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Interaction of genotype, water availability, and nitrogen fertilization on the mineral content of wheat grain

Cristina Caldelas [a], Fatima Zahra Rezzouk [a], Nieves Aparicio Gutiérrez [b], Maria Carmen Diez–Fraile [b], José Luis Araus Ortega [a,*]

[a] Section of Plant Physiology, University of Barcelona, Barcelona & AGROTECNIO (Center of Research in Agrotechnology), Lleida, Spain
[b] Agro-Technological Institute of Castilla y León (ITACyL), Valladolid, Spain

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ABSTRACT

The aim of this study was to test the hypothesis that genetic variability is the key driver of mineral concentration in wheat grain in Mediterranean conditions. We grew 12 modern winter wheat varieties in semi-arid conditions and alkaline soils, in two consecutive years of contrasting water availability, and at three rates of *N*-fertilization: 64, 104, and 130 Kg N/ha. The genotype was the main driver of [Ca], [K], [Mg], and [S] in wheat grain, while the environmental conditions were more relevant for [Fe] and [Zn]. The nitrogen fertilization rate had little effect. The thousand-grain weight correlated negatively with the mineral concentration in the grain, revealing the importance of grain shape. CH-Nara grains were highly nutritious making this variety a potential source of germplasm. The knowledge gained from this study will guide future breeding and agronomic practices and guarantee food safety in the region in the advent of climate change.

1. Introduction

Several factors control mineral concentration in wheat grains: environmental factors, agronomic practices, plant traits, and physiological responses. Among the environmental factors, soil properties are key. The mineral composition, pH, clay content, cation exchange capacity, and redox activity of the soil have all been shown to affect nutrient bioavailability (Naidu, Bolan, Megharaj, Juhasz, Gupta, Clothier, & Schulin, 2008), and hence the nutritional content of the grain. The climate is also crucial. Water scarcity during the growth period, especially during grain-filling, increases the concentration of micronutrients in the grain (Zhao, He, Wang, Wang, & Lin, 2009). Further, agronomic practices such as liming to increase soil pH, and fertilizing can also impact the nutrient content of the grain (Chauhan, Sankhyan, Sharma, Singh, & Gourav, 2020). Nitrogen (N) fertilization can increase nutrient mobility in the soil and stimulate root growth, improving nutrient uptake by plants (Rekaby, Eissa, Hegab, & Ragheb, 2019). Nitrogen fertilization can also promote the allocation of N to *N*-rich compounds such as ribulose bisphosphate carboxylase and chlorophylls, hence increasing the plants' photosynthetic performance (Hamnér, Weih, Eriksson, & Kirchmann, 2017). Moreover, N is thought to play an important role in the mechanisms of mineral uptake, root-to-shoot transport, and loading in the grain (Shi et al., 2010).

The nutritional content of wheat grains is further controlled by genetic variability. Tall varieties and landraces typically have higher iron (Fe), copper (Cu), magnesium (Mg), and zinc (Zn) content relative to the semi-dwarf modern varieties (Fan et al., 2008). Even among varieties of the same stature and antiquity, there is great variability from one variety to another (Murphy, Reeves, & Jones, 2008). It is not possible to associate the genetic variability in grain mineral concentration with just a few quantitative trait loci (QTLs) and many minerals seem to locate together, which makes genetic modification for this trait difficult (Wang et al., 2021). The mineral concentration of the grain is controlled by a complex network of interconnected processes, from root uptake to the loading of nutrients and toxic metals in the grain. For this reason, breeding has been proposed as the best strategy to increase the mineral concentrations of wheat grains (so-called biofortification). To breed for more nutritious wheat, it is essential to first identify modern varieties of interest for the nutrient content of their grains, especially Fe and Zn. However, there is very little information in the literature about the nutritional content of the high-yielding wheat varieties that are in regular use in semi-arid environments with alkaline soils. These conditions

* Corresponding author.
E-mail addresses: criscaldelas@ub.edu (C. Caldelas), fz.rezzouk@ub.edu (F.Z. Rezzouk), apagutni@itacyl.es (N. Aparicio Gutiérrez), dieframa@itacyl.es (M.C. Diez–Fraile), jaraus@ub.edu (J.L. Araus Ortega).

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are common in the Mediterranean region, which is home to 480 million people, and are also experienced in many other areas of the world. Considering the yield gap that afflicts regions with similar levels of water scarcity (de Lima, Gracia-Romero, Rezzouk, Diez-Fraile, Araus-Gonzalez, & Kamphorst, 2021), and the potential to improve agricultural productivity and climate change resilience for many communities, this void in our knowledge must be addressed.

This study aims to compare the importance of genetic variability versus water scarcity and N fertilization as drivers of mineral concentration in the grain of high-yielding winter wheat varieties in the Mediterranean climate. Further, we aim to identify varieties with highly nutritious grains and diagnose any nutritional deficiencies and toxicities that may reduce grain yield in the region. To do so, we defined four research objectives:

i) To evaluate grain mineral concentration in 12 bread wheat varieties in response to water scarcity and three different levels of top-dressing *N*-fertilization,
ii) To identify which high-yielding varieties have the highest levels of nutrients and the lowest levels of toxic metals in the grain,
iii) To diagnose potential nutritional deficiencies and toxicities that might affect grain yield and quality locally and in similar settings, and
iv) To examine the associations between the mineral concentration and the yield, yield components, and C and N stable isotopes in the grain.

This research is associated with the European Consortium for Open Field Experimentation (ECOFE), which covers all major climatological regions of Europe. ECOFE aims to develop systematic investigations of the interactions between plant genotype, environment, and agricultural management, i.e., studies of high-yielding wheat varieties across a range of farming practices and locations under highly standardized conditions. For this reason, the present study was performed at one of the project's experimental stations.

2. Hypotheses

The study tested four working hypotheses: 1) The genotypic variability determines the mineral concentration of winter wheat grains grown in Mediterranean conditions; 2) water deficit increases the mineral concentration in the grain; 3) increased N–fertilization increases the concentration of some elements in the grain, especially micronutrients like Fe, Mn, or Zn; and 4) grain yield has a negative correlation with the concentration of micronutrients in the grain. The knowledge gained from this study will contribute to guiding future breeding and agronomic practices in the region and guarantee food safety in the advent of climate change.

3. Materials and methods

3.1. Experimental design

The field trials were conducted at the Castile-Leon Agriculture Technology Institute (ITACyL), in the Experimental Station of Zamadueñas (41° 39́ 8́ N and 4° 43‘24“ W, 690 m.a.s.l.), sited in Valladolid (Spain). The main properties of the soil are listed in Table 1. We surveyed 12 high-yielding winter bread wheat varieties widely cultivated in different European countries and previously tested in Spain: Benchmark, Bennington, Bologna, Chambo, CH-Nara, Henrik, Hondia, JB Diego, Julius, KWS-Siskin, RGT-Reform, and Soberbio (see Table 1 from de Lima et al., 2021). These were grown under three contrasting N fertilization rates: N1 = 64 Kg N ha^{-1}, N2 = 104 Kg N ha^{-1} (optimal fertilization rate), and N3 = 130 Kg N ha^{-1} (Table S.1). The experimental design consisted of a split-plot design with N treatments allocated to the main plot and varieties to subplots. The experiment was replicated in two consecutive seasons, harvested in 2019 and 2020. Each trial consisted of three replicates (individual plots) per genotype (12 varieties) and nitrogen rate (three rates), totalling 108 plots.

Table 1
Main characteristics of the soil. Topsoil samples (0–10 cm) were collected before applying fertilizers and sowing. The number of replicates was n = 3, each replicate corresponding to a soil sample analysed once.

	2018	2019
pH in water (1:5)	8.52	8.62
Clay (%)	17.5	12.0
Sand (%)	35.0	33.0
Silt (%)	48.0	55.0
Texture	loam	silty loam
Phosphorus (mg P_2O_5 Kg^{-1})	64.2	45.4
Total carbon (%)		1.00
Organic carbon (%)	1.35	
Organic matter (%)	2.44	1.17
Total nitrogen (%)	0.065	0.067
Total nitrogen (mg Kg^{-1})	650	670
Nitrates (mg Kg^{-1})		67
Ammonia (mg Kg^{-1})		1.2
Potassium (mg K_2O g^{-1})	0.373	0.250
Potassium (mg Kg^{-1})	373	250
Active lime (%)	4.97	1.66
Electrical conductivity (μS cm^{-1})	117	136
Humidity (%)	1.43	1.62
Carbonates (%)		<3

For the first trial, plots were 12 m long and 1.5 m wide, with 7 rows sown 21 cm apart (totalling 18 m^2 per plot). For the second trial, plots were 8 m long and 1.5 m wide, with 7 rows sown 21 cm apart (totalling 12 m^2 per plot). The trials were sown on 29/11/2018 (first trial) and 28/10/2019 (second trial), at a rate of 450 seeds per m^2. Phytosanitary treatment included the spraying of fungicides (Amistar, Syngenta, Basel, Switzerland; Prosaro, Bayer, Leverkusen, Germany), insecticide (Karate Zeon, Syngenta), and herbicide (Amadeus top, Syngenta) at the recommended rates during the elongation, booting, and heading stages as needed. Plants reached maturity in the second half of June. Plots were harvested mechanically on 17/07/2019 for the first trial and 21/07/2020 for the second trial. During the first trial, the temperatures ranged from −7.7 to 40.7 °C and the daily mean was 10.3 °C (Fig. 1). Rainfall was very scarce with only 158 mm accumulated over the growing season, so the conditions of this trial were those of intense drought. During the second trial, the temperatures ranged from −4.8 to 36.2 °C, the daily mean was 11.2 °C, and the accumulated rainfall was 469.8 mm, which represented an average year.

3.2. Yield and yield components

At ripening, the plots were harvested mechanically, and grain yield (GY) was determined for each plot and adjusted to a 10 % moisture rate. A grain subsample was taken from each plot to determine the thousand-grain weight (TGW). For the first trial, the grain weight per spike (GW_spike), the number of grains per spike (NGSP) and the number of spikes per area (NSPM2) was determined in subsamples of 10 plants. For the second trial, the data set for these parameters was only determined in the N2 treatments.

3.3. C and N content and stable isotopes

Grain samples were dried at 60 °C until constant weight and finely ground using a ball mill (MM 400, Retsch GmbH, Haan, Germany). Approximately 1 mg of the powdered samples were weighed in tin capsules (3.3–5 mm, Cromlab, Barcelona, Spain). The samples were taken to the facilities of the Scientific and Technical Centres of the University of Barcelona (CCiTUB, Barcelona, Spain) for C and N analyses. The total C and N content and $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were determined using an Elemental Analyser (EA) interfaced with an Isotope

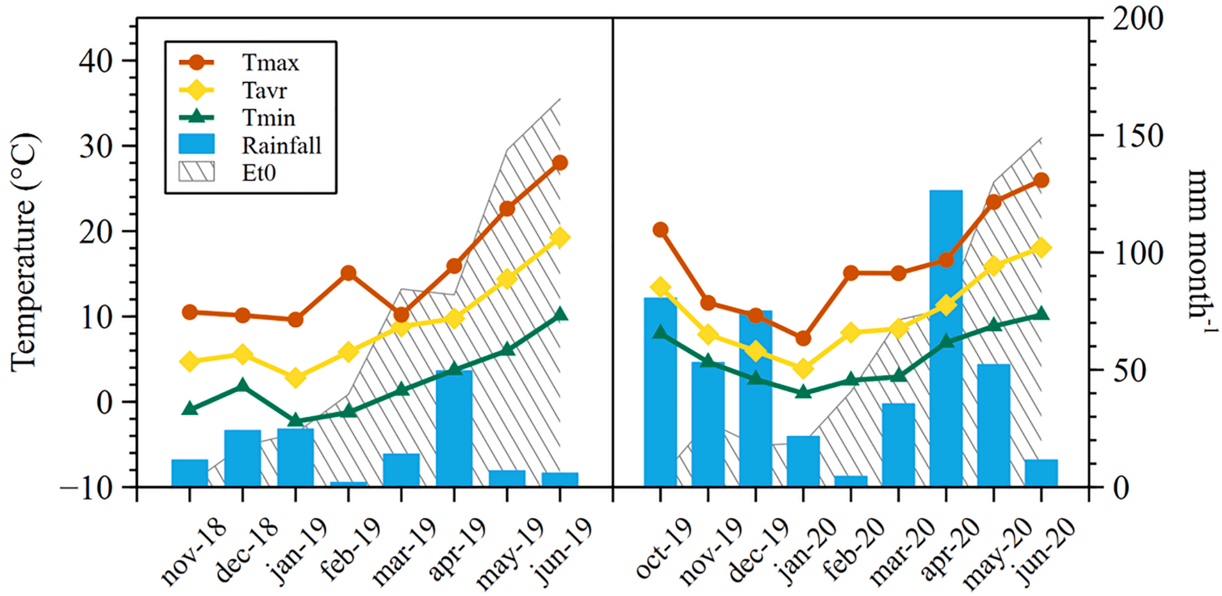

Fig. 1. Monthly temperatures, accumulated rainfall and evapotranspiration recorded during the two field trials. Tmax = monthly maximum temperature; Tavr = monthly mean temperature; Tmin = monthly minimum temperature; Rainfall = monthly accumulated rainfall; Et0 = monthly accumulated evapotranspiration calculated by the Penman-Monteith method. Climatic data were collected from a meteorological station located at the experimental site, managed by the SIAR (Service of Agroclimatic Information for Irrigation, www.siar.es).

Ratio Mass Spectrometer (IRMS). For the first trial this was a Flash EA-1112-DeltaC IRMS and for the second trial a Flash EA-IRMS-Delta-V Advantage (both ThermoFisher Scientific, Waltham, MA, USA). Total C and N contents in grains were expressed as the percentage (%) on a dry matter basis. Stable isotope values were expressed in delta notation (δ), following equation (1):

$$\delta X_{sample(‰)} = \left[\frac{R_{sample}}{R_{standard}} - 1\right] \times 1000$$

Where δX_{sample} is the isotope composition of the sample expressed in ‰ ($\delta^{13}C$ or $\delta^{15}N$); R_{sample} is the isotope ratio of the sample ($^{13}C/^{12}C$ or $^{15}N/^{14}N$), and $R_{standard}$ is the isotope ratio of the secondary standard calibrated against the primary standard. The primary standards were Pee Dee Belemnite for C and N_2 from the air for N. The secondary standards for C were IAEA–CH7 (International Atomic Energy Agency, Austria), UCGEMA K, UCGEMA CH, and fructose (in–house standards); and for N, IAEA N1, IAEA 600, UCGEMA K, UCGEMA CH, and UCGEMA. The analytical precision of the $\delta^{13}C$ and $\delta^{15}N$ analyses was 0.2 ‰.

3.4. Mineral concentration analyses

To determine the mineral concentration in the grain, 0.1 g of wheat flour were weighed in Teflon® beakers and digested in 2 ml HNO_3 and 1 ml H_2O_2 at 90 °C overnight at the CCiTUB. Digests were diluted in 30 ml of MilliQ water (18.2 Ω) and refrigerated until analysis. The mineral concentration in the digests was determined by Induced Coupled Plasma Optical Emission Spectrometry for Ca, Fe, K, Mg, Na, P, S (ICP-OES, Optima 8300, Perkin Elmer, Waltham, MA, USA) and for Si (Optima 3200rl, Perkin Elmer); and by Induced Coupled Plasma Mass Spectrometry for B, Cd, Cu, Mn, Na, Pb, and Zn (Elan6000, Perkin Elmer). For every 11 samples a procedural blank and an aliquot of certified reference materials were analysed in the same manner. The reference materials were BCR–60 (aquatic plant, Joint Research Centre, Brussels, Belgium), BCR-62 (olive leaves, JCR), and BCR–279 (sea lettuce, JRC). Recoveries were 90–94 % for Cd, Cu, Mn, Pb, and Zn relative to the certified values. The digestion beakers were acid-washed and rinsed in Milli-Q water before use. The quality of the acids was 69–70 % Baker Instra – Analyzed Reagent 9598.34 (Thermo Fisher Scientific, Waltham, MA, USA) for HNO_3, and 30 % Suprapur 1.07298.1000 (Merck, Darmstadt, Germany for H_2O_2. Plastic tubes and caps were rinsed three times in the digest before filling them for analysis.

3.5. Statistical analyses

The statistical analyses were conducted using R software version 3.4.0 for Windows (R Core Team, 2013) with the FSA package version 0.8.2660. For each dependent variable, the best mixed model was selected using the Akaike Information Criterion (AIC) and a three-way ANOVA was applied to the model with the lowest AIC score (Table S.2). We screened the data for outliers using Rosner's test and removed them from the dataset before analyses. In some cases, the conditions of normal distribution and equal variances were not met completely. Following Knief and Forstmeier (2021), we trusted the robustness of the three-way ANOVA test. To separate statistically significant groups, we used Tukey's post-hoc contrasts. The non-parametric Kruskal-Wallis (KW) ranks test and Dunn's test with Benjamini-Hochberg adjustment (Dunn) were also applied to each factor separately, to confirm the results of the ANOVA and Tukey's test. The scientific plotting package Veusz 1.23.2 (Sanders, 2015) was used to create the graphs.

4. Results

4.1. Yield and yield components

The grain yield (GY, t ha^{-1}) was strongly affected by the year, the variety, and their interaction (all three $P < 0.001$) (Table 2). The mean GY was 3.3x higher in 2020 than in 2019 (2.5 ± 0.1 t ha^{-1} vs 8.3 ± 0.1 t ha^{-1}, mean of all varieties and nitrogen treatments), and this was likely due to the drought. The variety Soberbio was the best performer across the two years, with a mean GY of 6.1 ± 0.8 t ha^{-1}. By contrast, CH-Nara had the lowest GY (4.3 ± 0.5 t ha^{-1}), which was 30 % lower on average than Soberbio. The interaction year:variety showed that the yields of CH-Nara and Bologna did not increase as much as other varieties in response to the increased rainfall in 2020 (Fig.S.1). Similarly, the thousand–grain weight (TGW, g) was strongly affected by the year ($P < 0.001$), the variety ($P < 0.001$), and their interaction ($P = 0.005$). The year:nitrogen interaction had some effect on TGW as well, but was less

Table 2
Grain yield (GY) and thousand-grain weight (TGW) averaged per variety, year of sowing, and nitrogen treatment. Values are reported as means ± standard errors. The number of replicates was n = 60, 71, and 69 for nitrogen treatments N1, N2, and N3 respectively; n = 17 for all cultivars, except Hondia (16) and Bologna (14); and n = 107 and 93 for the 2018/2019 and 2019/2020 growing seasons, respectively. Different letters indicate statistically significant differences between groups according to Tukey's post-hoc test.

	GY (t ha^{-1})	TGW (g)
genotype		
Benchmark	5.13 ± 0.83^{bcde}	30.7 ± 1.5^{abc}
Bennington	5.67 ± 0.88^{efg}	33.8 ± 1.2 ^{cd}
Bologna	4.35 ± 0.58^{a}	24.8 ± 1.5^{a}
Chambo	5.57 ± 0.75^{defg}	32.7 ± 1.5^{bcd}
CH-Nara	4.33 ± 0.50^{a}	29.5 ± 1.0^{a}
Henrik	5.96 ± 0.82 ^{fg}	35.1 ± 0.9^{d}
Hondia	4.88 ± 0.77^{abc}	34.4 ± 1.2^{d}
JB Diego	5.25 ± 0.82^{cdef}	33.2 ± 1.0^{bcd}
Julius	4.46 ± 0.67^{ab}	33.3 ± 1.1^{bcd}
KWS Siskin	5.66 ± 0.80^{defg}	30.4 ± 1.3^{ab}
RGT Reform	5.00 ± 0.73^{abcd}	32.4 ± 1.3^{abcd}
Soberbio	6.07 ± 0.78 ^{g}	34.2 ± 1.7^{d}
year		
2018/2019	2.53 ± 0.08^{a}	28.2 ± 0.4^{a}
2019/2020	8.29 ± 0.13^{b}	36.6 ± 0.3^{b}
nitrogen		
N1	4.60 ± 0.37^{a}	30.9 ± 0.8^{a}
N2	5.40 ± 0.37^{a}	32.5 ± 0.7^{a}
N3	5.53 ± 0.38^{a}	32.8 ± 0.6^{a}
year	<0.001***	<0.001***
nitrogen	ns	ns
genotype	<0.001***	<0.001***
year:nitrogen	ns	0.04
year:genotype	<0.001***	<0.01**
nitrogen:genotype	ns	ns
year:nitrogen:genotype	ns	ns

significant ($P = 0.04$). Similar to GY, the mean TGW was higher in 2020 (36.6 ± 0.3 g) than in 2019 (28.2 ± 0.4 g), but this reduction was much smaller than for GY. The varieties Hondia, Soberbio, and Henrik reached the highest TGW at an average of 34.4 ± 1.2, 34.1 ± 1.7, and 35.1 ± 0.9 g, respectively. By contrast, CH-Nara had the lowest mean TGW (29.5 ± 1.0 g), which was 16 % lower than Henrik. The interaction year:variety indicated that Soberbio had a much larger increase in TGW in 2020 than the other varieties. Further, the interaction year:nitrogen indicated that N3 led to the highest TGW in 2019, but the lowest in 2020. In view of the low contribution of the TGW to the increase in GY in the second season, we compared the grain weight per spike (GW_spike), the number of grains per spike (NGSP), and the number of spikes per area (NSPM2) for both years, and calculated the number of grains per area (NGM2) (Table S.3). The four variables increased more than the TGW between 2019/2020 and 2018/2019, and particularly the NGM2 (177 %).

4.2. Element content – Effect of the variety, year, and the N rate.

The variety was the most important factor to explain the element content in the grain. When the data from 2019 and 2020 were analysed together, the variety had a significant effect on all the elements quantified except C, Na, and Pb (Fig. 2). Overall, CH-Nara showed the highest mean content for Ca, Fe, Mg, Mn, N, P, S, and Zn. Moreover, KWS-Siskin had the highest K, Bologna the highest Cu, and both Bennington and JB Diego the highest B content. Regarding the toxic metals, Cd content was highest in Hondia and lowest in Benchmark. The year of the trial was the second most important factor affecting the mineral concentration in the grain. The grain collected in 2019 during an intense drought showed higher contents of B, C, Ca, Cd, Cu, Fe, K, N, S, and Zn than the grain collected in 2020, which was a typical year. The same was probably true for Na, although the results were only significant according to the KW test ($P < 0.001$). By contrast, Mg was higher in the 2020 samples. The Mn, P, and Pb contents were also higher in 2020 according to the KW test ($P < 0.001$), although the ANOVA was not significant ($P = 0.12$, 0.09, and 0.25, respectively).

The N fertilization treatment had no effect on the mineral concentration of the grain when the two years were analysed together (Fig. 2). However, some interactions with the year or the variety were significant. The interaction year:variety was significant for K, Mg, Ca, Fe, Mn, Cu, and Cd (interactions for minerals not shown for the sake of brevity, available upon request). This interaction is indicative of the varieties that were most affected by water scarcity and consequently showed a greater variation in mineral concentration between the two years. JB Diego had the greatest reduction in Cu, Fe, Mg, and Mn contents in 2020 relative to 2019. Bologna showed the strongest reduction in K and the highest increase in Cd in 2020. Benchmark increased Ca content in 2020, while the other varieties decreased it or maintained it. By contrast, the K content of Julius grains decreased the least in 2020 compared to the other varieties. Further, the interaction year: nitrogen enabled determination of the N treatments that best preserved the nutritional value of the grain each year. This interaction was only significant for K and P, which were supplied together with N in the NPK fertilizer. Plants grown in the N3 treatment did not have such notable reductions in K content in 2020. Besides, plants grown with the N2 and N3 treatments showed an increase in P content in 2020. Finally, the interaction nitrogen:variety was only significant for S. Most varieties had a higher S content under the N1 treatment, except for the following: RGT-Reform and KWS-Siskin (N3), and Julius and Chambo (N2).

Next, we analysed the data for each year separately to identify any year-specific trends that did not appear in the joint analysis. In 2019, the variety had a significant effect on the grain content of all the elements analysed except B, C, Cd, N, P, Pb, and Zn (Tables S.4–6). The variety CH-Nara showed the highest mean Ca, Mg, Mn, and S in 2019. Furthermore, KWS-Siskin had the highest K and Bologna and Julius the highest Cu. For Na, the ANOVA showed a significant effect of the variety ($P = 0.04$) that was not present in 2020. However, the post hoc tests did not separate any statistically different groups. Finally, the P in the grain in 2019 decreased with increasing nitrogen ($P = 0.04$), again an effect that was only observed during that year. In 2020, the variety had a significant effect on all the elements analysed except C, Cd, and Na (Tables S.8–10). The variety CH-Nara showed the highest mean Ca (together with Benchmark), Fe, Mg (together with Soberbio), Mn, N, P, S, and Zn. Moreover, KWS-Siskin had the highest K, Bologna the highest Cu, and Bennington the highest B. Interestingly, in 2020 the variety had a significant effect on Pb accumulation in the grain ($P = 0.04$), but this was not evident when the two years were pooled together. The Pb content was highest in Bologna grains and lowest in Hondia, Bennington, and Soberbio. Besides, the interaction nitrogen:variety was significant for Pb ($P < 0.001$) due to Bologna showing an increased Pb in response to the N3 treatment. Finally, in 2020 the Cd accumulation in the grain increased with increasing N fertilization ($P = 0.002$).

4.3. Stable isotopes

The $\delta^{13}C$ was isotopically lighter in 2020 than in 2019 ($P < 0.001$) (Table 3). Additionally, there were clear differences between varieties ($P < 0.001$), with CH-Nara showing the highest $\delta^{13}C$. By contrast, the $\delta^{15}N$ was isotopically lighter in 2019 ($P < 0.001$) (Table 3) and did not differ across varieties. The N fertilization treatment had a significant effect on $\delta^{15}N$ ($P = 0.013$). However, the post hoc tests did not separate any significant groups (Table 3). The interaction year:variety seemed significant for $\delta^{13}C$ ($P = 0.049$) and the interaction year:nitrogen was significant for $\delta^{15}N$ ($P = 0.0104$). However, a closer inspection of the interaction plots did not reveal any biologically relevant effect (results not shown, available upon request). When the two years were analysed

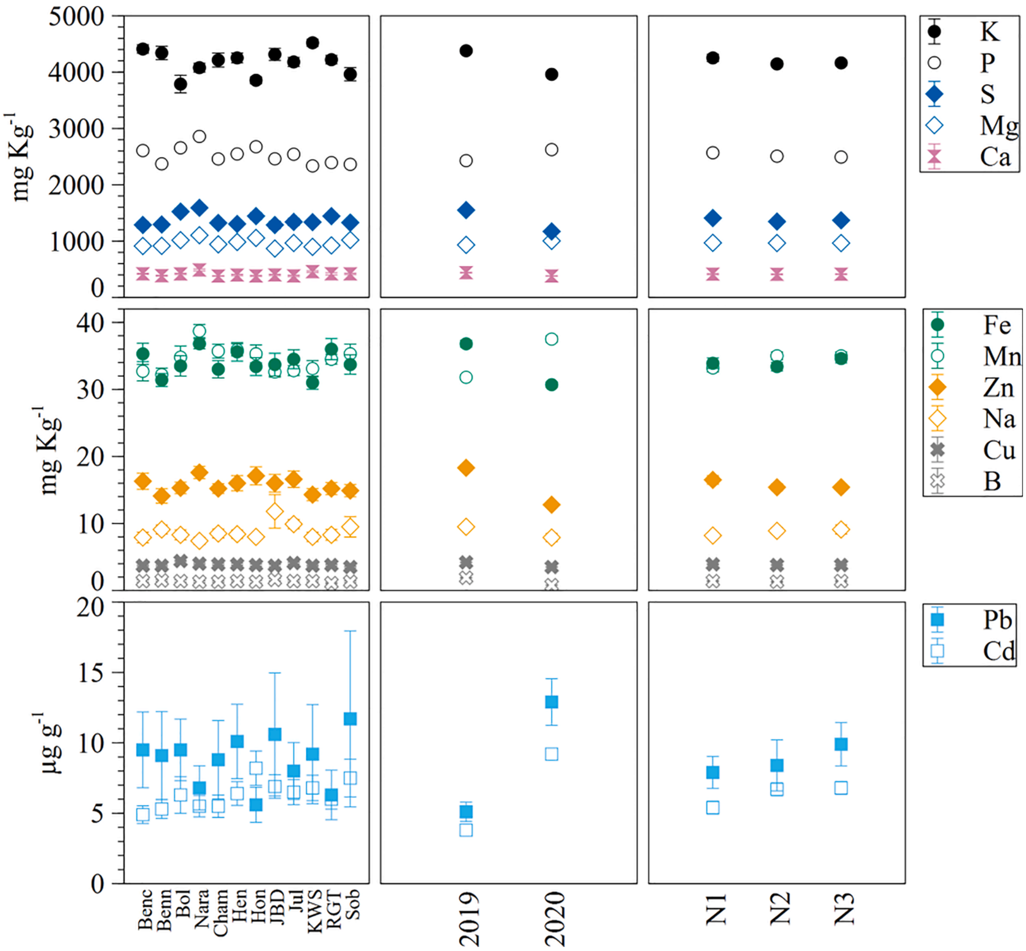

Fig. 2. Mineral content in the grain per wheat variety (left) and year of sowing (right). Values are reported as means ± standard errors. The rates of nitrogen fertilization were 64 (N1), 104 (N2), and 130 (N3) Kg N/ha. The abbreviations for the genotypes are Benc (Benchmark), Benn (Bennington), Bol (Bologna), Nara (CH-Nara), Cham (Chambao), Hen (Henrik), Hon (Hondia), JBD (JB Diego), Jul (Julio), KWS (Kawasaki Siskin), RGT (RGT Reform), and Sob (Soberbio). The number of replicates was n = 17 for all cultivars, except Hondia (16) and Bologna (14); and n = 107 and 93 for the 2018/2019 and 2019/2020 growing seasons, respectively. Each replicate consisted of one individual plant analysed once. The results of the three-way ANOVA are presented in supplementary Table S.4.

separately, we still observed significant differences in the δ^{13}C between varieties ($P < 0.001$), with CH-Nara having the highest δ^{13} in both in 2019 and 2020 (Tables S.7 and S.10). By contrast, δ^{15}N only showed significant differences between varieties in 2020 ($P = 0.013$), when CH-Nara had the highest δ^{15}N. Further, δ^{15}N decreased with increasing nitrogen fertilization in 2019 alone ($P = 0.0011$).

4.4. Correlations

The physiological and agronomic parameters used in this study displayed several significant correlations with the mineral concentration of the grain (Fig. 3). The GY, TGW, and δ^{15}N correlated negatively with B, C, Ca, Cu, Fe, K, N, S, and Zn. The same three parameters showed positive correlations with Cd and Mn, and even with Mg and Pb if we include the weak correlations. By contrast, the δ^{13}C and C% showed exactly the opposite trends as GY, TGW, and δ^{15}N. Furthermore, we observed several correlations between the nutrients, which were generally positive, except when either Cd, Pb, or Mn were involved. Sulfur and N correlated positively with Cu, Zn, and B. Zinc and Cu were also strongly correlated, as were P and Mg. All the correlations described in this paragraph were highly significant ($P < 0.001$).

When the correlations were calculated separately for 2019 and 2020, the results obtained were different. In 2019, even fewer correlations were observed between the agronomic and physiological parameters and the mineral concentration in the grain (Fig. S.1). The GY only showed a moderate negative correlation with Zn (–0.341, $P < 0.001$) and no relevant associations were observed for TGW. In terms of the stable isotope composition, the δ^{13}C correlated positively with Cu, Mn, Mg, N, P, S, and Zn (0.3–0.6, $P < 0.001$) and the δ^{15}N correlated positively with N, P, S, and Zn (0.3–0.4, $P < 0.01$). Besides, we observed several positive correlations between nutrients. P correlated with B, C, Cu, Fe, K, Mg, Mn, N, S, and Zn (0.4–0.8, $P < 0.001$); S with C, Ca, Cu, Fe, Mg, Mn, N, P, and Zn (0.4–0.7, $P < 0.001$); and N with C, Cu, K, Mg, Mn, P, S, and Zn (0.3–0.7, $P < 0.001$). Further, both Fe and Mg correlated positively with Cu, Zn, and Mn (0.3–0.6, $P < 0.01$), and Zn and Cu had a strong positive correlation (0.70, $P < 0.001$). In 2020, the GY only showed a moderate negative correlation with P (-0.304, $P < 0.01$) and a moderate positive correlation with TGW (0.389, $P < 0.001$) (Fig.S.2). The TGW correlated negatively with the concentration of Ca, P, S, Cu, Zn, Fe, and N in grains (-0.3 to −0.6, $P < 0.001$). Regarding the isotopic signatures, δ^{13}C correlated negatively with the nutrients N, S, P, and Zn (-0.3 to −0.6, $P < 0.001$), while δ^{15}N did not show any relevant associations. Finally, in 2020, all minerals correlated positively with one another except B, Cd, K, Pb, and Na.

5. Discussion

5.1. Nutritional value of the grain. Potential deficiencies and toxicities

The mineral concentrations in this study are in line with the literature on wheat cultivated in semi-arid environments for B, Cd, Cu, Fe, K, Mg, Mn, Na, Pb, and Zn (Ciudad-Mulero, Barros, & Fernandes, 2020; Marcos-Barbero, Pérez, Martínez-Carrasco, Arellano, & Morcuende, 2021; Nyachoti, Adebayo, & Godebo, 2021). The mean [Cu], [Fe], [K], [Mg], [Mn], and [P] in the grain are enough to make nutritional food claims following Regulation No 1169/2011 (EU., 2011), and to consider grain weight as a foodstuff valuable for human nutrition. This agrees with Ciudad-Mulero et al. (2020), who found high [Cu], [Fe], [K], [Mg],

Table 3
Carbon and nitrogen content and stable isotope composition in wheat grains averaged per variety and year of sowing. Values are reported as means ± standard errors. The number of replicates was n = 60, 71, and 69 for nitrogen regimes N1, N2, and N3 respectively; n = 17 for all cultivars, except Hondia (16) and Bologna (14); and n = 107 and 93 for the 2018/2019 and 2019/2020 growing seasons, respectively. Different letters indicate statistically significant groups according to Tukey's post-hoc test.

	C (%)	N (%)	δ^{13}C (‰)	δ^{15}N (‰)
genotype				
Benchmark	40.3 ± 0.99^{a}	2.19 ± 0.14^{a}	−24.11 ± 0.56^{abc}	2.09 ± 0.19^{a}
Bennington	41.6 ± 0.75^{a}	2.14 ± 0.14^{a}	−24.37 ± 0.59^{ab}	1.97 ± 0.16^{a}
Bologna	42.4 ± 0.53^{a}	2.43 ± 0.17^{a}	−23.89 ± 0.63^{d}	1.72 ± 0.21^{a}
CH-Nara	41.5 ± 0.64^{a}	2.54 ± 0.11^{b}	−23.51 ± 0.59^{d}	2.11 ± 0.17^{a}
Chambo	41.4 ± 0.69^{a}	2.19 ± 0.13^{a}	−24.19 ± 0.64^{abc}	1.69 ± 0.20^{a}
Henrik	41.7 ± 0.54^{a}	2.20 ± 0.12^{a}	−24.06 ± 0.54^{abc}	1.99 ± 0.21^{a}
Hondia	41.6 ± 0.54^{a}	2.14 ± 0.13^{a}	−24.05 ± 0.60 ^{cd}	1.89 ± 0.23^{a}
JB Diego	41.5 ± 0.58^{a}	2.23 ± 0.14^{ab}	−24.09 ± 0.63^{abc}	2.13 ± 0.17^{a}
Julius	42.0 ± 0.54^{a}	2.39 ± 0.15^{ab}	−23.85 ± 0.57 ^{cd}	2.15 ± 0.18^{a}
KWS Siskin	41.0 ± 0.60^{a}	2.20 ± 0.12^{a}	−24.33 ± 0.53^{abc}	2.03 ± 0.18^{a}
RGT Reform	41.8 ± 0.59^{a}	2.39 ± 0.12^{ab}	−23.98 ± 0.57^{bcd}	2.06 ± 0.19^{a}
Soberbio	41.4 ± 0.64^{a}	2.24 ± 0.14^{ab}	−24.49 ± 0.60^{a}	2.03 ± 0.17^{a}
year				
2019	43.3 ± 0.15^{b}	2.67 ± 0.036^{b}	−21.95 ± 0.057^{b}	1.46 ± 0.057^{a}
2020	39.5 ± 0.22^{a}	1.82 ± 0.030^{a}	−26.53 ± 0.049^{a}	2.61 ± 0.037^{b}
nitrogen				
N1	41.9 ± 0.3^{a}	2.35 ± 0.08^{a}	−23.8 ± 0.31^{a}	2.07 ± 0.08^{a}
N2	41.8 ± 0.23^{a}	2.23 ± 0.06^{a}	−24.3 ± 0.28^{a}	2.04 ± 0.09^{a}
N3	40.9 ± 0.41^{a}	2.25 ± 0.06^{a}	−24.1 ± 0.28^{a}	1.88 ± 0.10^{a}
year	<0.01**	<0.001***	<0.001***	<0.001***
nitrogen	ns	ns	<0.01**	<0.05*
genotype	ns	<0.001***	<0.001***	ns
year:nitrogen	ns	ns	ns	<0.05*
year:genotype	ns	ns	ns	ns
nitrogen: genotype	ns	ns	ns	ns
year:nitrogen: genotype	ns	ns	ns	ns

[Mn], and [Zn] in wholemeal flour from wheat grown in similar conditions to ours. However, many samples in our study did not reach the nutritional claim threshold for Zn (15 mg Kg^{-1}). Moreover, some samples were below the nutritional deficiency thresholds for Fe, P or Zn in wheat (21 mg Kg^{-1}, 2000 mg Kg^{-1}, and 16 μg Kg^{-1}, respectively) (Celletti, Pii, Mimmo, Cesco, & Astolfi, 2016, Reuter & Robinson, 1997). The soil pH was high, which is the main cause for low Fe, P, and Zn bioavailability (Rengel, 2015). This factor alone can fully explain the low Fe, P, and Zn concentrations in some of our grain samples. The EDTA-extractable Zn of these soils is 1.3 mg Kg^{-1}, and in fact soils with < 1.5 are considered Zn-deficient (De Groote, Tessema, Gameda, & Gunaratna, 2021). Moreover, Zn and Fe in the grain are mostly stored in the aleurone layer, bound to phytate (a phosphate-rich compound) and sequestered in the protein storage vacuoles (Borrill et al., 2014). Therefore, P deficiency in wheat can reduce [Fe] and [Zn] in the grain.

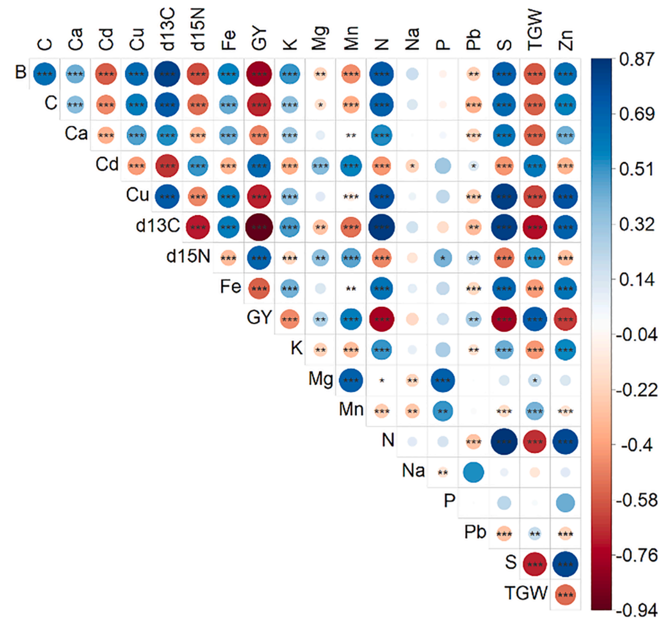

Fig. 3. Pearson's correlation matrix for wheat grain mineral content, grain yield (GY), thousand-grain weight (TGW), δ^{13}C (d13C), and δ^{15}N (d15N) in both the 2018/2019 and 2019/2020 growing seasons. Significance levels are reported as P < 0.001 (***), P < 0.01 (**), and P < 0.05 (*).

Finally, the high GY of modern wheat varieties causes a dilution effect in the grain that reduces the nutrient concentration, a trend that started several decades ago (Fan et al., 2008). Fertilization with P, Zn, and Fe could prove a useful strategy to improve wheat nutritional status in our region, and in general to increase GY and quality in wheat grown in alkaline soils and semi-arid conditions (Sánchez-Rodríguez et al., 2021).

The safety threshold for [Cd] in wheat flour is < 0.2 mg Kg^{-1} (FAO/WHO, 2018). In this study, [Cd] in the grain was always very low and within the legal limits. Moreover, [Cd] increased with GY, and was higher in some varieties, but was not affected by the N fertilization rate. Ata-Ul-Karim and co-workers (2020) reported that [Cd] in wheat grains increased with increasing N fertilization, reaching up to 0.26 mg Kg^{-1}. The conditions of their experiment were very different to ours: acidic soil (pH 6.0), humid climate (>1800 mm year^{-1}, 78 % humidity), and higher N application rates (up to 300 Kg N ha^{-1}). The semi-arid climatology, alkaline soil, and lower N rates in our experiment might have lowered Cd loading in the grain, by either reducing Cd availability in the soil or limiting bulk flow. Of the varieties tested, Benchmark showed the lowest Cd contents in the grain and could be interesting as a source of germplasm for this trait. The [Pb] limit for wheat is also < 0.2 mg Kg^{-1} (FAO/WHO, 2018). In most of our samples, Pb was either very low or below the detection limit. However, we found a few samples with abnormally high [Pb] (≈ 0.1 mg Kg^{-1}). This can be caused by random Pb sources in the soil, like old birdshot. Still, all the samples were within the safe limit and none of the varieties stood out for having high [Pb] in the grain.

5.2. Effect of the genotype on the mineral concentration in the grain

The genotype was the factor that most influenced mineral concentration in the grain. The 12 high-yielding varieties were selected because they are popular with farmers from different European regions. They adapt well to our conditions according to our previous studies (Fernandez-Gallego et al., 2019; de Lima et al., 2021). Unfortunately, none of the highest-yielding varieties stood out for their highly nutritious grains. The variety CH–Nara showed the highest mineral concentration overall, while its yield was low compared to the other varieties. Developed in Switzerland, CH–Nara produces top-quality grain with high protein content. This variety could be an interesting source of germplasm to increase the grain mineral concentration of future wheat cultivars. Since we found no records in the literature reporting nutrient levels in the grain for this variety, we compared our results with other genotypes grown in similar environments. Marcos-Barbero et al. (2021) recently examined the mineral composition of grain from 10 genotypes cultivated in growth chambers simulating Mediterranean conditions. As compared to the genotype with the highest concentration for each nutrient in that study, CH-Nara had higher [Ca], [Fe], and [S] (26 %, 62 %, and 16x more, respectively); lower [Cu], [Mg], [P], and [Zn] (25 %, 16 %, 48 %, and 54 % less, respectively); and similar [B], [K], and [Na]. It must be noted that the S content in wheat grains in Marcos-Barbero et al. (2021) was exceptionally low. Similarly, in comparison to the maximum values for the variety Cajeme (Ciudad-Mulero et al., 2020), CH-Nara showed higher [Ca], [Fe], [K], and [Mn] in grains (2.6x, 47 %, 3.3x, and 6.5x more, respectively); lower [Cu], [Na], and [Zn] (60 %, 98 %, and 41 % less, respectively); and a similar [Mg]. Further, Vázquez, Efraín, José, and Elena (2018) reported the [Fe] and [Zn] in the grain for several landraces and commercial wheat varieties in a wide range of soil types. The average [Fe] values reported here for CH-Nara are within the range given for both landraces and commercial varieties. However, the [Zn] was much lower (91 % and 72 % less Zn, respectively for landraces and commercial wheat) (Vázquez et al., 2018). In summary, CH-Nara grains are high in Ca and Fe, but low in Zn, in comparison to other wheat varieties in similar conditions.

The effect of the genotype was not equally strong for all the minerals analysed: Ca, K, Mg, and S differed greatly between varieties; B, Fe, P, and Zn showed a low variability across the genotypes; and Na and Pb did not show any statistical differences between varieties. For B, Fe, Na, P, Pb, and Zn, we propose that the environmental conditions (e.g., soil pH) might be more relevant as drivers of their accumulation in the grain. Marcos-Barbero et al. (2021) found that the variation in B, Fe, Na, and Zn across 10 wheat genotypes did not reach statistical significance. Guttieri and co-workers (2015) reported that the heritability of the mineral concentration in the grain was high for Cd, Li, and Ca; moderate for Ni, Mn, Cu, and Fe; and low for Zn. The same authors observed very low heritability for the mineral concentration of the grain in one of the trial sites. The genetic basis underlying the diversity in grain mineral concentration across genotypes is currently poorly understood. However, some alleles have been linked to increased mineral allocation to the grain. In Swedish spring wheat, varieties carrying the wildtype allele of the gene *NAM-B1*, involved in leaf senescence and nutrient remobilization to the grain, accumulate more P and K in the grain (Asplund, Bergkvist, Leino, Westerbergh, & Weih, 2013). Recently, a genome-wide association study on 205 winter wheat accessions identified 101 loci affecting the content of Ca, Fe, Zn, Se, Cu, Mn, Cd, As, and Pb in the grain (Wang et al., 2021). In summary, our results and the literature suggest that there is a strong influence of the genotype on the mineral concentration in the grain, but also that the environment can be more relevant for some locations and minerals. A strategy of plant breeding considering the environmental constraints at the local level might be the most effective way to maximize the nutritional value of modern wheat varieties while maintaining yield.

5.3. Relationship of the yield and yield components with the mineral concentration in the grain

To understand which traits might lead to increased mineral accumulation in some varieties but not in others, we will now discuss the relationship between the mineral concentration in the grain and the GY yield components. An increase in GY is generally followed by a decrease in the mineral concentration (Fan et al., 2008). This agrees with our results because the mineral concentration in the grain decreased with increasing GY and TGW when the two years were analysed together. Further, the variety with the highest mineral concentration (CH-Nara) also had the lowest GY. However, the GY and the mineral concentration did not strongly correlate when we separated the data from different years. Moreover, the TGW correlated negatively with several nutrients in 2020 (the normal year) but not in 2019 (the dry year). The most suitable explanation is the location of the minerals in the grain. The mineral concentration is higher in the outer layers of the grain than in the starchy endosperm (Borrill et al., 2014). Therefore, the varieties with bigger and rounder grains due to their increased starch accumulation will have higher TGW but a lower nutrient concentration. A higher TGW does not always lead to higher GY. The total number of grains produced, rather than the TGW, is usually more important for determining GY. As we have seen, the GW_spike, NSPM2, NGSP, and NGM2 increased greatly in 2020. This could explain why, within each year, the GY generally did not strongly correlate with the mineral concentration of the grain. The total weight of the grains per spike recorded in the first trial followed the same trend as the GY. Hence, we propose that the mineral concentration of wheat grain in our conditions is mostly influenced by the shape of the grain, which can be a trait associated with the variety. However, this effect disappears under drought, as can be seen by the absence of correlations of TGW with the minerals in the grain in 2019. The most likely reason for this is that grain filling could not be completed in these unfavourable conditions, reducing the roundness of the grains in all varieties. If our hypothesis is correct, a good strategy to tackle the dwindling nutritional value of wheat crops could be to breed for long grains that maintain a high surface to volume ratio even after grain filling.

5.4. Effect of the N fertilization rate on the mineral concentration in the grain

There is conflicting evidence in the literature about the effect of N fertilization on the mineral concentration in wheat grains. Nitrogen enhances root growth and the synthesis of storage proteins, increasing the plant's capacity to acquire nutrients and accumulate them in the grain (Rekaby et al., 2019; Zhen et al., 2020). However, N fertilization also increases yield, causing a dilution effect when the proportion of starch increases relative to the nutrients accumulated (Smith, Janzen, & Ellert, 2017). Field data show that increasing the dose of N fertilizer can promote the accumulation of minerals in the grain in some instances (Svecnjak, Jenel, Bujan, Vitali, & Dragojević, 2013), decrease it in others (Smith et al., 2017; Andruszczak, 2018), or have little influence (Jaskulska et al., 2018). The N fertilization rates tested in our study barely had any effect on the mineral composition of the grain. From the literature, we could not identify any single factor (pH, organic matter, etc.) that provided a consistent explanation for the variable effects of N fertilization on the mineral concentration in the kernels. We conclude that the impact of N fertilization on the mineral concentration in wheat grains is highly site-specific, and probably the result of complex interactions between several factors, such as the environmental conditions, management schemes, cultivars used, etc. In our case, the most likely explanation for the low response to N fertilization is that N was not a key limiting factor for nutrient acquisition in our conditions, the main issues being water scarcity and high soil pH. Studies dealing with the nutritional content of wheat grain have mostly focussed on temperate climates and acidic-neutral soils, and wheat cultivars that grow well in those conditions (Hamnér et al., 2017). Given the strong influence of environment on the mineral concentration in the grain, it is crucial to increase the number of studies in arid and semi-arid environments and alkaline soils.

5.5. Effect of environmental conditions on the mineral concentration in the grain

In 2019, maximum temperatures were higher and it rained less than in 2020. Moreover, wheat in 2020 was sown one month earlier, so plants were exposed to heat at a later developmental stage. Drought increases the mineral concentration in the grain because it reduces grain filling

(Wang & Liu, 2021) and nutrient uptake (Nawaz, Ahmad, Waraich, Naeem, & Shabbir, 2012). Heat stress tends to decrease mineral accumulation in the grain, but this effect varies with the genotype and is less prominent or even opposite in heat-tolerant varieties (Wang & Liu, 2021). Concerning the soil, we found less P, K, active lime and organic matter (SOM) (41 %, 49 %, x2, and x3 respectively) in 2020 relative to 2019. Active lime and nutrients can be washed away with the rain, which explains the lower concentration in 2020. Further, drought reduces microbial activity in the soil (Manzoni & Katul, 2014), which could have led to more SOM remaining in the soil in 2019. There was not a constant link between the nutrient concentration in the soil and in the grain. The lower [K] in the grain in 2020 is consistent with the [K] of the soil that year. However, the [P] in grains was higher in 2020. Further, the SOM promotes nutrient uptake by plants (Uyanöz et al., 2006), so the lower SOM in 2020 could have contributed towards decreasing the mineral content of the grain.

5.6. *Effect of water availability and N fertilization on C and N stable isotopes*

As discussed before, the drought conditions of 2019 increased the mineral concentration of the grain. We attribute this to a change in the geometry of the grain caused by insufficient grain filling, which limited the dilution effect of starch accumulation. At the same time, the $\delta^{13}C$ correlated positively with the mineral concentration when the two years were analysed both together and separately. The $\delta^{13}C$ varies with the internal CO_2 concentration of the leaves, which greatly depends on the stomatal aperture (Farquhar, Ehleringer, & Hubick, 1989). Hence, lower $\delta^{13}C$ values indicate higher transpiration rates and GY (Foulkes, DeSilva, Gaju, & Carvalho, 2016). Higher transpiration rates could potentially increase nutrient acquisition through bulk flow. However, that does not necessarily mean that the nutrient content of the grain should increase. The higher GY and the subsequent dilution effect will tend to lower the mineral concentration in the grain (Fan et al., 2008). When crop growth is very fast, the mechanisms for mineral uptake, transport and allocation to the grain cannot increase in the same proportion as the synthesis of photoassimilates (Jarrell & Beverly, 1981). Our $\delta^{13}C$ data are consistent with the dilution effect being more relevant for explaining the mineral concentration of the grain than the transpiration rate. The mineral concentration correlated positively with $\delta^{13}C$ and was lower in 2020, even if this was a relatively wet year that allowed plants to maintain higher transpiration rates.

In 2019, $\delta^{15}N$ decreased with increasing N application and was lower than in 2020. The relationship between $\delta^{15}N$ and N fertilization was not significant in 2020. This points towards the plants acquiring N from different pools in 2019 and 2020. Soils amended with chemical fertilizers exhibit lower $\delta^{15}N$ than those fertilized with compost, where the N is derived from decomposition of organic matter (Choi et al., 2003). The activity of soil bacteria, including the biological fixation of nitrogen, is inhibited by intense drought (Manzoni & Katul, 2014). Hence, a possible interpretation of the $\delta^{15}N$ data is that most of the N taken up by plants in 2019 originated from the fertilizer because the soil bacteria were affected by drought. In contrast, the higher $\delta^{15}N$ in 2020 and lack of response to increasing fertilizer rates indicates that plants were using additional sources of N, such as N from organic matter decomposition. However, N metabolism in plants is very complex, and many steps during N uptake, assimilation, and remobilization could have altered the $\delta^{15}N$ of the original N source. Therefore, we should explore alternative explanations for these results. Unlike the $\delta^{13}C$, the $\delta^{15}N$ decreases in response to stress (Yousfi, Serret, & Araus, 2013). In C3 plants such as wheat, water deficit restricts stomatal aperture and reduces the intracellular CO_2/O_2 ratio, increasing photorespiration rates (Yousfi et al., 2013). Photorespiration is strongly connected to N assimilation and acts as the intersection between N and C assimilation. In agreement, lower $\delta^{15}N$ values were associated with higher mineral concentrations in the grain and lower GY and TGW.

6. Conclusions

Of the four working hypotheses tested, our findings support hypotheses 1, 2, and 4. The genotype and water availability were key to determining the mineral concentration of wheat grain. The genotype was the main driver of the mineral concentration in the grain, especially for Ca, K, Mg, and S, but the environmental conditions can be more relevant for other minerals such as Fe and Zn. To maximize the nutritional value of modern wheat varieties in Mediterranean semi-arid conditions and alkaline soils, the most effective strategy could be plant breeding, but with consideration of the local constraints (water, soil pH). Our results also support the idea of a dilution effect as the most likely mechanism for the dwindling nutritional value of the grain in modern wheat varieties. However, our data are not compatible with hypothesis 3. We did not observe that higher N–fertilization rates had any beneficial effect on the concentration of micronutrients like Fe and Zn in the grain. We attribute this to the fact that N was not a key limiting factor for nutrient acquisition in our conditions, the main issues being water scarcity or other nutritional deficiencies instead. We identified three potential nutritional deficiencies (P, Zn, and Fe), likely caused by the high soil pH. Correcting these deficiencies might improve wheat GY and Fe and Zn content in the grain in our region and others with similar conditions (semi-arid, alkaline soils). We propose that specific fertilization to provide these three elements and soil amendments that aim to reduce soil pH could help to close the yield gap and improve the grain nutrient concentration in this type of setting. We identified CH-Nara as a variety of interest for the high nutritional value of its grain while maintaining an adequate yield by modern standards. We also provided data on the mineral concentration in the grain for 12 high-yielding bread wheat varieties for the first time. The knowledge gained from this study will contribute to guiding future agronomic practices in the region and other areas with similar climate and soil properties, and guarantee food safety and quality for local communities.

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Data availability

The data supporting the findings of this study are available from the first author, Cristina Caldelas, upon request.

CRediT authorship contribution statement

Cristina Caldelas: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Fatima Zahra Rezzouk:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. **Nieves Aparicio Gutiérrez:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Maria Carmen Diez–Fraile:** Conceptualization, Formal analysis, Investigation, Resources, Methodology. **José Luis Araus Ortega:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134565.

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