Evolution of Water Use Efficiency, Heat Tolerance, and Carbon Isotope Discrimination Among Canadian Spring Wheat Cultivars

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Article

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Abstract

Background: Climate projections for western Canada predict reduced water availability and more frequent heatwaves, underscoring the need to improve water-use efficiency and heat tolerance to sustain crop productivity and grain quality. Materials and Methods: A total of 198 historical and modern Canadian spring wheat cultivars were evaluated under water-deficient and high- temperature conditions. Measurements included whole-plant and leaf-level WUE, carbon isotope discrimination ($\delta^{13}C$) in flag leaves, and physiological traits such as leaf water potential, photosynthetically active radiation, and chlorophyll fluorescence parameters (F_0, FV/FM, FM, FV, φDo, and ETR) across six growth stages. Results: WUEWP showed a weak relationship with $\delta^{13}C$, indicating strong environmental and genetic influences and limiting its reliability as a proxy across conditions. Spring wheat cultivars exhibited low genetic diversity for WUEWP and heat tolerance, suggesting limited adaptive capacity to increasing stress. Multivariate analyses (PCA and clustering) effectively captured trait variation and differentiated cultivars. Chlorophyll fluorescence traits sensitively reflected reductions in photosynthetic efficiency under drought and heat stress. Conclusion: Overall, the results indicate meaningful genotypic variation but limited genetic diversity and weak relationships among WUE, $\delta^{13}C$, and related traits, highlighting the need for new germplasm and integrated phenotyping to enhance selection efficiency and develop more climate-resilient spring wheat.

Keywords: genetic diversity; spring wheat; stable isotope; abiotic stress tolerance

1. Introduction

Wheat (*Triticum aestivum* L.) is the largest cereal crop in the Canadian Prairies; however, seasonal variations in moisture and temperature are one of the factors affecting plant development, resulting in significant yield losses [1]. Climate projections by Environment Canada indicate increasing drought frequency, higher temperatures, and reduced water availability across the region, posing major threats to wheat productivity and sustainability [2]. In recent years, producers have frequently experienced severe moisture deficits during the growing season, resulting in recurrent drought and heat stress. These extreme conditions reduce yield and grain quality, and in severe cases, cause partial or complete crop failure, thereby reducing farm profitability [3]. According to Statistics Canada, total wheat production declined by 38.5% due to moisture deficit, particularly in the 2021 and 2023

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growing seasons [4]. Zhao et al. reported an average wheat yield loss of 50–60% in a water-limited environment [5]. High temperatures exacerbate yield losses by inhibiting photosynthesis, degrading chlorophyll, and disrupting key physiological processes, leading to extensive cellular damage [6]. Increases of only 3–4 °C above the optimum during grain filling can reduce wheat yield by 10–50%, depending on the cultivar [7,8]. These challenges underscore the urgent need to develop high-yielding, resilient cultivars with enhanced drought and heat tolerance.

Crop water use efficiency (WUE) is a major trait for sustaining productivity in water-limited environments, where crop growth depends on the efficient use of available water resources [9]. WUE reflects how effectively a plant converts water into biomass or grain yield, balancing carbon assimilation with water loss through transpiration [10]. Developing spring wheat cultivars with high WUE could enhance crop resilience, stabilize yields, and support sustainable production under increasingly variable climatic conditions. However, accurately quantifying WUE remains challenging because it integrates complex biological and physical processes that vary spatially and temporally, and are strongly influenced by environmental factors [11,12].

Various direct and indirect methods have been developed to estimate WUE [13,14], yet each approach has limitations in accuracy, scale dependency, and data requirements, leading to inconsistent results across environments. Traditionally, WUE has been estimated from leaf-level gas exchange measurements of photosynthesis and transpiration, assuming these are representative of whole-plant performance [15–17]. In practice, WUE can be assessed at multiple scales, ranging from the leaf and whole-plant levels to population or field scales (biomass-based WUE), each capturing different physiological and ecological aspects of plant water use.

At the leaf level, several physiological parameters have been proposed as indirect indicators of WUE and drought tolerance; among these, carbon isotope discrimination ($\delta^{13}C$) is particularly promising. $\delta^{13}C$ provides a rapid and integrative measure of WUE by quantifying the ratio of ^{13}C to ^{12}C in plant tissue relative to atmospheric CO_2 [18–20]. It reflects the balance between carbon assimilation and stomatal conductance, making it a reliable indicator of a plant's capacity to use water efficiently. In wheat breeding programs, $\delta^{13}C$ has been effectively used to identify genotypes with superior WUE and yield potential under drought conditions [21–25]. Under non-limiting water conditions, positive correlations between $\delta^{13}C$, grain yield, and biological yield have also been reported [26].

The ratio of mesophyll to stomatal conductance (g_m/g_s) represents a key determinant of CO_2 uptake efficiency, while respiration rate was identified as another major factor influencing WUE because increased respiration reduces net carbon assimilation [27,28]. Other physiological traits, such as leaf water potential (LWP), have also been suggested as useful indicators for screening crops for drought tolerance and WUE [29]. The LWP reflects the plant's overall water status, and higher LWP values are associated with mechanisms to avoid dehydration. Leaf-level WUE is a complex trait influenced by multiple physiological processes affecting photosynthesis and transpiration [28].

At the whole-plant or population level, WUE is defined as the ratio of biomass or grain yield produced per unit of water consumed [30–33]. This parameter is affected by genetic factors that influence either biomass production or transpiration efficiency. Substantial genetic variation for WUE has been reported in several crops, including barley [34–36], grapevine [37,38], cowpea [39], peanut [40], sorghum [41], soybean [42,43], cotton [44,45], and wheat [46–48].

Genetic diversity is fundamental for improving WUE because it provides the basis for selecting genotypes with favorable combinations of water-use traits [49]. Conventional breeding has relied on field-based evaluations of biomass production and grain yield under contrasting soil moisture conditions. At the same time, recent advances in high-throughput phenotyping have allowed precise measurements of WUE-related traits across large populations [50]. However, previous studies on WUE and $\delta^{13}C$ in wheat often involved limited numbers of genotypes, restricting the assessment of broader genetic diversity.

Understanding the extent of genetic variation for WUE, $\delta^{13}C$, and heat tolerance among Canadian spring wheat cultivars is therefore essential for breeding climate-resilient varieties. The

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objectives of this study were to: (a) assess the genetic diversity and relationships among whole-plant water-use efficiency (WUE_{WP}), $\delta^{13}C$, and related physiological traits linked to drought and heat tolerance in Canadian spring wheat cultivars registered between 1905 and 2018; and (b) quantify the extent of genetic differentiation among Canadian spring wheat cultivars.

2. Results

2.1. Variation in WUE_{WP}, $\delta^{13}C$, Biomass Accumulation and Water Use per Plant

Significant differences were detected among the Canadian spring wheat cultivars for whole-plant water-use efficiency ($WUE_{(WP)}$) ($P < 0.001$). Mean $WUE_{(WP)}$ values ranged from 2.99 g L^{-1} in the cultivar 'Minnedosa' to 7.81 g L^{-1} in 'WR859' (Table 3). Among breeding programs, cultivars from Syngenta Canada Inc. exhibited the highest average $WUE_{(WP)}$, while the lowest average values were observed in cultivars developed by the AAFC program (**Figure 1a**). Variation in $WUE_{(WP)}$ was not uniform across all programs; cultivars from Canterra, NDSU, USDA, and the group categorized as "Others" displayed relatively high genetic diversity for this trait, whereas cultivars from AAFC and University of Saskatchewan, despite including the largest number of lines, generally showed narrower variation (**Figure 1a**).

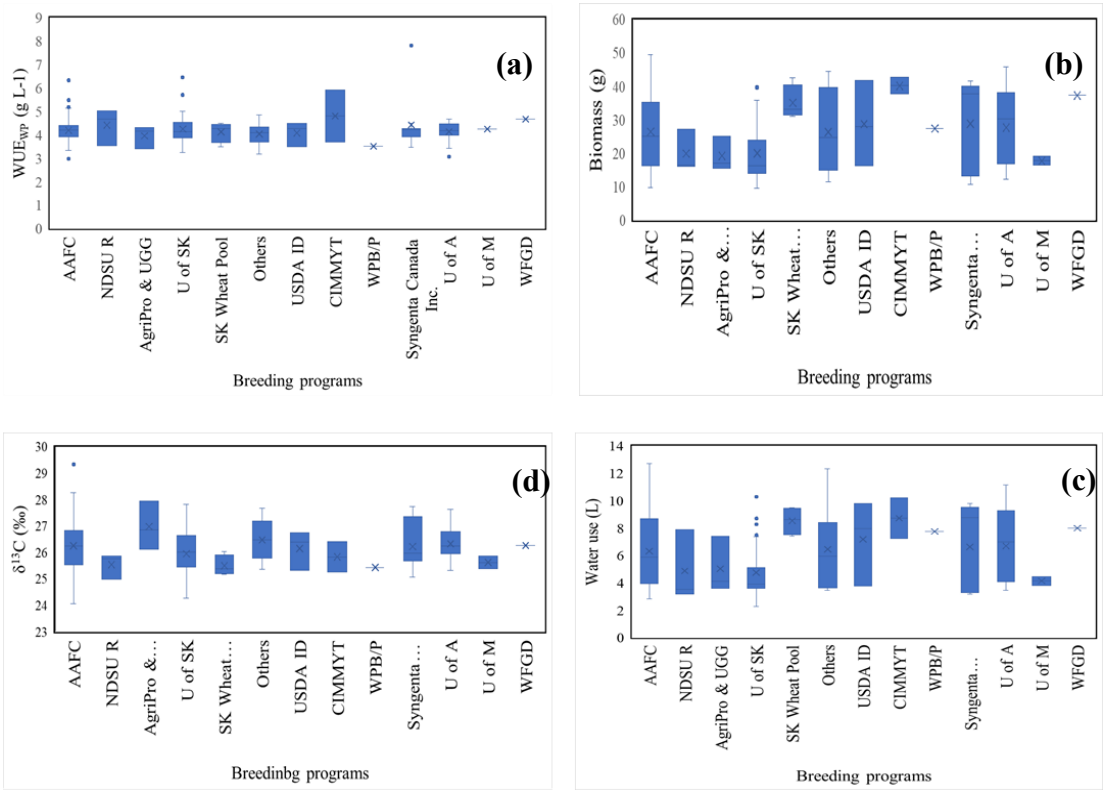

Figure 1. Phenotypic diversity for WUE_{WP} (a),, whole plant biomass accumulation (b), whole plant water use (c), and $\delta^{13}C$ (d) among 198 Canadian spring wheat cultivars derived from various breeding programs AAFC: Agriculture and Agri Food Canada; U of A: University of Alberta; U of SK: University of Saskatchewan; U of M: University of Manitoba; WFGD: Western Feed Grain Development Inc' CIMMYT: International Maize and Wheat Improvement Center; NDSU: North Dakota State University, USDA ID: United States Department of Agriculture, WPB/P: Wiersum Plant Breeding / Plantomar, AgriPro & UGG: AgriPro / United Grain Growers.

Biomass accumulation per plant differed markedly among cultivars, ranging from 9.50 g in 'CDC Plentiful' to 50.60 g in 'Wildcat', with an overall mean of 25.76 g (**Table 3, Figure 1b**). Total water use per plant also varied significantly, ranging from 2.28 L in 'CDC Plentiful' to 12.34 L in 'Wildcat' (**Table 3, Figure 1c**). Notably, 'Wildcat' produced 35.22 g of biomass while using 12.34 L of water, whereas 'CDC Plentiful' produced 21.20 g of biomass with only 2.28 L of water, highlighting

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differences in water-use efficiency among cultivars. Across breeding programs, both biomass accumulation and water use showed significant variation, indicating diverse physiological responses under the applied water-deficit and heat stress conditions (**Figure 1b, c**).

$\delta^{13}C$ values ranged from 24.06‰ to 29.33‰ among the cultivars, reflecting substantial differences in carbon isotope composition and suggesting variation in photosynthetic and water-use processes (**Figure 1d**). The distribution of $\delta^{13}C$ values among cultivars varied across breeding programs, consistent with observed differences in $WUE_{(WP)}$ and biomass accumulation.

When cultivars were grouped by Canadian wheat class, no significant differences were observed for $WUE_{(WP)}$, $\delta^{13}C$, biomass accumulation, or total water use per plant (**Figure 2**). This indicates that, despite variation among individual cultivars, wheat classes showed broadly similar responses under water-limited and heat stress conditions. Collectively, these results demonstrate that physiological traits related to water use and biomass production vary significantly at the cultivar level and among breeding programs, while remaining largely consistent across wheat classes.

The Shannon-Weaver diversity index (H') was used to compare phenotypic diversity among the studied traits. The lowest values were observed for WUE_{WP} (H'= 1.88), biomass accumulation (H'= 1.57), and water use per plant (H'=1.43) in experiment 1, indicating that the cultivars exhibited a narrow range of genetic diversity for these traits. Plant health, slightly affected by excessive fertilizer application during the early growth stage, may be a cause of low or reduced genetic diversity. In experiment 2, the highest values of Shannon-Weaver diversity index for WUE_{WP} (H'=2.02), biomass accumulation (H'= 2.29), and water use per plant (H'=2.15) indicated greater diversity in WUE_{WP}, biomass accumulation, and water use per plant, suggesting a wider range of genetic diversity within the cultivars. The low level of diversity might indicate a narrow genetic base, and a small sample size contributed significantly to the low diversity index. The overall Shannon-Weaver diversity indices for the whole plant WUE_{WP}, biomass accumulation, $\delta^{13}C$, and water use per plant (H' = 2.52; 2.52; 2.61 and 1.98, respectively) confirmed the existence of a moderate diversity among the spring wheat cultivars.

The coefficient of variance has been repeatedly reported as a good estimator of genetic variability across different traits. **Table 3** shows the mean values, maximum, minimum, standard deviation (stdev), and coefficient of variance (CV), and Shannon Diversity Index (H') for whole plant WUE_{WP}, biomass accumulation, water use per plant among the cultivars in two experiments differed by their sets of cultivars. The CV values were moderate to high for the traits, ranging from 9.48 – 11.98% for WUE_{WP}, 12.13–13.48% for biomass accumulation, and 11.41–19.87% for water use per plant (**Table 3**). The range of variation for WUE_{WP} appears to be the lowest, while that of water use appears to be the highest, indicating greater dispersion within the values for those traits. The genetic variation observed for WUE_{WP} among cultivars depends on the number of cultivars and the breeding program's origin.

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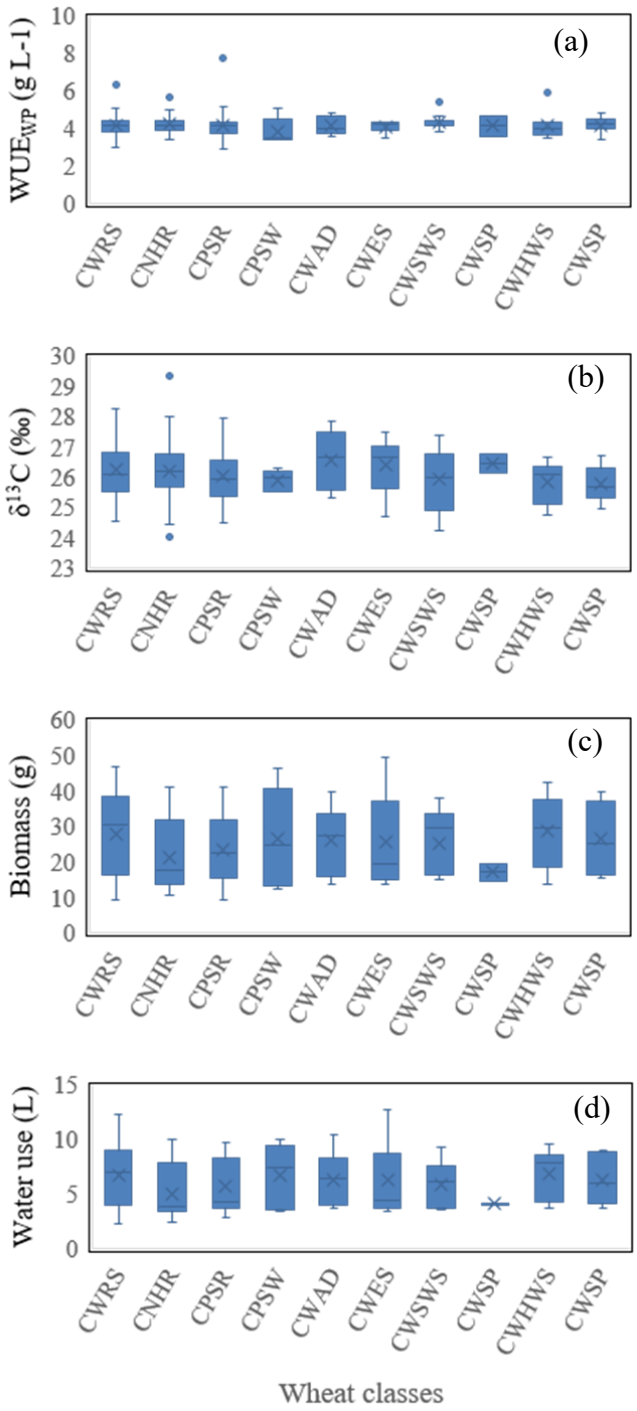

Figure 2. Phenotypic diversity for WUE_{WP} (a), $\delta^{13}C$ (b), whole plant biomass (c), and whole plant water use (d) among 198 Canadian spring wheat cultivars derived from various wheat classes; CNHR: Canada Northern Hard Red; CPSR: Canada Prairie Spring Red; CPSW: Canada Prairie Spring White; CWAD: Canada Western Amber Durum; CWES: Canada Western Extra Strong; CWHWS: Canada Western Hard White Spring; CWRS: Canada Western Red Spring; CWSP: Canada Western Special Purpose; CWSWS: Canada Western Soft White Spring.

2.2. Frequency Distribution of WUE_{WP}, $\delta^{13}C$, Biomass Accumulation and Water Use per Plant

The 198 wheat cultivars were split into two experimental groups, each comprising 99 unique cultivars, due to the limited growth chamber space available. Both experimental groups were exposed to the same experimental conditions, allowing comparison of the cultivars' performance under similar environments. The cultivars tested in this study showed significant differences in several key traits, including WUE_{WP}, biomass production efficiency, $\delta^{13}C$, and water use per plant

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(**Table 3**). The observed bimodal distribution for biomass accumulation and water use per plant indicated that the cultivars fell into two distinct groups, each with a different level of biomass accumulation and water use per plant, rather than being evenly distributed across a single range (**Figure 3**). This may be due to variations in pest control (aphids) that affected plant health by creating uneven pest pressure, leading to slight differences in plant growth, biomass accumulation, and water use per plant. However, the unimodal distributions of WUE_{WP} and $\delta^{13}C$ showed a mode that represents the most common or typical value, indicating a single, dominant process or condition influencing these variables (**Figure 3**). The clustering of data points around a single central tendency, with less variability, suggested that the factors affecting WUE_{WP} and $\delta^{13}C$ were relatively consistent and unaffected by slight differences in plant health, leading to unified and predictable patterns in the measured parameters among the cultivars studied.

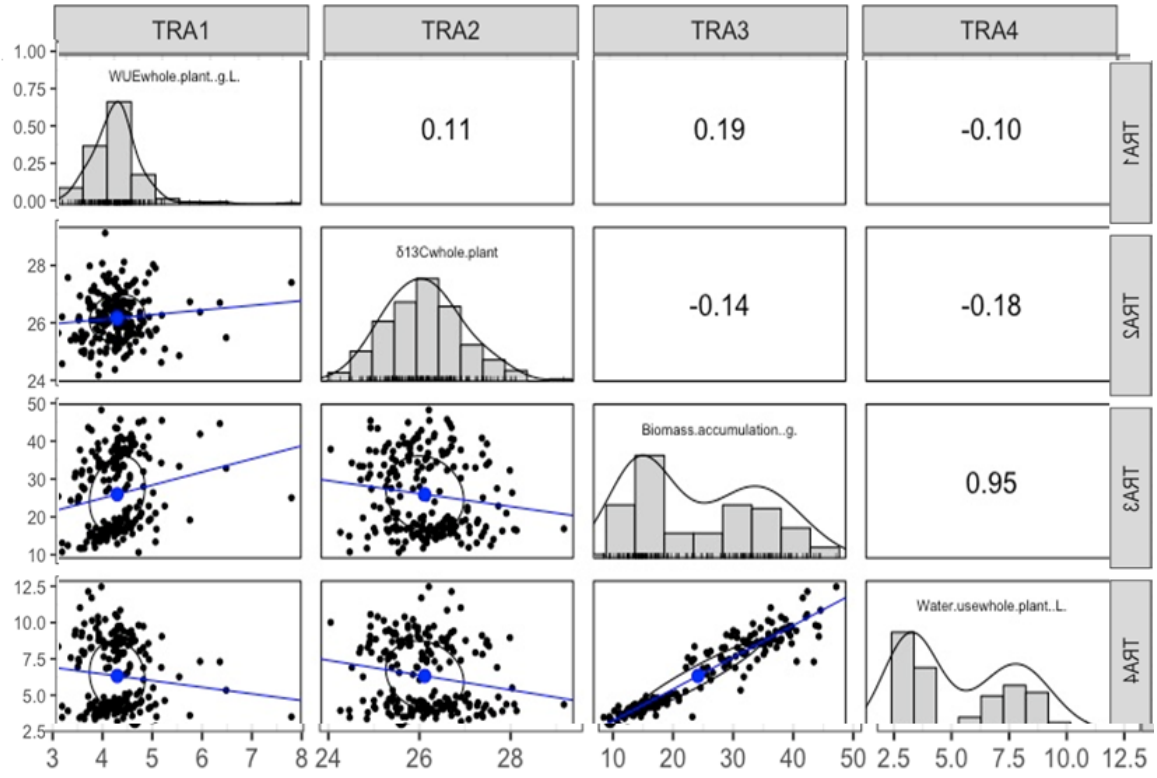

Figure 3. Variation and spearman scatter plots correlations among traits. istograms for WUE_{WP}, $\delta^{13}C$, biomass accumulation, and water use per plant values measured are displayed along the diagonal. The frequency distribution of each variable is shown on the diagonal. On the bottom of the diagonal: the bivariate scatter plots with a fitted line are displayed. On the top of the diagonal: the values of the correlations plus the significance level as 'ns',*,** and *** are significance level 0.1, 0.05, 0.001 (not-significant, significant, very significant, and highly significant, respectively. The experiments were conducted in a BioChamber's plant growth room, Conviron BioChamber LTRB Growth Room, at the University of Alberta in Edmonton, Alberta. The values were measured on the whole plant in two replicates.

2.3. Trait Correlation Analysis

Correlation analysis among the four traits (**Figure 3**) demonstrated significant associations. Biomass accumulation and water use exhibited a very strong positive correlation ($r = 0.95$, $p < 0.001$), indicating that higher biomass is closely linked to greater water consumption. WUE_{WP} (TRA1) showed a weak but significant positive correlation with biomass ($r = 0.19$, $p < 0.01$) and a negative correlation with $\delta^{13}C$ ($r = -0.14$, $p < 0.05$) and water use ($r = -0.18$, $p < 0.05$). These results suggested that genotypes with higher WUE_{WP} tend to use less water and exhibit slightly lower $\delta^{13}C$ values, while biomass accumulation is primarily driven by water availability.

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2.4. Cluster Analysis of Wheat Cultivars

Hierarchical clustering of 198 wheat cultivars based on four physiological traits, WUE_{WP}, $\delta^{13}C$, biomass accumulation, and water use per plant, revealed distinct grouping patterns (**Figure 4**). The heatmap indicates two major clusters, with sub-clusters differentiating genotypes exhibiting high biomass and water use from those with higher WUE and $\delta^{13}C$ values. Cultivars with extreme trait values, particularly for biomass and water use, were concentrated in specific clusters, suggesting strong trait-based differentiation. These clusters represented groups of cultivars slightly affected by plant health during the experiment.

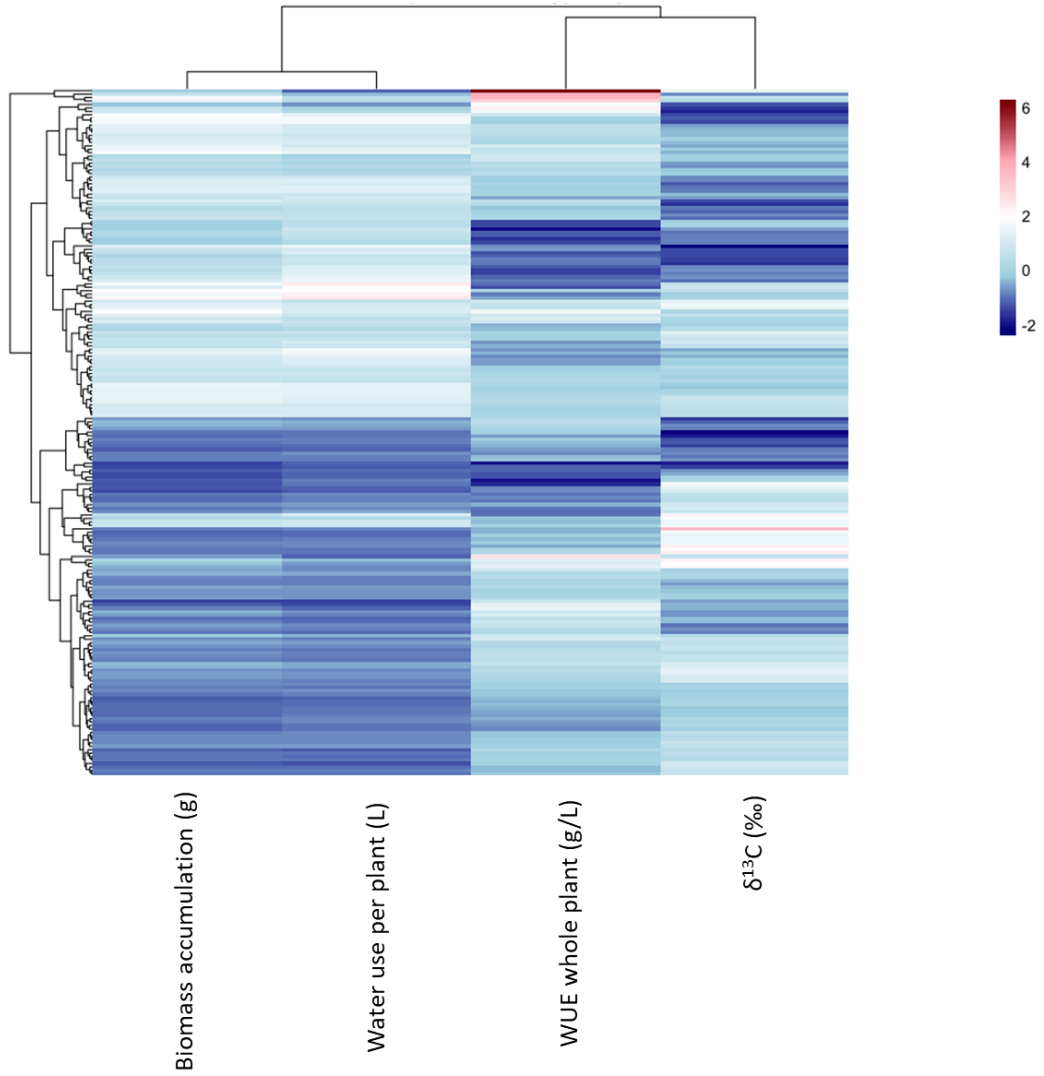

Figure 4. Hierarchical clustering heatmap for WUE_{WP}, biomass accumulation, water use per plant of 198 spring wheat cultivars. Red and blue colors in the columns denote high and low values, respectively, for each trait, with color intensity associated to trait values.

2.5 Variation in Leaf Water Potential (LWP) and Chlorophyll Fluorescence Parameters

To further characterize the physiological responses of the 198 spring wheat cultivars, we measured several chlorophyll fluorescence parameters, including LWP (F_M/F_0), F_M, F_0, F_V ($F_M - F_0$), F_V/F_M [$(F_M - F_0)/F_M$], φDo (F_0/F_M), relative electron transport rate (ETR), and photosynthetically active radiation (PAR) across six developmental stages: stem elongation (BBCH 30–36 and BBCH 37–40), booting (BBCH 41–44 and BBCH 45–49), and inflorescence emergence (BBCH 50–59 and BBCH 60–69). Box plots (**Figure 5**) were used to illustrate the variation in physiological parameters across different growth stages. An increasing trend was observed in LWP, PAR, ETR, and the maximum

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quantum efficiency of PSII (F_V/F_M) from the stem elongation to the booting stage. These parameters subsequently declined under water-deficient and heat-stressed conditions during the booting growth stage. A recovery phase was later observed, suggesting that the plants had adapted to the imposed stress (**Figure 5**). In contrast, F_O, F_M, and the F_O/F_M ratio exhibited the opposite pattern, increasing from the stem elongation stage and then decreasing during the booting stage, indicating distinct regulatory responses of the photosynthetic apparatus under stress (**Figure 5**).

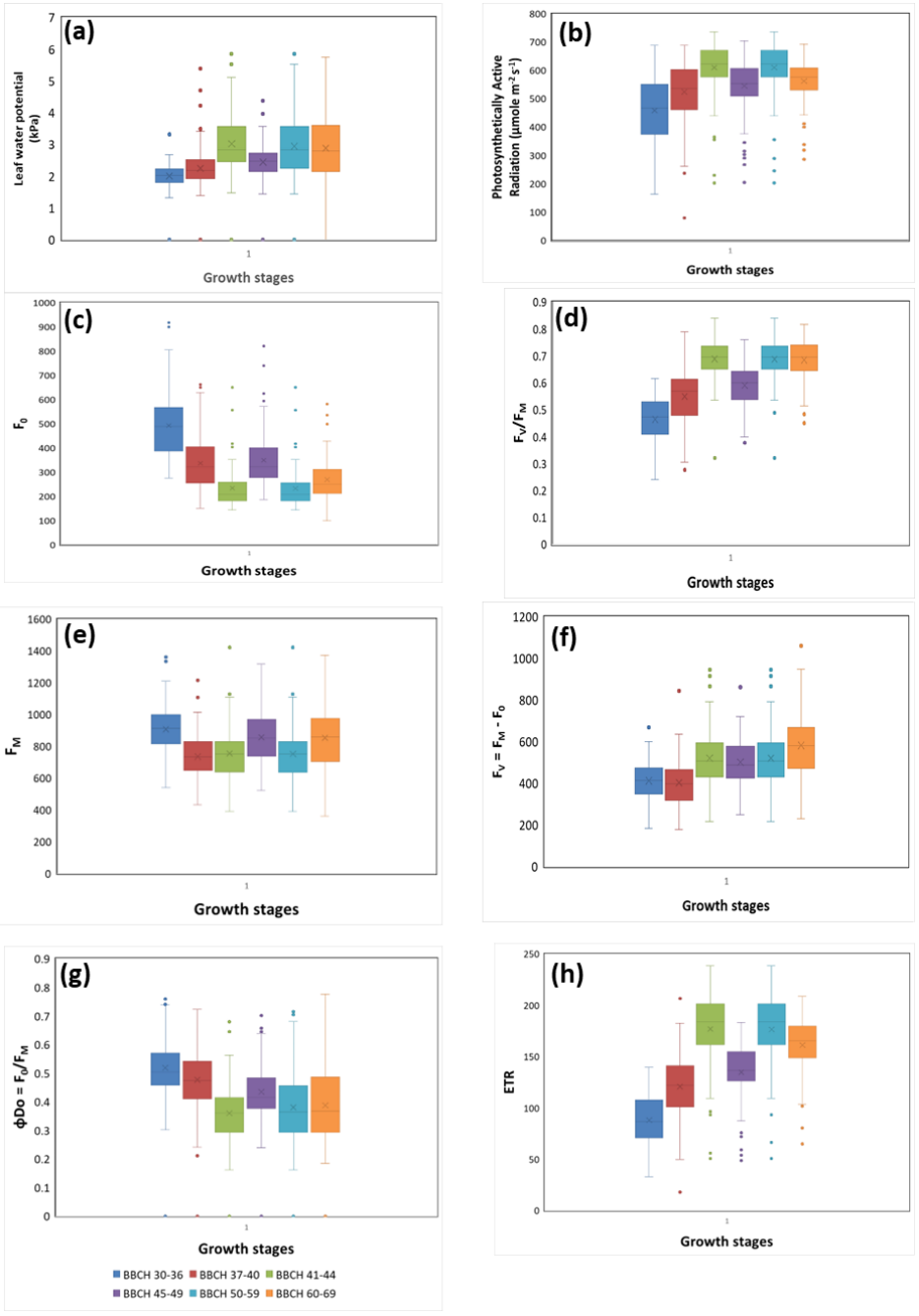

Figure 5. Box plots showing mean performances for the LWP and chlorophyll fluorescence parameters across six growth stages: stem elongation (BBCH 30-36 and BBCH 37-40), booting growth stage (BBCH 41-44 and BBCH 45-49), and inflorescence emergence (BBCH 50-59 and BBCH 60-69). (a) LWP, (b) PAR, (c) F_0, (d) F_V/F_M, (e) F_M, (f) F_V, (g) φDo, (h) ETR. Plants were subjected to water-deficit and heat stress treatment for 7 days at the booting growth stage (BBCH 41-44 and BBCH 45-49). The temperature was raised from 22°C to 35°C.

During the stem elongation stage, PAR showed the greatest genetic variation among growth stages, despite lower mean PAR values (**Figure 5**). This suggests that, at this developmental phase,

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the spring wheat panel possesses a broader spectrum of genetic diversity influencing PAR, thereby providing a larger pool of allelic variation for selection to act upon. Variations in F_V, F_M, and ETR were similar across all growth stages (Figure 5), whereas F_V/F_M showed a consistent trend at the stem elongation and booting stages under water deficit and elevated temperature conditions. Under these stress conditions, the cultivar 'CDC Teal' displayed the highest F_V/F_M, indicating superior tolerance, whereas 'Super' exhibited the lowest value, suggesting greater sensitivity to the combined stresses. The F_V/F_M reflects the maximum quantum efficiency of photosystem II (PSII), a critical determinant of photosynthetic performance, with higher values indicating reduced photoinhibition and better maintenance of photosynthetic function under stress. Notably, the relative ranking of cultivars shifted under stress, which may be attributed to evolutionary forces such as natural selection and genetic drift, leading to stochastic changes in allele frequencies.

Overall, the values of the photosynthesis-related parameters F_V/F_M, PAR, LWP, ETR, and F_V showed an upward trend from stem elongation to the booting growth stage, indicating strong photosynthetic activity before declining under stress, followed by slight recovery as the plants recovered from stress. On the other hand, the values of F_O, F_M, and φDo showed a downward trend, suggesting the plant's ability to absorb light energy and perform photochemistry was impaired. This may be due to a potential nutrient deficiency during the plant's stem elongation phase, a critical growth period where inadequate nutrients could lead to symptoms like slight yellowing observed on the leaf tip, which vary depending on the specific nutrient deficiency.

2.6. Principal Component Analysis (PCA)

Figure 6 presents a biplot integrating both cultivar and trait data, revealing patterns of phenotypic similarity and the relationships among traits across the evaluated wheat cultivars. The first principal component (PC 1) accounted for 50.1% of the total variance, while the second component (PC 2) explained an additional 28.1%, jointly capturing 78.2% of the overall variation in the dataset. The PC 3 and PC 4 contributed marginally (21.6% and 0.2%, respectively). Cultivars positioned closely together exhibited similar phenotypic profiles, suggesting comparable physiological or morphological responses. Conversely, cultivars plotted at opposite ends of the biplot displayed contrasting trait combinations, indicative of differing adaptive strategies. The strong loadings of specific traits along PC 1 and PC 2 axes highlight the major factors driving phenotypic differentiation, providing insights into the multivariate structure of cultivar performance. Overall, the PCA biplot underscores substantial phenotypic diversity and helps identify cultivars with favorable trait associations for potential use in breeding programs aimed at improving drought and heat resilience.

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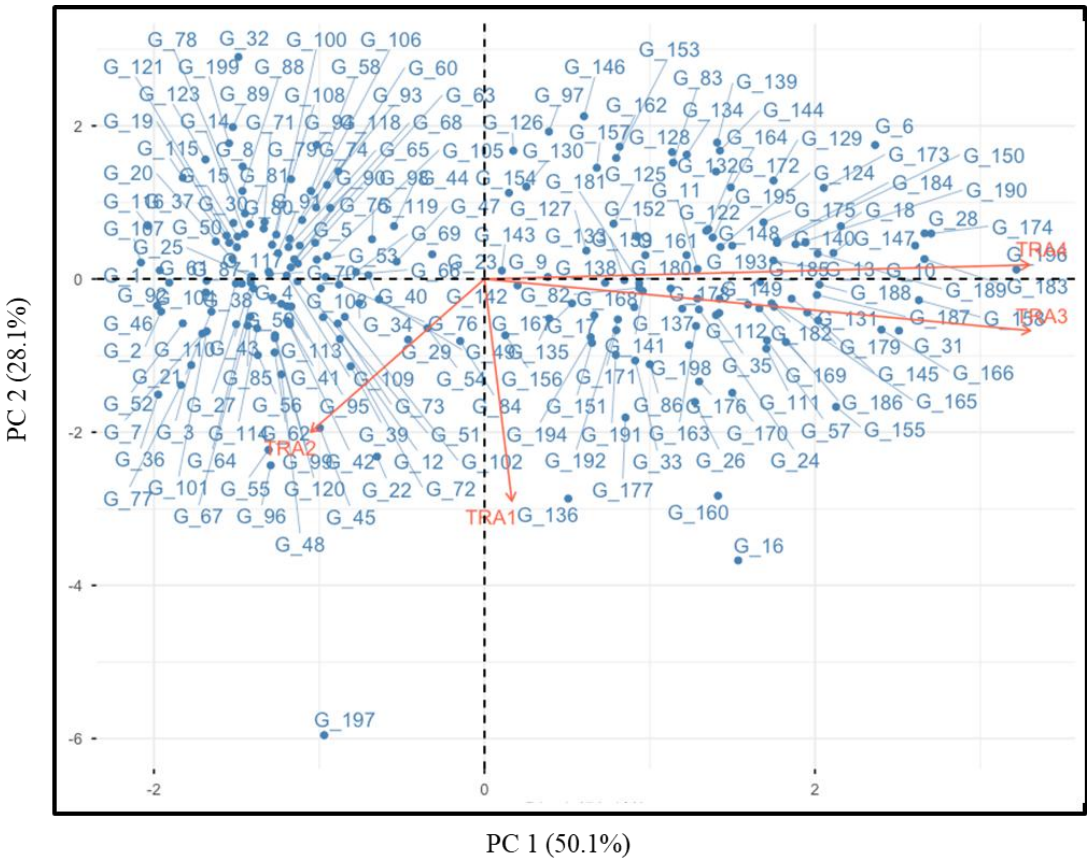

Figure 6. PCA analysis of 198 Canadian spring wheat cultivars derived from various breeding programs. The x-axis and y-axis represent principal component 1 (PC 1) and principal component 2 (PC 2) with the proportions. Presented here are the following: WUE_{WP} (TRA1), $\delta^{13}C$ (TRA2), biomass (TRA3) and water use per plant (TRA4).

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Table 1. The breeding program origins of 198 historical and modern Canadian spring wheat cultivars registered in western Canada between 1905 and 2018 used in the current study. CDC: Crop Development Centre; WPB: Wiersum Plant Breeding; CIMMYT: International Maize and Wheat Improvement Center; NDSU: North Dakota State University, USDA ID: United States Department of Agriculture.

Breeding program	No.	Cultivars							
AAFC	108	AAC AwesomeVB	AAC Iceberg	AC Abbey	Enchant	Peace	AC Barrie	Columbus	
		AAC Bailey	AAC Innova	AC Andrew	Fieldstar	Pembina	AC Cora	Grandin	
		AAC Brandon	AAC Jatharia	AC Cadillac	Garnet	PT472	AC Crystal	Katepwa	
		AAC Cabri	AAC Penhold	AC Corinne	Glencross	PT479	AC Domain	Lancer	
		AAC Cameron	AAC Prevail	AC Intrepid	GoodeveVB	RL6077	AC Elsa	Laura	
		AAC Castle	AAC Proclaim	AC Meena	Helios	Sadash	AC Foremost	Leader	
		AAC Chiffon	AAC Raymore	AC Phil	HY320	Sinton	AC Karma	Lillian	
		AAC Cirrus	AAC Redwater	AC Snowbird	Kanata	Snowhite475	AC Majestic	Pasqua	
		AAC Connery	AAC Ryley	AC Vista	Kane	Snowstar	AC Michael	Roblin	
		AAC Crossfield	AAC Spitefire	Alvena	Manitou	Stettler	AC Minto	Somerset	
		AAC Crusader	AAC Tenacious	Burnside	Marquis	Superb	AC Reed	Vesper	
		AAC Current	AAC Tradition	Canuck	Minnedosa	Unity	AC Splendor	Wildcat	
		AAC Durafield	AAC Viewfield	Carberry	MuchMore	Waskada	AC Taber		
		AAC Elie	AAC W1876	CDN Bison	Napayo	Whitehawk	Benito		
		AAC Entice	AAC Whitefox	Conquer	Neepawa	Cardale	Biggar		
		AAC Foray	AC 2000	Cypress	Park	AC Eatonia	Bluesky		
CDC / U of S	31	CDC Carbide	CDC Kernen	CDC Stanley	CDC Fortitude	CDC Bradwell	CDC Plentiful	CDC Walrus	Kenyon
		CDC Abound	CDC Merlin	CDC Teal	CDC Cordon	CDC Go	CDC	CDC	Moats
		CDC Alsask	CDC NRG003	CDC Thrive	CDC TERRAIN	CDC Hughes	Primepurple	Whitewood	BW970
		CDC Imagine	CDC Osler	CDC Utmost	CDC VR	CDC Bounty	CDC Rama	Conway	Pro
					Morris		CDC Titanium	PT595	
University of	19	Alikat	Cutler	PT771	Ellerslie	Coleman	Laser	BYT1419	
Alberta		BW1039	Go Early	PT778	Tracker	Thorsby	Parata		
		BYT1411	GP168	PT780	Jake	RedNet	Zealand		

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University of Manitoba	2	Amazon	Glenlea						
WPB / Plantomar	1	Pasteur							
	3	Cardale	Infinity	Lovitt					
AgriPro & UGG	3	5700PR	5701PR	Invader					
Syngenta Canada Inc.	11	5604HR CL	GP112	SY 433	SY637	SY985	SY995		
		5605HR CL	SY087	SY479 VB	5702PR	WR859 CL			
WFGD Co-op	1	WTF603							
	1	Harvest							
NDSU	2	Faller	Prosper						
SK Wheat Pool	4	Prodigy	McKenzie	Oslo	Journey				
CIMMYT	2	Pitic62	SAAR						
USDA ID	3	Owens	Springfield	Fielder					
Others	8	Bhishaj	FL62R1	Red Bobs	Sumai3	BW493	NRG007	SWS52	BW278

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Table 2. Terms, formulas and description of the chlorophyll fluorescence parameters used in the study.

Terms and Formulas	Description
PAR	Photosynthetically active radiation
F_0	Minimum yield of Chla fluorescence measured in a dark-adapted state
$F_V = F_M - F_0$	Variable fluorescence
F_M	Maximum yield of Chla fluorescence measured in a dark-adapted state
$F_V/F_M = (F_M - F_0)/F_M$	Maximum quantum yield of photochemistry in PSII, measured in a dark-adapted state
$ETR = \Phi_P = \Delta F/F_M = (F_{M'}-F)/F_{M'}$ X (PPFD) or 40. $ETR = Y(II) \times PAR \times 0.84 \times 0.5$	Relative electron transport rate, is the product of the effective photochemical yield of PSII, $\Phi_P = \Delta F/F_{M'} = (F_{M'}-F)/F_{M'}$ and photosynthetic photon flux density (PPFD) Electron transport rate (ETR), estimated from chlorophyll fluorescence, is a widely-used indicator of photosynthetic activity.
$\varphi Do = F_0/F_M$	Quantum yield (at t = 0) of energy dissipation. Quantum yield for heat dissipation of PSII
$LWP = F_M/F_0$	Leaf water potential (LWP)

Table 3. Mean value, maximum, minimum, standard deviation (s), coefficient of variance (CV), and Shannon Diversity Index (*H'*) of whole plant WUE, biomass, water use and $\delta^{13}C$ measured in 198 historical and modern wheat cultivars under controlled environment.

Studied character	Experiment 1							
	Mean	Max	Min	CV (%)¶	$LSD_{(0.05)}$	Stdev	Pr > F	*H'*
$\delta^{13}C$ (‰)	26.37	29.33	24.28	-	-	0.92	-	2.62
WUE_{wp} (g L^{-1})	4.17	5.71	3.07	9.48	0.79	0.43	< 0.001	1.88
Biomass (g)	16.10	23.85	9.50	12.13	3.89	3.36	< 0.001	1.57
$Water\ use_{wp}$ (L)	3.92	5.04	2.27	11.41	0.87	0.71	< 0.001	1.43
	Experiment 2							
	Mean	Max	Min	CV (%)	$LSD_{(0.05)}$	Stdev	Pr > F	*H'*
$\delta^{13}C$ (‰)	26.00	28.16	24.06	-	-	0.80	-	2.49
WUE_{wp} (g L^{-1})	4.12	7.81	3.11	11.98	0.98	0.69	< .0001	2.02
Biomass (g)	35.24	50.60	24.80	12.33	8.68	0.32	< .0001	2.29
$Water\ use_{wp}$ (L)	8.73	12.34	3.17	18.42	3.21	1.59	0.10	2.15
	Combined experiments 1 and 2							

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	Mean	Max	Min	CV (%)	$LSD_{(0.05)}$	Stdev	Pr > F	*H'*
$\delta^{13}C$ (‰)	26.18	29.33	24.06	-	-	0.88	-	2.61
WUE_{wp} (g L^{-1})	4.15	7.81	3.07	11.44	0.94	0.57	< .0001	2.52
Biomass (g)	25.69	50.60	9.50	13.48	6.87	10.79	< .0001	2.52
Water use_{wp} (L)	6.30	12.34	2.27	19.87	2.48	2.57	< .0001	1.98

3. Discussion

The experimental materials provided high-throughput data, which were analyzed to reveal genetic information related to WUE_{WP}, $\delta^{13}C$, biomass accumulation, water use per plant, and chlorophyll fluorescence parameters. Thus far, there have been no relevant reports on the evolution of whole plant and leaf WUE, heat tolerance, and $\delta^{13}C$ among historical and modern Canadian spring wheat cultivars. This study used phylogenetic analysis, photosystem II (PSII) system, chlorophyll fluorescence capacity, Shannon diversity index, and coefficient of variation to explain how variations in whole plant and leaf WUE, heat tolerance, and $\delta^{13}C$ can be used for the improvement of cultivar resilience, which is important for breeding programs. The Shannon diversity index, which measures both the number and evenness of genotypes, revealed genetic diversity, while chlorophyll fluorescence parameters provided insights into cultivar performance at the physiological level.

Biomass accumulation varied from 9.5 to 50.6 g per plant, and water use ranged from 2.27 to 12.34 L per plant. WUE_{WP} ranged from 3.07 to 7.81 g L^{-1}, while δ13C values ranged from 24.06‰ to 29.33‰. Despite substantial differences in biomass and water use among cultivars, WUE_{WP} and $\delta^{13}C$ exhibited relatively narrow and consistent ranges, indicating that the efficiency of water and carbon conversion into biomass is a stable trait. These findings suggested that WUE is cultivar-specific, reflecting both genetic potential and growth conditions, with some cultivars inherently more efficient at producing biomass per unit of water. Zhang et al. [51], Kemanian et al. [52] reported that the average WUE for wheat ranged from 3.34 to 4.70 g dry matter per kg of water used, indicating variability in efficiency depending on environmental conditions and management practices. WUE in wheat varied significantly, with values ranging from 0.5 to 13.8 per kg of water used [53,54], depending on factors such as growth stage, cultivar, and water stress levels. These studies highlighted the variability in wheat water-use efficiency across environments and suggest potential for improvement in Canadian cultivars.

The variation in genetic diversity across breeding programs reflects a combination of the germplasm used, the traits under selection, the environmental conditions targeted, and the intensity and methodology of selection. Programs with broader objectives and more diverse parental lines typically maintain greater diversity in physiological traits such as WUE, $\delta^{13}C$, biomass, and water use per plant. The AAFC breeding program contributed the largest number of cultivars to the panel; however, these cultivars exhibited a relatively narrow range of genetic variation for WUE_{WP}. This suggests that, despite the greater sample size, the AAFC germplasm may share similar genetic backgrounds or selection histories for WUE-related traits. Similar patterns have been reported in other studies, where modern wheat breeding programs, despite releasing a large number of cultivars, often exhibit reduced genetic and phenotypic variation due to selection bottlenecks and the recurrent use of elite parental lines [55–62].

The $\delta^{13}C$ measurements, obtained from flag leaves exposed to heat and water stress, provided an integrated measure of photosynthetic and transpiration dynamics during stress exposure. The broad range observed (24.06‰–29.33‰) indicated substantial genotypic variation and suggested differences in stomatal regulation, mesophyll conductance, and photosynthetic capacity among cultivars. Such variation suggested that the plants exhibited differential physiological responses to drought and heat stress, reflecting differences in stomatal regulation, mesophyll conductance, and photosynthetic capacity. Plants with low $\delta^{13}C$ values likely exhibit conservative water-use behavior under heat stress, characterized by reduced stomatal conductance and enhanced WUE, whereas those with higher $\delta^{13}C$ values may favor a more acquisitive strategy aimed at sustaining higher carbon

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assimilation rates despite greater water loss. These physiological trade-offs underscore the complexity of breeding for drought and heat tolerance, as the optimal balance between productivity and water conservation depends on target environment and breeding objectives [63,64].

The negative relationship between WUE_{WP} and $\delta^{13}C$, commonly reported in previous studies, may be due to physiological mechanisms that regulate $\delta^{13}C$ during photosynthesis. During CO_2 fixation, plants preferentially assimilate the lighter carbon isotope (^{12}C) over the heavier isotope (^{13}C). When stomatal conductance is high and intercellular CO_2 concentration (Ci) approaches ambient levels, discrimination against ^{13}C increases, resulting in higher $\delta^{13}C$ values. Conversely, when stomatal conductance is reduced under stress conditions, Ci declines, leading to lower discrimination and more negative $\delta^{13}C$ values. As a result, lower $\delta^{13}C$ values are generally associated with higher intrinsic WUE, reflecting a balance between maintaining photosynthetic carbon assimilation and minimizing water loss through transpiration [65,66].

The phylogenetic tree showing differences in WUE_{WP}, $\delta^{13}C$, biomass accumulation, and water use per plant among cultivars suggested that genetics contributed to determining these traits, and that selection for one trait may influence others. These differences indicated that the ability to use water efficiently was not uniform across cultivars, and the tree provides a visual representation of how these traits are distributed within a group of related plants based on their evolutionary history. These traits are interconnected, with $\delta^{13}C$ serving as a proxy for WUE_{WP}, which reflects how efficiently a plant uses water for biomass accumulation. Therefore, the discernible differences suggested that multiple factors interacted and influenced each other, indicating that the cultivars share a recent common ancestor.

Canadian wheat cultivars were categorized according to official marketing classes, defined primarily by grain quality traits such as grain color, hardness, kernel size, baking and milling quality, dough or gluten strength, grain protein content, and intended end-use. We hypothesized that WUE_{WP}, $\delta^{13}C$, biomass, and water use might vary across these classes due to underlying physiological differences. However, no significant differences were detected among classes for any of these traits, indicating a shared genetic background and parallel breeding histories. Because Canadian wheat breeding has historically emphasized grain yield, grain quality, and disease resistance, traits related to WUE_{WP} have not been direct selection targets. Consequently, the classes exhibit similar physiological responses to water limitation and comparable photosynthetic adaptations to the temperate agroclimatic conditions of the Canadian Prairies.

The Shannon diversity index values in the wheat panel ranged from 1.43 to 2.62, indicating low to medium diversity (1 = low, 2.5 = medium). The higher the values, the greater the diversity of traits [67,68], indicating the genetic potential of spring wheat cultivars and the presence of desirable genes for improving WUE_{WP}, drought tolerance, and biomass production. These results suggested a moderate level of diversity for traits related to WUE_{WP}, $\delta^{13}C$, biomass accumulation, and water use per plant, consistent with previous reports in wheat [69,70]. Although the observed genetic diversity among the evaluated cultivars may be adequate for specific breeding objectives, the effective improvement of WUE_{WP} and associated physiological traits would likely require the incorporation of additional genetic variation. Expanding the breeding pool through the strategic introduction of international germplasm could strengthen the adaptive potential of Canadian spring wheat under increasingly variable climatic conditions. However, the continuing erosion of genetic diversity among modern, high-yielding varieties remains a critical concern, reinforcing the urgency of global and regional research initiatives aimed at broadening the genetic base and safeguarding long-term crop resilience [71–76].

Among the chlorophyll fluorescence parameters, the maximum quantum efficiency of photosystem II (F_V/F_M) is one of the most important and sensitive chlorophyll fluorescence parameters for assessing moisture and temperature stresses as it reflects the maximum efficiency of energy conversion in the photosynthetic system and provides an early and non-invasive indicator of plant stress by showing a decline in photosynthetic efficiency [77–82]. These parameters are indirectly related to WUE_{WP} through their influence on photosynthetic capacity and stomatal regulation. The

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F_V/F_M, together with F_M and ETR, was assessed across different growth stages of wheat cultivars under combined water deficit and heat stress. While the F_V, F_M, and ETR showed similar variations across stages, the F_V/F_M remained the most consistent and informative indicator. The cultivar 'CDC Teal' maintained the highest F_V/F_M, indicating superior tolerance, whereas 'Superb' displayed the lowest values, suggesting greater stress sensitivity.

The F_V/F_M is a sensitive indicator of PSII photochemical efficiency and photosynthetic performance under stress. Higher F_V/F_M values reflect reduced photoinhibition and better maintenance of photosynthetic function [78,83]. Consistent with previous reports, cultivars with higher F_V/F_M under drought or heat stress demonstrate improved tolerance, maintaining photosynthetic activity and potentially supporting higher biomass accumulation [84,85]. Variation in F_V/F_M among cultivars under stress likely arises from genetic differences influencing PSII stability and efficiency. Evolutionary forces, including natural selection and genetic drift, can produce shifts in allele frequencies that affect stress-responsive traits. Selection may favor alleles enhancing stress tolerance, while drift can generate stochastic changes in trait distributions independent of fitness [86,87].

These results highlight the importance of PSII efficiency in determining cultivar-specific responses to combined water deficit and heat stress. Understanding genetic variation in F_V/F_M can inform breeding programs aimed at improving abiotic stress resilience in cereal crops. The observed variations in photosynthetic and fluorescence parameters across growth stages reflect the differential physiological responses of wheat to combined water deficit and heat stress. The initial increase in LWP, PAR, ETR, and F_V/F_M from stem elongation to booting suggests an enhancement in photosynthetic performance and photochemical efficiency during early stress exposure, probably due to short-term acclimation mechanisms such as osmotic adjustment and activation of photoprotective pathways [88–90]. The subsequent decline in these parameters at the booting stage indicates that prolonged or intensified stress impaired photosynthetic electron transport and PSII efficiency, leading to a reduction in carbon assimilation capacity. Conversely, the increase in F_0 and FM followed by their decline suggests transient structural perturbations and subsequent partial recovery of PSII reaction centers, which are typical responses to photo-oxidative stress [91,92]. The decline in F_V/F_M and ETR at booting indicates photoinhibition and reduced photosystem II efficiency, which are common responses to cumulative stress effects that impair the photosynthetic apparatus [83,84]. The increase in F_0 under severe stress at booting could indicate damage to PSII reaction centers or dissociation of light-harvesting complexes, leading to reduced energy use efficiency [78]. These patterns align with reports that water deficit and high temperature synergistically exacerbate oxidative stress, leading to chlorophyll degradation, impaired electron transport, and photodamage [93,94]. These patterns collectively imply that wheat plants exhibit dynamic adjustments of photochemical processes under stress, with the observed recovery phase reflecting activation of adaptive mechanisms.

The PCA results demonstrated differences among the studied wheat cultivars based on key physiological and agronomic traits such as WUE_{WP}, $\delta^{13}C$, biomass accumulation and water use per plant, reflecting substantial phenotypic diversity within the panel. The high proportion of variance explained by the first two components (78.2%) indicated that a few major traits largely account for cultivar variability, consistent with previous findings in Canadian spring wheat populations [1,71]. The biplot revealed that biomass accumulation and water use were strongly aligned with PC1, indicating their major contribution to overall variability. The WUE_{WP} and $\delta^{13}C$ were more associated with PC2, suggesting these traits captured a different dimension of variation. Cultivars clustering near traits associated with higher biomass and WUE_{WP} may possess adaptive advantages under water-limited conditions, whereas those aligned with higher $\delta^{13}C$ values or reduced photosynthetic parameters may be more sensitive to stress. The observed trait associations highlighted potential trade-offs between productivity and water conservation, underlining the importance of selecting cultivars that balance these physiological dimensions for future breeding efforts under increasing drought and heat stress in the Canadian Prairies.

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4. Materials and Methods

4.1. Plant Material

A spring wheat diversity panel consisting of 198 historical and modern Canadian cultivars registered in western Canada between 1905 and 2018 was used in this study (**Table 1**). These cultivars represent releases from more than eleven breeding programs across Canada. The panel encompasses a wide range of genetic backgrounds and end-use quality types, capturing the historical and contemporary genetic diversity of Canadian spring wheat. Cultivars were classified according to their official marketing classes, which are defined by functional characteristics such as grain color, hardness, kernel size, baking and milling quality, dough or gluten strength, grain protein concentration, and end-use (**[95]**, https://www.grainscanada.gc.ca/en/grain-quality/grain-grading/wheat-classes.html). The classes are Canada Northern Hard Red (CNHR), Canada Prairie Spring Red (CPSR), Canada Prairie Spring White (CPSW), Canada Western Amber Durum (CWAD), Canada Western Extra Strong (CWES), Canada Western Hard White Spring (CWHWS), Canada Western Red Spring (CWRS), Canada Western Special Purpose (CWSP), and Canada Western Soft White Spring (CWSWS) (**Supplementary Table S1**). Among these, the CWRS class is the most widely cultivated, accounting for approximately 60% of Canada's total wheat production, followed by CWAD, CPSR, and CESRW (**[96]**. The inclusion of cultivars from these classes ensured comprehensive representation of genetic and phenotypic variation in agronomic and physiological traits related to water use efficiency, drought tolerance, and heat stress response.

4.2. Growth Conditions

The experiment was conducted in a Conviron BioChamber LTRB growth room at the University of Alberta, Edmonton, Canada. Plants were grown under controlled conditions with a photoperiod of 18 h, light intensity of 1000 μmol m^{-2} s^{-1} (provided by sodium halide bulbs), day/night temperatures of 22/16 °C, and relative humidity of 55%/70% (day/night). Plants were grown individually in 1-gallon pots filled with 1.9 kg of a soil mixture composed of field soil and peat moss (Promix BX) at a 1:3 (v/v) ratio. Before sowing, all pots were flushed with tap water and drained overnight to determine field capacity. Three seeds were sown per pot at 1.5 cm, and plants were thinned to one seedling per pot two weeks after emergence. To reduce direct soil evaporation, the soil surface was covered with a 2-cm layer of perlite. Plants were maintained at field capacity by daily water replenishment based on pot weight until the start of the drought and heat treatments. Each genotype was subjected to water-deficient treatments in two biological replicates. Water-deficit and heat stress treatments, in which the temperature was increased from 22 to 35 °C, were imposed for 7 days at the booting stage (BBCH 41–49), according to the Zadoks scale (**[97])**. After the stress period, pots were re-watered to field capacity and maintained until grain maturity.

Drought stress was induced by withholding irrigation. Once the target soil water content (SWC) was reached, plants were maintained under constant stress for 7 days by daily replenishment of the exact amount of water lost, determined gravimetrically. The SWC was calculated as:

SWC = (pot weight − minimum pot weight) / (maximum pot weight − minimum pot weight) × 100).

The SWC decreased from 70–90% (well-watered) to 10–15% (water-deficient). Soil moisture was also monitored using Soil Moisture Equipment Corp. (Santa Barbara, CA, USA) probes (20-mm stainless steel, 3-rod configuration). During the water-deficit treatment, the temperature was increased from 22 °C to 35 °C in a single step for 7 days. Chlorophyll fluorescence parameters were measured before and after the treatments, and at the beginning and end of the stress period. Flag leaves that developed during the stress treatment were collected at maturity for carbon isotope discrimination analysis. Two control pots containing the same soil mixture and perlite, but without plants, were used to estimate soil surface evaporation using successive weight differences. At the end of the 7-day WD treatment (average soil water potential ≈ −80 kPa), plants were maintained until maturity and harvested.

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4.3. Determination of WUE at the Whole Plant Level

Plant water consumed over the growth period until maturity was estimated from the sum of the daily water consumption determined by pot weight as follows:

Whole Plant Water Consumption = (Pot Weight) Field capacity – (Pot Weight) Daily Weight

At the plant maturity stage (BBCH 83), WUE was estimated as the ratio of total above-ground biomass accumulation to total water applied during the experiment, less water lost due to evaporation. WUE was determined as follows:

WUE_{WP} (g L^{-1}) = (dry weight of final biomass) / total water consumed

4.4. Determination of Leaf Water Potential

Leaf water potential was estimated using chlorophyll fluorescence parameters. Chlorophyll fluorescence in leaves was measured in the growth chamber using a portable photosynthesis yield analyzer (MINI-PAM, Walz, Effeltrich, Germany) and a pulse-amplitude-modulated (PAM) fluorometer (TEACHING-PAM, Walz, Effeltrich, Germany). The fluorometer was connected to a Leaf Clip Holder (2030-B, Walz) fitted with a microquantum sensor and a thermocouple for monitoring the leaf temperature and relative air humidity, respectively. These chlorophyll fluorescence parameters include ΦPSI and $F_V/F_M = (F_M – F_0)/F_M$. The F_V/F_M ratio was used to assess stress tolerance under field conditions. This parameter measures the efficiency of excitation energy capture by open PSII reaction centres **([98])**. Variable fluorescence (F_V) was calculated by subtracting F_0 from F_M (Table 2). This parameter measures the efficiency of excitation energy capture by open PSII reaction centres **([98])** and represents the maximum capacity for light-dependent charge separation in PSII. The terms, formulas, and descriptions of the chlorophyll fluorescence parameters used in the study are presented in Table 2.

Leaf water potential was calculated as follow:

$LWP = F_M / F_0$ (kPa) **([99])**.

Where F_M is the maximum yield of Chlorophyll a (Chla) fluorescence measured in a dark-adapted state, and F_0 is the minimum yield of Chla fluorescence measured in a dark-adapted state. kPa is the unit of pressure of leaf water potential in the International System of Units.

At the booting growth stage, water-deficit treatment was imposed for 7 days by withholding watering, followed by re-watering to restore the pots to field capacity. Drought stress levels were monitored with a soil moisture sensor. When the soil volumetric water content dropped to 5% (or at the wilting point), which occurred in three to four days after withholding water. The temperature was raised from 22°C to 35°C for 7 days. The chlorophyll fluorescence parameters were measured before and after the water deficit and temperature treatments, and at the beginning and end of the stress treatments, corresponding to six different growing points: stem elongation (BBCH 30–36 and BBCH 37–40), booting growth stage (BBCH 41–44 and BBCH 45–49), inflorescence emergence (BBCH 50–59 and BBCH 60–69).

At the booting and inflorescence growth stages, measurements were taken in the centre of the flag leaf of each cultivar. Flag leaves of wheat, regarded in crop production as the 'functional leaves', are the main organs for photosynthesis, and contribute 45–58% of photosynthetic performance during the grain-filling stage **([100,101])**. At the stem elongation growth stage, measurements were taken in the centre of the last completely unfolded leaf.

4.5. Determination of Carbon Isotope Discrimination ($\delta^{13}C$) in Flag Leaf

For $\delta^{13}C$ measurement, five flag leaves from each plant subjected to drought and heat stress were collected for $\delta^{13}C$ determination. All flag leaves were harvested and bulked for the determination of $\delta^{13}C$. Leaf samples were air-dried and ground into powder. Subsamples of 2 mg were sent to the Analytical Service Laboratory, Faculty of Agricultural, Life and Environmental Sciences – Renewable Resources (University of Alberta, Edmonton, AB T6G 2J7, Canada) and analyzed for $\delta^{13}C$. The $\delta^{13}C$ v VPDB plant was determined by flash combustion. There are two naturally occurring stable isotopes

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of both carbon ^{12}C (98.89%) and $\delta^{13}C$ (1.11%). An aliquot of the sample was combusted in oxygen, and the carbon in the sample was converted to CO_2, which was separated by chromatography and then analyzed by continuous-flow IRMS. Working standards were calibrated against the International Reference scale (i.e. $\delta^{13}C$ vs. VPDB). Raw data from the mass spectrometer was then referenced to VPDB using a linear regression calculated from the working standard results. Instrument used included a Thermo Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS), Thermo Scientific Inc., Bremen, Germany (2016), Thermo FLASH HT Plus 2000 Organic Elemental Analyzer, and ConFlo IV (for CF-IRMS).

Farquhar and Richards (1984) **[102]** defined $\Delta^{13}C$ as:

$$\Delta^{13}C\ (‰) = \frac{Ra-Rp}{Rp} \times 1000 = \frac{\delta a - \delta p}{1+\delta p} \times 1000 \text{ (Eqn } \mathbf{1}\text{)}$$

where R_a is the $^{13}C/^{12}C$ ratio of CO_2 in air, and R_p is that of plant carbon. In the second form of the equation, δ_a is $\delta^{13}C$ of CO_2 in air, and δ_p is that of plant carbon. The $\delta_a - \delta_p$ refers to the C isotope ratios of atmospheric CO_2 (−8‰) and plant tissue, respectively (**[102]**).

The $\delta^{13}C$ is defined with respect to a standard as:

$$\delta^{13}C_{\text{ sample}}\ (‰) = \frac{R_{sample}-R_{std}}{R_{std}} \text{ Eqn } \mathbf{2}$$

where $\delta^{13}C_{sample}$ is that of the sample of interest, R_{sample} is its $^{13}C/^{12}C$ ratio, and R_{std} is the $^{13}C/^{12}C$ ratio of a standard. The $\delta^{13}C$ values were referenced to a Pee Dee Belemnite standard, which is the internationally accepted standard for expressing stable carbon isotope ratios, with a $^{13}C/^{12}C$ of 0.0112372 (**[103]**). In order to avoid working with very small numbers, Δ and $\delta^{13}C$sample are typically multiplied by 1000 and expressed in parts per thousand (‰).

4.6. Data Analysis

Analyses of variance (ANOVA) were performed for WUEwp, biomass accumulation, and consumed water using SAS, version 10.0 (SAS Institute Inc. *SAS/STAT Software*, Version 10.0; Cary, NC, USA, 2010.Pairwise Pearson correlation analyses were performed to quantify linear associations among continuous variables. Correlation coefficients (r) and corresponding p-values were computed, with statistical significance determined at $\alpha = 0.05$. Pearson's correlation coefficients were estimated with regression analysis. As all correlations were based on the combination of two continuous variables, model II regression was used to estimate the equation parameters (**[104]**). All graphical outputs were produced using the ggplot2 package (Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016. https://doi.org/10.1007/978-3-319-24277-4, v3.4.2).

The Shannon Diversity Index was used to assess genetic diversity within the spring wheat panel. The Shannon Diversity Index was calculated as follows:

$$H' = -\sum(\text{Pi} \times \ln\text{Pi})$$

Where Σ is the summation symbol, ln is the natural logarithm, and Pi is the proportion of the entire panel represented by cultivar i. The higher the value of H', the greater the diversity of cultivars in the panel. A value of $H' = 0$ indicates no diversity. The Shannon diversity index typically ranges from 1.5 to 3.5 and rarely exceeds 4.5 (**[105]**). The maximum possible value depends on the number of cultivars in the panel. All diversity index analyses were performed using Microsoft Excel.

A heatmap was generated using the pheatmap package (Kolde, R. *pheatmap: Pretty Heatmaps (R package)*. CRAN, 2019. https://cran.r-project.org/package=pheatmap) based on z-score-standardized values across traits, employing hierarchical clustering with Euclidean distance and the complete linkage method. Principal component analysis was conducted to explore multivariate relationships among traits after centering and scaling the data. The proportion of variance explained by each principal component was calculated to assess their relative contributions.

5. Conclusions

Whole-plant water-use efficiency was not consistently associated with $\delta^{13}C$, highlighting the influence of both environmental conditions and genetic background on this relationship. Although a

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negative correlation is commonly expected, the weak association observed indicates that $\delta^{13}C$ alone may not serve as a reliable proxy for WUE_{WP} across diverse conditions. The spring wheat panel exhibited limited genetic diversity for WUE_{WP} and heat tolerance, suggesting restricted capacity to cope with increasing heat and drought stress. This limited variation likely contributes to the weak relationship between WUE_{WP} and $\delta^{13}C$ and constrains the effective use of these traits in breeding. Enhancing resilience will therefore require incorporation of novel genetic resources such as landraces, supported by marker-assisted selection and genomic selection approaches.

Multivariate analyses provided complementary insights into trait variation and cultivar performance. Principal component analysis explained 78.2% of total variation and revealed a clear structuring of traits, separating biomass and water use from WUE_{WP} and $\delta^{13}C$. In parallel, chlorophyll fluorescence measurements demonstrated that water deficit and heat stress significantly reduce photosynthetic efficiency, as reflected by declines in F_V/F_M, with responses varying across growth stages and cultivars.

Among analytical approaches, the Shannon Diversity Index was most effective for quantifying genetic diversity, revealing low to moderate variation across key traits. In contrast, PCA and hierarchical clustering were more informative for elucidating relationships among traits and grouping cultivars with similar profiles, while correlation analysis characterized pairwise associations. Together, these methods provide an integrated framework for assessing diversity, understanding trait interactions, and guiding the development of more resilient spring wheat cultivars.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: LJAC conceived the project, designed the experiments and wrote the article. MA and JZ reviewed and made critical revisions to the original project. MI and PH provided the wheat genotypes and edited the manuscript. SC, AE, MH contributed to manuscript editing, critical revisions, and improvement of the discussion. GHR supervised the execution of the project and revised the article. All authors contributed to the article and approved of the final submitted version.

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Conflicts of Interest: The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be interpreted as potential conflicts of interest.

List of Abbreviations

The following abbreviations are used in this manuscript:

ANOVA – Analysis of Variance

BBCH – Biologische Bundesanstalt, Bundessortenamt and CHemical industry scale (growth stage scale)

CF-IRMS – Continuous-Flow Isotope Ratio Mass Spectrometry

Chla – Chlorophyll *a*

CNHR – Canada Northern Hard Red

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CPSR – Canada Prairie Spring Red

CPSW – Canada Prairie Spring White

CWAD – Canada Western Amber Durum

CWES – Canada Western Extra Strong

CWHWS – Canada Western Hard White Spring

CWRS – Canada Western Red Spring

CWSP – Canada Western Special Purpose

CWSWS – Canada Western Soft White Spring

FV – Variable Fluorescence

F_0 – Minimum Fluorescence Yield (dark-adapted state)

FM – Maximum Fluorescence Yield (dark-adapted state)

FV/FM – Maximum Quantum Efficiency of Photosystem II

H′ – Shannon Diversity Index

IRMS – Isotope Ratio Mass Spectrometer

LWP – Leaf Water Potential

PAM – Pulse-Amplitude-Modulated (fluorometry)

PCA – Principal Component Analysis

PSII – Photosystem II

ΦPSI – Quantum Yield of Photosystem I

r – Pearson Correlation Coefficient

SWC – Soil Water Content

VPDB – Vienna Pee Dee Belemnite (carbon isotope standard)

WD – Water-Deficient (treatment)

WUE – Water Use Efficiency

WUE_{WP} – Whole-Plant Water Use Efficiency

Carbon Isotope–Related Terms

$\delta^{13}C$ – Carbon Isotope Composition (relative difference in $^{13}C/^{12}C$ ratio)

$\Delta^{13}C$ – Carbon Isotope Discrimination

R_a – $^{13}C/^{12}C$ Ratio of Atmospheric CO_2

R_p – $^{13}C/^{12}C$ Ratio of Plant Material

Rsample – $^{13}C/^{12}C$ Ratio of Sample

Rstd – $^{13}C/^{12}C$ Ratio of Standard

Statistical / Mathematical Terms

Ln – Natural Logarithm

Σ – Summation Symbol

Pi – Proportion of the *i*th genotype in the population

Units

°C – Degrees Celsius

kPa – Kilopascal (pressure unit)

μmol m^{-2} s^{-1} – Micromoles per square meter per second (light intensity)

g L^{-1} – Grams per liter

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