWUE, particularly when direct physiological measurements are not feasible in breeding programs. Grain-based measurements are particularly useful for large-scale screening because they reflect assimilate integration during yield formation [5, 14]. Furthermore, advances in molecular genetics enhance these applications. Quantitative trait loci (QTLs) linked to $\Delta^{13}C$ variation have been identified in multiple crops [86, 87], indicating multiple genomic regions regulate responses to water limitation. These loci may facilitate marker-assisted selection and genomic prediction approaches targeting improved water-use efficiency. Therefore, breeders should integrate $\Delta^{13}C$ with yield and complementary physiological traits and prioritize multi-environment trials to ensure that isotopic selection translates into agronomic gain.

5.3 Interaction with Nutrient Status and Other Stress Conditions

Nutrient availability, particularly nitrogen, strongly influences $\Delta^{13}C$ by modulating key physiological processes. Nitrogen functions as a core component of photosynthetic enzymes, and adequate supply enhances carbon fixation [86], increasing intercellular CO_2 concentration and altering $\Delta^{13}C$ values. Conversely, nitrogen deficiency constrains photosynthetic capacity [87], which can increase c_i/c_a and weaken the relationship between $\Delta^{13}C$ and iWUE. These interactions generate contrasting isotopic responses under combined drought × nitrogen stress, depending on whether stomatal limitation or photosynthetic capacity dominates. Additional abiotic stresses also affect $\Delta^{13}C$ through changes in stomatal conductance, assimilation, and metabolic activity [90, 91]. Salinity reduces stomatal conductance and photosynthetic efficiency, thereby producing isotopic signatures similar to drought stress [90]. Heat stress affects enzymatic activity and increases evapotranspiration [93, 94], which modifies $\Delta^{13}C$ through changes in stomatal behaviour. Biotic stresses, including pathogen infection, alter carbon allocation and sink–source relationships, which can influence isotopic composition independently of water status [93]. These observations demonstrate that $\Delta^{13}C$ reflects an integrated stress signal rather than a drought-specific indicator, which requires careful interpretation under multi-stress conditions.

5.4 Limitations of Using Carbon Isotope Discrimination in Crop Breeding

Despite its strong physiological foundation, several limitations constrain the application of $\Delta^{13}C$ in crop improvement programs. Sensitivity to environmental variation remains a major limitation, as factors such as vapor pressure deficit, soil moisture, and temperature can mask true genotypic differences [94]. These factors modify stomatal

behaviour and photosynthetic rates, thereby influencing $\Delta^{13}C$ expression across genotypes [95]. Consequently, isotopic measurements obtained under a single environment may not accurately reflect genotype performance across diverse agro-ecological conditions. Methodological limitations also restrict the widespread use of $\Delta^{13}C$. High-precision $\delta^{13}C$ measurements rely on isotope ratio mass spectrometry (IRMS), which requires specialized infrastructure, trained personnel, and high operational costs [98, 99]. High-throughput methods such as near-infrared spectroscopy (NIRS) enable rapid screening; however, their reliability depends on calibration against IRMS reference datasets that capture environmental and genetic variability

Species-specific variation in the relationship between $\Delta^{13}C$ and iWUE presents an additional limitation. C_4 cereals such as maize and sorghum exhibit weaker and more variable relationships due to CO_2-concentrating mechanisms and bundle sheath leakiness [98], which complicate interpretation and reduce predictive value. Weak or inconsistent correlations between $\Delta^{13}C$, yield, and iWUE in C_4 crops reduce their effectiveness as a standalone selection trait. C_3 cereals also show variable relationships between $\Delta^{13}C$ and yield across environments [101, 102], particularly when non-water-related stresses limit productivity. These constraints suggest that $\Delta^{13}C$ may be more effective when combined with complementary selection criteria.

Practical constraints in breeding programs further limit adoption. Early-generation populations require screening of thousands of lines, yet IRMS throughput is too low to support such a scale, while NIRS calibration often lacks robustness across genetically diverse populations. Trade-offs between $\Delta^{13}C$ and yield-related traits also complicate selection, as genotypes with high water-use efficiency may exhibit reduced biomass accumulation under favourable conditions. Targeted strategies are required to overcome these limitations. Multi-environment trials should be used to validate $\Delta^{13}C$ stability across environments and identify conditions where selection is reliable. Standardized sampling and analytical protocols should be developed to improve reproducibility across laboratories. Integration of $\Delta^{13}C$ with complementary physiological traits, such as stomatal conductance, mesophyll conductance, and photosynthetic capacity, can strengthen selection accuracy. Combining $\Delta^{13}C$ with genomic tools, including quantitative trait loci mapping and genomic selection, can further enhance breeding efficiency by linking isotopic traits to genetic markers.

6. Ideotypes and Carbon Isotope Discrimination in iWUE-Based Breeding

Carbon isotope discrimination captures the balance between CO_2 supply and demand by enzymes within chloroplasts [99], providing a time-integrated proxy for iWUE. Its mechanistic basis in the c_i/c_a ratio enables direct linkage between stomatal conductance, photosynthetic capacity, and water loss [100], making $\Delta^{13}C$ a powerful criterion for defining drought-adapted ideotypes [101]. Ideotypes adapted to water-limited environments require coordinated regulation of carbon assimilation and transpiration rather than maximisation of either process alone. The lower $\Delta^{13}C$ values in C_3 cereals reflect reduced c_i/c_a ratios, achieved through partial stomatal closure combined with sustained carboxylation capacity. Therefore, selection based on $\Delta^{13}C$ favours genotypes that maintain assimilation under restricted water supply [102], avoiding the yield penalties associated with excessive stomatal limitation. This distinction separates functional iWUE from conservative water use strategies that reduce productivity.

Trait integration determines the performance of $\Delta^{13}C$-based ideotypes, where stomatal regulation is coordinated with mesophyll conductance and enzymatic capacity to sustain CO_2 fixation. Variation in Rubisco efficiency and nitrogen status modifies carboxylation demand [103], while leaf anatomy and root system architecture regulate internal CO_2 diffusion and water uptake [104, 105], which stabilize $\Delta^{13}C$ under fluctuating conditions. Grain $\delta^{13}C$ provides an integrative measure during reproductive development, with lower values associated with sustained assimilation and improved carbon partitioning [20]. The application of $\Delta^{13}C$ in breeding depends on aligning analytical precision with population scale, where isotope ratio mass spectrometry establishes reference relationships and near-infrared spectroscopy enables high-throughput screening. Integration with genomic tools accelerates identification of allelic variation underlying $\Delta^{13}C$ and supports selection of stable ideotypes across environments [106, 107]. Environmental sensitivity remains a key constraint, requiring multi-environment evaluation and integration with complementary traits to ensure reliable selection under variable field conditions [66].

7. Conclusion

Carbon isotope discrimination ($\Delta^{13}C$) is a robust integrative trait for estimating iWUE in cereals because it reflects the long-term balance between stomatal conductance and photosynthetic carbon assimilation via the intercellular-to-atmospheric CO_2 ratio (c_i/c_a). Its physiological basis enables indirect selection for improved water-use performance under drought conditions, making it valuable in breeding programs targeting climate resilience. The practical

application of $\Delta^{13}C$ depends on reliable analytical approaches, primarily IRMS, NIRS, and laser-based techniques. IRMS provides high analytical precision, while NIRS and laser-based methods offer high-throughput alternatives when properly calibrated, enabling large-scale screening in breeding populations. The expression of $\Delta^{13}C$ is influenced by multiple interacting factors, including soil moisture availability, nutrient status, developmental stage and genotype × environment interactions. These factors regulate stomatal behaviour and photosynthetic activity, thereby affecting isotopic discrimination and its relationship with iWUE. For effective deployment in crop improvement, $\Delta^{13}C$ should be integrated with complementary physiological traits and genomic tools and evaluated across multiple environments. This integrated approach improves selection accuracy and supports the development of drought-resilient cereal ideotypes characterized by efficient carbon assimilation, regulated water loss, and stable performance under water-limited conditions.

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Author contributions

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Declarations

Ethical approval

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Consent to participate

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

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