

Article

Genetic gain for grain micronutrients and their association with phenology in historical wheat cultivars of Pakistan released between 1909 and 2018 in Pakistan

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Abstract: Wheat (*Triticum aestivum* L.) being a staple food crop is an important nutritional source providing protein and minerals. It is important to fortify staple cereals like wheat with essential minerals to overcome the problems associated with malnutrition. The experiment was designed to evaluate the status of 11 micronutrients including grain iron (GFe) and zinc (GZn) in 62 wheat cultivars released between 1911 and 2016 in Pakistan. Field trials were conducted over two years and GFe and GZn were quantified by both inductively coupled plasma optical emission spectroscopy (ICP-OES) and energy dispersive X-ray fluorescence spectrophotometer (EDXRF). The GZn ranged from 18.4 to 40.8 mg/kg by ED-XRF and 23.7 to 38.8 mg/kg by ICP-OES. Similarly, GFe ranged from 24.8 to 44.1 mg/kg by ICP-OES and 26.8 to 36.6 mg/kg by EDXRF. The coefficient of correlation was higher for GZn ($r=0.90$), compared to GFe ($r=0.68$). Modern cultivars like Zincol-16 and AAS-2011 showed higher GFe and GZn along with improved yield components. Old wheat cultivars WL-711, C-518 and Pothowar-70 released before 1970 also exhibited higher value of GFe and GZn, however their agronomic performance was poor. Multivariate analysis using ten micronutrients (Al, Ca, Cu, K, Mg, Mn, Na and P) along with agronomic traits, and genome-wide SNP markers identified the potential cultivar with improved yield, biofortification trait and wider genetic diversity. Genetic gain analysis identified significant increase in grain yield (0.4% year⁻¹), while there was negative gain for GFe (-0.11% year⁻¹) and GZn (-0.15% year⁻¹) over the span of 100 years. The Green Revolution *Rht-B1* and *Rht-D1* genes had strong association with plant height, and grain yield (GY), while semi-dwarfing alleles had negative effect on GFe and GZn contents. This study provided a valuable insight into biofortification status of wheat cultivars deployed historically in Pakistan and is a valuable source to initiate a breeding strategy for simultaneous improvement in wheat phenology and biofortification.

Keywords: Wheat, Biofortification, Iron, Zinc, Rht genes

1. Introduction

More than two billion people in the world are severely affected by the dietary deficiency of essential micronutrients such as zinc (Zn) and iron (Fe) [1]. Zinc deficiency leads to stunted growth and increased risk of child mortality, and currently 17% of global population is at risk of inadequate Zn intake. Fe deficiency leads to anemia which currently affects 800 million women and children (HarvestPlus, 2021). Other trace elements like copper (Cu), manganese (Mn), calcium (Ca) and selenium (Se) are also essential micronutrients because they take part in key metabolic reactions for both plant growth and human health [2]. The deficiency of Cu and Mn is not widespread among humans, but these trace elements play critical role in growth and development. Wheat (*Triticum aestivum* L.) is one of the most important cereals crop plant worldwide and ranked the third largest producing crop followed by rice and maize [3]. Wheat provides more than 20% of the calories for global population, especially for those living in developing countries. Therefore, increasing the micronutrient contents, known as biofortification, in wheat cultivars is a low-cost and sustainable strategy for alleviating micronutrient malnutrition.

Wheat cultivars usually have low amount of micronutrients including Fe and Zn [4]. It has been estimated that Zn concentration in wheat grain should be >50 µg per gram of dry weight, while current wheat grains contain about 25–30 µg Zn per gram dry weight on average [5]. The preliminary breeding target for primary target countries, Pakistan and northern India, is to increase Zn levels by 12 mg/kg, about 50% above the baseline, which is the mean of popular varieties currently grown in the region [4]. Velu et al. [4] reviewed that dietary supplements and agronomic practices involving the use of Fe- and Zn-containing fertilizers can help address the nutrient deficiency problem. However, sustainable and cost-effective approach to increasing essential mineral concentration is through genetic biofortification, which requires identification of cultivars with useful genetic variability for grain minerals and understanding of the physiological and genetic architecture of these minerals in wheat [6].

There is clear evidence that modern and old wheat cultivars differs significantly for grain micronutrients, and it was observed that grain Fe, Zn, Cu and Mg decreased significantly after 1965 with the introduction of semi-dwarf and high yielding wheat cultivars [7]. It is likely due to the reason that *Rht-B1b* and *Rht-D1b* genotypes have reduced growth of the root system affecting the ability to scavenge minerals from the soil, or the ability to store minerals in the vegetative tissues prior to redistribution to the grain [8]. Murphy et al [9] analyzed the mineral elements in 63 historical wheat cultivars released between 1842-1965 in Pacific Northwest US and concluded that all minerals except Ca significantly decreased over time. Although breeding for biofortified wheat is not main target in Pakistan and India, high Zn cultivars like Zincol-2016 and Zn Shakti have been released in Pakistan and India, respectively [10]. Previously, some cultivars with high grain Fe/Zn have been released like cv. Burnside in Canada with *Gpc-B1* gene [11]. Therefore, it is very important to analyze the status of minerals in historical wheat germplasm for better insight of selecting germplasm resources in breeding.

The present study was designed to evaluate the status of minerals contents in historical wheat cultivars released in Pakistan between 1911 to 2016. The main objectives include, a) to assess the temporal variation for grain mineral elements in historical wheat cultivars and rate of progress for improvement in grain mineral elements, b) to identify the important phenological traits associated with grain mineral elements in historical wheat cultivars of Pakistan, and c) to identify the allelic effects of important Green Revolution *Rht-1* genes and others such as *NAM-A1*, *TaSus2-2B*, *TaGW2-6B* and *TaGW2-6A* on agronomic traits and mineral contents in historical wheat cultivars from Pakistan.

2. Materials and Methods

2.1. Plant material and field trials

A set of 62 wheat cultivars released in Pakistan from 1911 to 2016 were selected for this study. The cultivar name, year of release and pedigree are given in Table 1. The cultivars were evaluated for two year 2018-2019 (later as 2018) and 2019-2020 (later as 2019) in

the field at National Agriculture Research Center (NARC), Islamabad, Pakistan using a randomized complete block design (RCBD) with two replications. The NARC site located at 33°43'N 73°04'E, and has soil electrical conductivity of 0.24dS/m. The date of sowing was 5 December in 2018 and 8 December in 2019.

Table 1. Pedigree of historical wheat cultivars evaluated for phenological parameters along with the grain iron and zinc content.

Cultivar	Release Year	Pedigree
T9	1911	Nil
C-518	1933	T9/8A
C-217	1944	C516/C591
C-271	1957	C230/IP165
C-273	1957	C209/C591
Dirk	1958	FORD//DUNDEE/BOBIN or FORD/DONDEE (I)
Mexipak-65	1965	PJ/GB55 or PJ62/GB55
Pothowar-70	1970	BURT/KENYA//QUETA(L)/3/NAD63
Pari-73	1973	CNO67//SN64/KLRE/3/8156
Parula	1973	Nil
WL-711	1978	S308/CHRIS//KAL
Pak-81	1981	KVZ/BUHO//KAL/BB
Barani-83	1983	BB/GLL/3/GTO/7C//BB/CNO67
Chakwal-86	1986	FORLANI/ACC//ANA or FlN/ACS//ANA
Khyber-87	1987	KVZ/TRM//PTM/ANA
Rawal-87	1987	MAYA/MON//KVZ/TRM
Inquilab-91	1991	WL 711/CROW "S"
Pasban-90	1991	INIA F66/TH.DISTICHUM//INIAF66/3/GENARO T81
Pastor	1993	PFAU/SERI-82//BOBWHITE
Bakhtawar-94	1994	AU/UP301//GLL/SX/3/PEW/4/MAI/MAYA//PEW
Parwaz-94	1995	V.5648/PARULA
Punjab-96	1996	SA42*2/4/CC/INIA//BB/3/INIA/HD832
Suleman-96	1996	F6.74/BUN//SIS/3/VEE#7 or F6-74/BUN//SIS/3/VEE#7
Tatara	1996	JUP/ALD'S'//KLT'S'
Chakwal-97	1997	BUC'S'/FCT'S'
MH-97	1997	NORD-DESPREZ(ND)/VG-9144//KALYANSONA/BLUEBIRD/3/YACO/4/VEERY-5
Margallah-99	1999	OPATA/BOW'S'
Auqab-2000	2000	CROW'S'/NAC//BOW'S'
Wafaq-2001	2001	OPATA/RAYON//KAUZ
AS-2002	2002	KHP/D31708//CM74A370/3/CNO79/4/RL6043/4*NAC
Bhakkar-2002	2002	P20102/PIMA/SKA/3/TTR'S'/BOW'S'
GA-2002	2002	DWL5023/SNB//SNB
Ufaq	2002	V.84133/V83150
Pirsabak-2004	2004	KAUZ/STAR
Pirsabak-2005	2005	MUNIA/CHTO//AMSEL

Fareed-2006	2006	PT'S/3/TOB/LFN//BB/4/BB/HD-832-5//ON/5/G-V/ALD'S//HPO
Seher-2006	2006	CHILL/2* STAR/4/BOW//BUC/PVN/3/2*VEE#10
Bathoor	2008	URES/JUN//KAUZ
Chakwal-50	2008	ATTILA/3/HUI/CARC//CHEN/CHTO/4/ATTILA
Faisalabad-2008	2008	PBW65/2*Pastor
Mairaj-2008	2008	SPARROW/INIA//V.7394/WL711/13/BAUS
Pirsabak-2008	2008	KAUZ/PASTOR
NARC-2009	2009	INQALAB 91*2/TUKURU
Atta-Habib	2010	INQALAB 91*2/TUKURU
Barsat-2009	2010	FRET2
AAS-2011	2011	PRL/PASTOR//2236(V6550/SUTLEH-86)
Dharabi-2011	2011	HXL-7573/2*BAGULA//PASTOR
Millat-2011	2011	CHENAB2000/INQ-91
NARC-2011	2011	OASIS/SKAUZ//4*BCN/3/2*PASTOR
Punjab-2011	2011	AMSEL/ATTILA//INQ-91/PEW'S'
Galaxy-2013	2013	PUNJAB-96/V-87094//MH-97
Pakistan-2013	2013	MEX94.27.1.20/3/Sokoll//Attila/3*BCN
Pirsabak-2013	2013	CS/TH.SC//3*PVN/3/MIRLO/BUC/4/MILAN/5/TILHI
Shahkar-2013	2013	CMH84.3379/CMH78.578//MILAN
Pakhtunkhwa-2015	2015	WBLL1*2/4/YACO/PBW65/3/KAUZ*2/TRAP//KAUZ
Ujala-2016	2015	KIRITATI/4/2*WEAVER/TSC//WEAVER/3/WEAVER
Ahsan-2016	2016	Pastor/3/Altar 84/Ae. squarrosa//Opata
Borlaug-2016	2016	Sokoll/3/Pastor//HXL7573/2*BAU
Gold-2016	2016	Nil
Johar-2016	2016	KAUZ/PASTOR//V.3009
Zincol-2016	2016	OASIS/ SKAUZ//4*BCN/3/2*PASTOR/4/T.SPELTA PI348449/5/BACEU#1/6/WBLL1*2/CHAPIO
Local-White	-	Nil

2.2. Phenotyping

The data were recorded for ten morphological parameters including tillers per plant (TPP), plant height (PH) in cm, spike length (SL) in cm, spikelet per spike (SNPS), grains per spike (GPS), thousand grain weight (TGW), grain length (GL), grain width (GW), grain diameter (GD), and grain yield (GY) in kg per m² per plot were recorded at different growth stages. Plant height from the ground to top of the spike without was recorded at late grain filling (Z77). Number of grains per spike was the mean of randomly selected spikes from 15 different plants per plot. Number of spikes per plot was counted at physiological maturity (Z96) and all plants in each plot were harvested manually and above-ground total biomass weight was recorded. Grain yield was measured as weight of grain harvested per plot. Thousand grain weight was based on three 200-grain samples from each plot. GL and GW were recorded for one hundred uniform seeds from each plot. Eleven mineral concentrations in the grains of sixty-two wheat cultivars were determined from each year and each replicate using inductively coupled plasma mass spectroscopy (ICP-OES). Briefly, grain samples were digested with concentrated HNO₃, hydrogen peroxide and hydrofluoric acid. The grain Fe and Zn were further evaluated with non-destructive high-throughput ED-XRF (Energy dispersive X-Ray fluorescence analysis).

method. This was performed by using an oxford instruments X-Supreme 8000 fitted with a ten place auto-sampler holding 40 mm aluminum cups. Fe and Zn were quantified and analyzed in 186 seconds in which the acquisition time was 60 seconds and dead time was also 60 seconds[12]. All the analyses were done at the central facility of Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.

2.3. Genotyping using KASP markers and GBTS

Total genomic DNA was extracted from each cultivar following a previously described protocol[13]. The KASP markers for genes *Rht-B1*, *Rht-D1*, *TaSus2-2B*, *TaGW2-6A*, and *TaGW2-6B* were used from our previous work[14]. Two KASP markers for NAM-A1 were used from Cormier et al. [15]. The PCR mix included 2 µl of 50-100 ng/µl template DNA, 2.5 µl of 2X KASP master mix, 0.07 µl of KASP assay mix and 2.5 µl of distilled water. PCR was performed in 384-well formats (S1000, Thermal Cycler, USA) by the following procedure: hot start at 95°C for 15 min, followed by 10 touchdown cycles (95°C for 20 s; touchdown at 65°C initially and decreasing at -1°C per cycle for 25 s), and then 30 additional cycles of annealing (95°C for 10 s; 57°C for 60 s).

DNA samples were also genotyped with a genotyping-by-targeted sequencing (GBTS) platform which include more than 100 SNPs distributed over all the wheat chromosomes. This assay was used for genetic diversity studies among cultivars. The design for the GBTS assay is not reported yet.

2.4. Statistical analysis

For each trait, the best linear unbiased estimator (BLUE) for each genotype was estimated using a mixed linear model across two environments. The full model was as follows;

$$Y_{ijkl} = \mu + geno_i + env_j + geno \times env_{ij} + Rep(env)_{jk} + \varepsilon_{ijkl}$$

where Y_{ijkl} is the average phenotypes of individual plot, μ is the grand mean, $geno_i$ is the fixed effect of the i th genotype, env_j is the random effect of the j th env (year in this case), $geno \times env_{ij}$ is the random effect of interaction between the i th genotype and the j th env, $Rep(env)_{jk}$ is the random effect of the k th Rep nested within the j th env, and ε_{ijkl} is the residual effect that was assumed to be independent and identically distributed following a normal distribution with a mean of zero and variance σ_e^2 . After removing the outliers for each phenotype, an iterative mixed liner model was fitted in *lmerTest* (R package) (Gilmour et al., 2009) with the full model. For each phenotype, the model was used to calculate the BLUE values for each genotype and estimate the variance components for broad-sense heritability (H^2) on a line basis as well as standard error by delta method (Lynch and Walsh, 1998; Holland et al. 2003).

To assess the degree of the association between BLUE values for each pair of traits, the pairwise Pearson's correlation coefficients were estimated via R package *Hmisc* and plotted using R package *PerforanceAnalytics*. The mean, range, and standard deviation of BLUEs were calculated via R statistics standard functions in the *stats* and the *pysch*.

For each trait, the genetic gain over time was estimated by a simple linear regression in R function *lm*. The model was as follows:

$$y_i = \beta_0 + \beta_1 x_i + \varepsilon_i$$

where y_i is the BLUE value of the i th genotype, x_i is the year of release of the i th genotype, β_0 is the intercept of the regression line, β_1 is the regression coefficient, and ε_i is the residual effect. The regression coefficient was used to estimate the genetic gain (Gao et al. 2017). The t test on the regression coefficient β_1 was carried out to examine the significance of the regression with the null hypothesis: $\beta_1 = 0$ and the significance level is 0.05.

SNP markers from GBTS assay were used to conduct principal component analysis (PCA) and estimates of unweighted paired group arithmetic mean (UPGMA) based on the neighbor joining (NJ) method using TASSEL version 5.0. The SNP markers with more

than 5% missing data and minor allele frequency were removed. The KASP markers were used to assess the allelic effects on the individual traits using Student's t-test.

3. Results

The historical wheat cultivars showed significant variations for morphological traits and 11 micronutrients evaluated in this study. Moreover, the allelic effects of some of the genes were also significant on the micronutrient traits. The result are given below in each subsection.

3.1. Variation in micronutrients and morphological traits

All 62 wheat cultivars were grouped into three categories according to the year of release. First group included seven cultivars released during 1911 to 1965, second group included 21 cultivars released during 1965-2000, and third group included 34 cultivars released after 2000. Descriptive statistics for all morphological and traits and micronutrients across three groups are described in Table 2. The coefficient of variation was highest for Na contents (75.4%), and the lowest CV% was observed for GD (3.7%). The mean and range for all traits in cultivars released in three breeding eras are also described in Table 2. The GY progressively increased from 1.3 t/ha to 2.09 t/ha, and 2.44 t/ha in three breeding eras, respectively. Contrastingly, TKW was higher (43.4 g) in old cultivars, compared to 39.1 g in mid-era and 41.3 g in cultivars released after 2000 (Table 2; Figure 1). Similarly, PH was 111 cm in old cultivars, 97.6 cm in cultivars released in 1965-2000 and 93.7 cm in post-2000 cultivars. Among the micronutrients, there was no clear pattern for GFe and GZn, however, K was significantly higher and, Se, Mg and Cu were significantly higher in cultivars released before 1965 (Table 2; Figure 1).

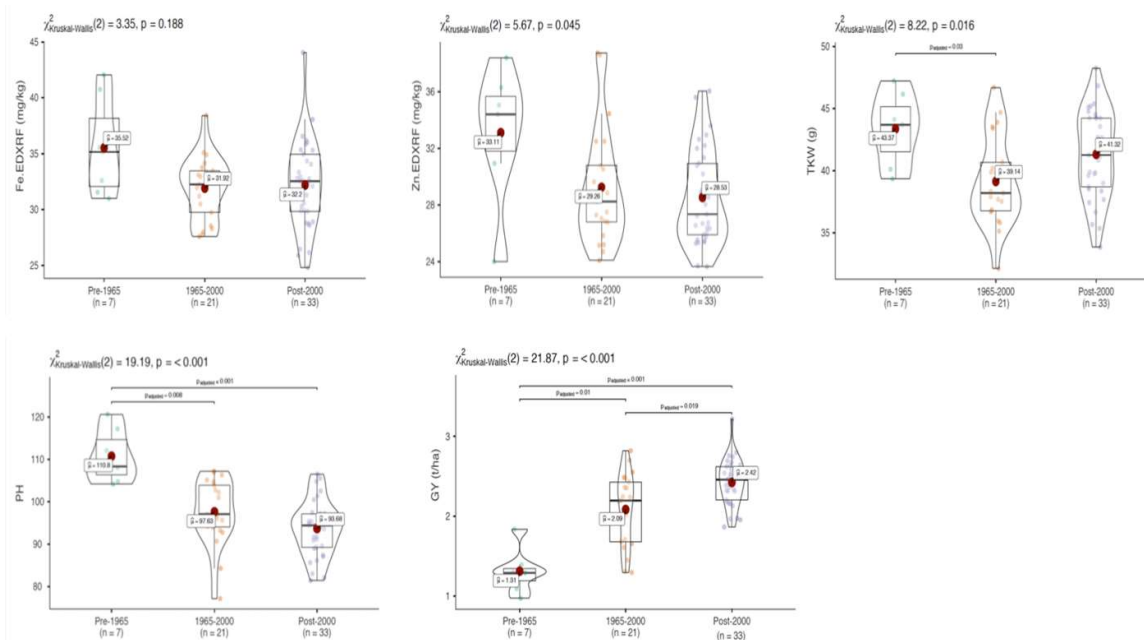


Figure 1. Box plots showing the variation among important micronutrients and yield related traits distributed over three selection breeding periods. The significance between mean performance of three breeding periods is shown as p.value of Kruskal-Wallis test.

Table 2. Descriptive statistics of grain mineral elements and morphological traits in sixty two wheat cultivars classified into three distinct breeding eras

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Traits	Pre-1965 (n=7)				1965-2000 (n=21)				Post-2000 (n=34)				Overall (n=62)			
	Min	Mean	Mean	CV(%)	Min	Max	Mean	CV(%)	Min	Max	Mean	CV(%)	Min	Max	Mean	CV (%)
Fe.EDXRF (mg/kg)	31	42	35.5	12.34	27.6	38.4	31.9	8.9	24.8	44	32.2	12.39	24.8	44	32.5	11.66
Zn.EDXRF (mg/kg)	24	38.4	33.1	14.14	24.1	38.8	29.3	14.2	23.6	36	28.5	11.72	23.6	38.8	29.3	13.62
Fe.ICPOES (mg/kg)	30.5	38.6	32.7	8.96	26.9	37.3	32.8	6.83	26.8	37.6	33.7	6.5	26.8	38.6	33.3	6.91
Zn.ICPOES (mg/kg)	18.8	33.1	28.8	16.63	20.4	40.8	29.8	14.7	23.9	37.2	28.5	11.82	18.8	40.8	28.9	13.43
Al (mg/kg)	2.69	4.78	3.87	21.06	2.44	7.78	4.48	23.21	2.06	4.97	3.58	18.91	2.06	7.78	3.92	23.37
Ca (mg/kg)	453	635	547	11.99	476	701	588	9.73	415	720	563	11.26	415	720	570	10.91
Cu (mg/kg)	3.81	5.28	4.68	10.51	3.25	5.69	4.16	17	3.06	5.47	4.08	14.24	3.06	5.69	4.18	15.22
K (mg/kg)	3591	4118	3892	4.83	3762	4891	4385	6.07	3732	5344	4279	8.72	3591	5344	4271	8.19
Mg (mg/kg)	984	1288	1181	8.64	923	1318	1134	8.06	970	1293	1130	7.22	923	1318	1137	7.69
Mn (mg/kg)	24.4	37.4	32.2	13.45	25.6	37.8	32	9.53	26.3	38.2	32.7	9.88	24.4	38.2	32.4	10.06
Na (mg/kg)	8.75	47.3	28.7	55.4	10	159	43.1	87.01	8.94	79.9	32.1	59.5	8.75	159	35.4	75.42
P (mg/kg)	2752	3349	3108	7.53	2862	3918	3361	7.94	2719	3657	3150	7.71	2719	3918	3217	8.33
Se (mg/kg)	0.199	0.258	0.232	8.84	0.132	0.24	0.199	18.69	0.135	0.26	0.212	14.29	0.132	0.26	0.21	15.71
TPP	3.13	4.25	3.58	12.04	2.83	4.34	3.53	12.32	2.44	4.08	3.23	11.8	2.44	4.34	3.37	12.76
PH (cm)	104	121	111	5.6	77.1	107	97.6	9.54	81.4	107	93.7	11.11	77.1	121	97	8.85
SL	12.8	18.5	15.8	12.72	15.1	22.2	17.2	9.42	14.1	19.2	16.9	6.98	12.8	22.2	16.9	8.70
SpPS	18.1	20.3	19.3	4.13	18.1	20.9	19.7	3.88	17.6	21.1	19.2	4.29	17.6	21.1	19.4	4.27
GPS	39.3	52.3	45.9	11.18	48.6	61.3	53.3	6.42	44.1	64	54.2	8.03	39.3	64	53	9.11
GY (t/ha)	0.975	1.84	1.31	20.76	1.3	2.82	2.09	21.77	1.87	3.22	2.44	12.05	0.975	3.22	2.19	22.74
TKW (g)	39.4	47.2	43.4	6.66	32.1	46.7	39.1	9.26	33.9	48.2	41.3	8.45	32.1	48.2	40.8	9.02
GL (mm)	1.11	1.36	1.25	7.01	1.16	2.04	1.46	14.11	1.16	2.23	1.38	16.09	1.11	2.23	1.39	15.32
GW (mm)	1.97	2.44	2.11	7.91	2.15	2.73	2.35	5.62	2.02	2.52	2.27	5.55	1.97	2.73	2.27	6.56
GD (mm)	5.86	6.65	6.16	4.69	5.89	6.65	6.36	3.25	5.77	6.8	6.38	3.65	5.77	6.8	6.34	3.77

Fe calculated by EDXRF, Zn calculated by EDXRF, Fe calculated by ICPOES and Zn calculated by ICPOES, aluminum (Al), calcium (Ca), copper (Cu), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorous (P) and selenium (Se), Plant height (PH), Grains per spike (GPS), Thousand grain weight (TGW), Tillers per plant (TPP), Spike length (SL), Spike number per spike (SpPS), Grain number per spike (GpS), Grain yield (GY), Grain length (GL), Grain width (GW), Grain diamter (GD)

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3
4

Table 3. Analysis of variance for morphological traits and micronutrient contents in historical wheat cultivars of Pakistan

	Replication	Genotype (G)	Year (Y)	G x Y interaction	
df	2	61	1	61	
Traits	Means squares				Heritability
TPP	1.162*	0.7386***	0.0413ns	0.9095***	0.35
PH	203.1443ns	465.0146***	256.8787ns	156.766	0.67
SL	1.2842ns	8.6695***	13.518*	5.5964**	0.49
SNPS	222.2401***	4.5472***	141.0344***	1.6726ns	0.48
GNPS	675.4824***	93.4753***	598.6102***	51.7566ns	0.52
GY	0.1032***	0.9954***	2.3278***	0.1575**	0.68
TKW	0.3556ns	54.2871***	0.0429ns	19.7962ns	0.66
GL	7.9117***	0.2239***	0.0032ns	0.0451ns	0.80
GW	8.6088***	0.1296***	0.0147ns	0.0455ns	0.65
GD	8.329***	0.2752***	0.0022ns	0.0579ns	0.78
Al	68.6896***	3.1559***	0.127ns	0.3507ns	0.52
Ca	35341.2148***	11898.9395***	81.7116ns	75.8745ns	0.86
Cu	8.2654***	1.1161***	0.5172ns	0.066ns	0.72
Fe.EDXRF	4.5506**	40.2081***	0.1301ns	37.163***	0.51
Fe.ICPOES	14.5627***	15.3527***	120.9392***	8.7979***	0.62
K	112509.1797*	455115.7812***	756099.6875***	113190.0078***	0.79
Mg	6691.2295*	25110.6953***	52088.6211***	8330.1592***	0.73
Mn	5.0568*	35.1404***	5.8521*	11.7127*	0.74
Na	23.8786ns	2031.3976***	6190.4634***	2507.9097***	0.43
P	49623.0234*	205982.9375***	390194.75***	120418.9297***	0.61
Zn.EDXRF	11.2164**	39.5774***	11.9913*	34.2843***	0.52
Zn.ICPOES	8.4438**	41.8175***	594.0781***	18.7137***	0.68
Se	7.3049***	0.045ns	0.0319ns	0.0518ns	0.0057

* Significant (P <0.05); ** Significant (P <0.01); *** Significant (P <0.001); ns Non-significant (P>0.05)

Analysis of variance (ANOVA) showed variation in nearly all the traits except grain Se contents (Table 3). All the traits showed significant variations for genotypes, year and genotype x year interaction with some exceptions. No significant variation was observed for planting years in TPP, PH, TKW, GL, GW, Al, Ca, Cu, and Se contents (Table 3). Among the micronutrients, the heritability for Fe and Zn was 0.62 and 0.68, respectively (Table 3). The heritability for important yield related traits like TKW, TPP and GY was 0.66, 0.35 and 0.68, respectively.

Among the micronutrient, two methods were used to phenotype GFe and GZn. GFe ranged between 24.8 mg/kg (Auqab-2000) to 44.0 mg/kg (Zincol-16), with an average of 32.5 mg/kg using EDXRF, while it ranged from 26.8 mg/kg to 38.6 mg/kg with an average of 33.3 mg/kg with ICP-OES (Table 2). The correlation coefficient between the two methods was $r = 0.68$ (Figure 2). Similarly, the GZn content ranged between 23.65 mg/kg (Auqab-2000) to 38.8 mg/kg (WL-711) with a mean value of 29.30 mg/kg by EDXRF, and ranged from 18.4 mg/kg (Rawal-87) to 40.8 mg/kg (Pothowar-70) with an average of 28.9 mg/kg

by ICP-OES. The correlation coefficient between the two methods was $r = 0.90$ (Figure 2). Other important micronutrients like Ca ranged from 415 to 720 mg/kg with an average of 570 mg/kg. Similarly, Mn ranged between 24.4 to 38.2 mg/kg with an average of 32.4 mg/kg (Table 2).

3.2. Correlation between traits and multivariate analysis

The coefficient of correlation is reported in Table 4 between all morphological traits and micronutrients. The coefficient of correlation between GFe and GZn was positive with $r=0.31$. GFe has strong positive correlation with Ca ($r=0.28$), Mg ($r=0.35$), Mn ($r=0.3$), and Se ($r=0.26$), while its correlation was non-significant with any morphological trait. GZn had relatively higher correlation with Ca ($r=0.61$), Cu ($r=0.6$), K ($r=0.4$), Mg ($r=0.74$), and P ($r=0.63$). While GZn had strong negative correlation with GD ($r=-0.48$). GY had negative correlation with TPP ($r=-0.3$) and positive correlation with GPS ($r=0.4$). The highest correlation among the morphological traits was between SL and SpPS ($r=0.46$).

The PCA biplot clearly separated the cultivars into three groups consistent with three breeding era defined previously (Figure 3a). The first two principal components explained 19 and 12.1% of the total variation. The pre-1965 cultivars were separated on the lower side of the PC2 in admixture some Chakwal-86 and Rawal-87. The dendrogram showed two major clusters, cluster I and cluster II. Cluster I consisted of 20 cultivars mostly released after 1965, except Dirk. While cluster II consisted of 42 cultivars and was further subdivided into three subclusters. The clustering was consistent with the breeding eras except that C-273 was in admixture with some modern cultivars (Figure 3b). The dendrogram generated from the genome-wide SNP marker also corroborated the diversity pattern showed by the phenotypic analysis (Figure 3c).

Table 4. Coefficient of correlation between morphological traits and grain micronutrients in historical wheat cultivars of Pakistan

Traits	Fe (mg/kg)	Zn (mg/kg)	Al (mg/kg)	Ca (mg/kg)	Cu (mg/kg)	K (mg/kg)	Mg (mg/kg)	Mn (mg/kg)	Na (mg/kg)	P (mg/kg)	Se (mg/kg)	TPP	PH (cm)	SL (cm)	SpPS	GPS	GY (t/ha)	TKW (g)	GL (cm)	GD (cm)
Zn.ICPOES (mg/kg)	0.313*	—																		
Al (mg/kg)	-0.041	0.166	—																	
Ca (mg/kg)	0.278*	0.613***	0.234	—																
Cu (mg/kg)	0.197	0.601***	0.359**	0.378**	—															
K (mg/kg)	0.149	0.399**	0.13	0.551***	0.188	—														
Mg (mg/kg)	0.354**	0.747***	0.058	0.521***	0.536***	0.278*	—													
Mn (mg/kg)	0.299*	0.24	-0.121	0.226	0.088	0.042	0.412***	—												
Na (mg/kg)	-0.01	0.299*	0.286*	0.253*	0.331**	0.38**	0.321*	-0.171	—											
P (mg/kg)	0.126	0.637***	0.195	0.498***	0.417***	0.555***	0.667***	0.353**	0.279*	—										
Se (mg/kg)	0.26*	0.373**	0.132	0.402**	0.42***	0.174	0.536***	0.296*	0.302*	0.101	—									
TPP	-0.134	0.11	0.355**	-0.083	0.315*	0.027	0.043	-0.071	0.281*	0.203	-0.024	—								
PH (cm)	-0.093	0.114	-0.004	-0.094	0.291*	-0.317*	0.114	0.053	0.051	0.004	0.131	0.296*	—							
SL (cm)	0.156	0.191	-0.019	0.187	-0.018	0.118	0.09	0.084	-0.02	0.282*	-0.127	0.181	0.252*	—						
SpPS	-0.041	0.153	0.207	0.078	0.114	0.019	-0.07	-0.055	0.059	0.148	-0.059	0.359**	0.344**	0.459***	—					
GPS	0.113	0.012	-0.046	0.02	-0.065	0.211	-0.137	0.103	-0.069	0.123	-0.147	-0.111	-0.09	0.282*	0.457***	—				
GY (t/ha)	0.2	-0.149	-0.354**	-0.176	-0.462***	0.07	-0.227	0.17	-0.169	-0.108	-0.248	-0.329**	-0.216	0.187	-0.073	0.393**	—			
TKW (g)	-0.005	-0.084	-0.152	-0.103	-0.013	-0.093	0.124	0.111	-0.042	-0.117	0.324*	-0.157	0.175	-0.015	-0.257*	-0.115	0.011	—		
GL (cm)	0.205	0.072	0.053	0.141	-0.166	0.108	0.113	-0.145	-0.03	0.162	-0.07	-0.108	-0.027	0.195	-0.023	0.061	0.166	-0.128	—	
GW (cm)	0.19	0.129	0.211	0.331*	0.17	0.503***	0.067	-0.238	0.301*	0.229	0.11	-0.026	-0.185	0.173	0.077	0.232	0.086	-0.124	0.37**	—
GD (cm)	-0.059	-0.488***	-0.081	-0.372***	-0.404**	-0.027	-0.309	-0.044	-0.004	-0.179	-0.246	0.045	0.026	0.179	-0.073	0.259*	0.359**	0.304*	0.215	0.278*

Note. * $p < .05$, ** $p < .01$, *** $p < .001$

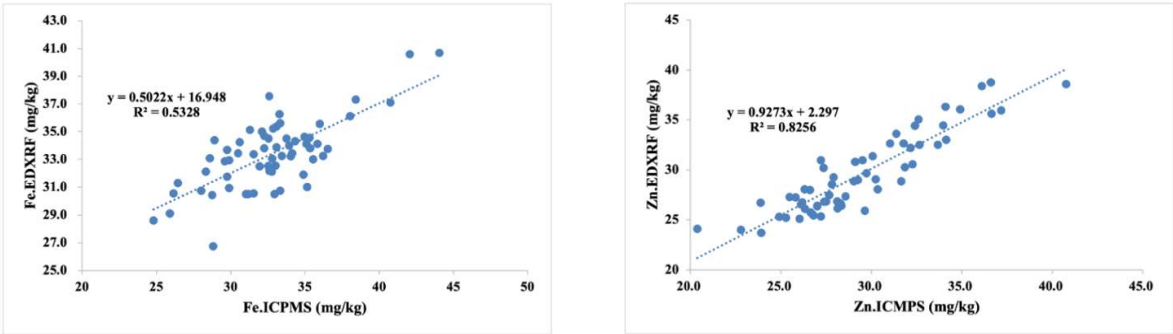
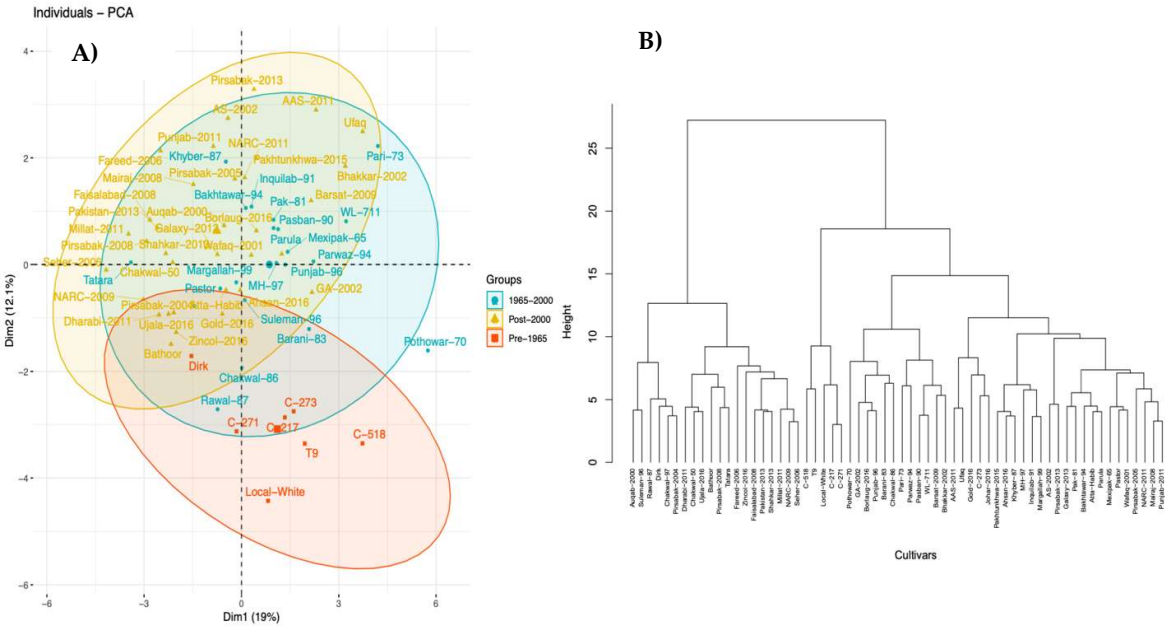


Figure 2. Coefficient of determination between two ICP-OES and EDXRF methods to predict grain iron (GFe) and zinc (Zn) in historical wheat cultivars released between 1911 and 2016

3.3. Genetic gain for micronutrients and morphological traits

The genetic gain analysis identified the traits significantly changed with the release year (Table S1). Among the morphological traits, there was significant change in TPP, PH, and SNPS which reduced significantly over the time. While GY and GpS were significantly increased over the time. The highest yielding cultivar Punjab-2011 (3.2 t/ha) yielded almost thrice compared to lowest yielding cultivar T9 (0.97 t/ha). The increase in genetic gain 0.41% over the period of 105 years, while the increase was highest in the recent period after 2000. The change in TKW, GL and GW remained non-significant over the years. Among the micronutrients, GZn significantly reduced during the course of breeding to -0.05 mg/kg/year (0.12%), while GFe also reduced at the rate of -0.02 mg/kg/year but change was non-significant.



C)

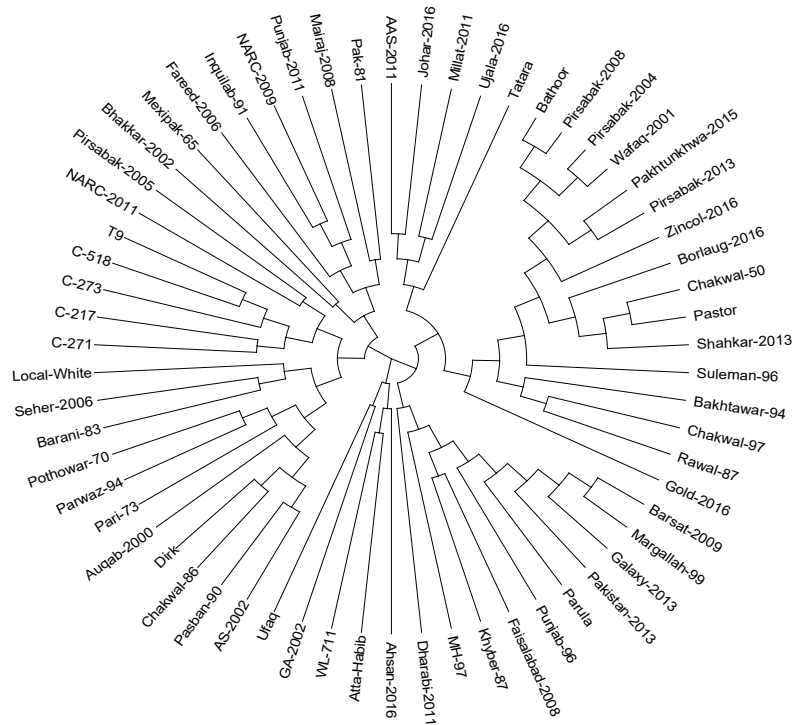


Figure 3. Multivariate analysis of historical wheat cultivars based on morphological traits and micronutrients, and genome-wide SNP markers, a) principal components analysis based biplot showing scattering of wheat cultivars grouped based on three breeding periods, b) dendrogram showing clustering of wheat cultivars based on morphological traits and micronutrients, c) dendrogram showing clustering of wheat cultivars based on genome-wide SNP markers

3.4. Allelic variation for functional genes and association with traits

The KASP markers for six genes were used to identify the allelic variation in historical wheat cultivars. The *Rht-B1* and *Rht-D1* genes were combined to identify the *Rht-1* haplotypes in the wheat cultivars. The results revealed that semi-dwarfing alleles either *Rht-B1b* or *Rht-D1b* were introduced after 1965 and their frequency was 79%, compared to 21% frequency of *Rht-B1a/Rht-D1a* haplotype (Table 5). Wheat sucrose synthase gene *TaSus2-2B*, had two haplotypes and haplotype Hap-L had very high frequency of 85.5% compared to 14.5% of the Hap-H frequency. Similarly, two grain width related genes, *TaGW2-6A* and *6B*, were also surveyed. The frequency of haplotypes associated with higher TKW was Hap-I was 32.% at *TaGW2-6B*, while frequency of Hap-6A-A was 80.6%, which was associated with higher TKW. At the *NAM-A1* locus, two haplotypes *NAM-A1b* and *NAM-A1d* had frequency of 40.3% and 59.7%, respectively.

The association of the alleles with phenotypes revealed that *Rht-1* haplotypes had minor but significant effect on GFe and GZn contents (Figure 4). The *Rht-B1a/Rht-D1a* haplotypes had slightly higher GFe and GZn contents compared to haplotypes with any semi-dwarfing allele. Similarly, the effect of *Rht-1* haplotypes was much higher on PH and GY. The presence of *Rht-B1b/Rht-D1a* and *Rht-B1a/Rht-D1b* haplotypes reduced the PH from 103.3 cm to 96.1 cm and 90.7 cm, respectively. Contrastingly, these haplotypes significantly improved GY from 1.72 t/ha to 2.33 and 2.22 t/ha, respectively. The *TaSus2-2B* haplotype Hap-H had significant and positive effect on TKW.

Table 5. Allelic frequencies for important functional genes in historical wheat cultivars of Pakistan

Genes	Alleles	Cultivar release era			
		Pre-1965	1965-2000	Post-2000	Overall
<i>Rht-8</i>	<i>Rht8</i>	8.1	30.6	50.0	88.7
	<i>rht-8</i>	3.2	3.2	4.8	11.3
<i>Rht-1</i>	<i>Rht-B1a/Rht-D1a</i>	9.7	6.5	4.8	21.0
	<i>Rht-B1a/Rht-D1b</i>	0.0	4.8	8.1	12.9
	<i>Rht-B1b/Rht-D1a</i>	0.0	24.2	41.9	66.1
<i>TaSus2-2B</i>	Hap-H	11.3	1.6	1.6	14.5
	Hap-L	0.0	32.3	53.2	85.5
<i>TaGW2-6B</i>	Hap-I	1.6	16.1	14.5	32.3
	Hap-II	9.7	17.7	40.3	67.7
<i>TaGW2-6A</i>	Hap-6A-A	11.3	24.2	45.2	80.6
	Hap-6A-G	0.0	9.7	9.7	19.4
<i>NAM-A1</i>	NAM-A1b	4.8	11.3	24.2	40.3
	NAM-A1d	6.5	22.6	30.6	59.7

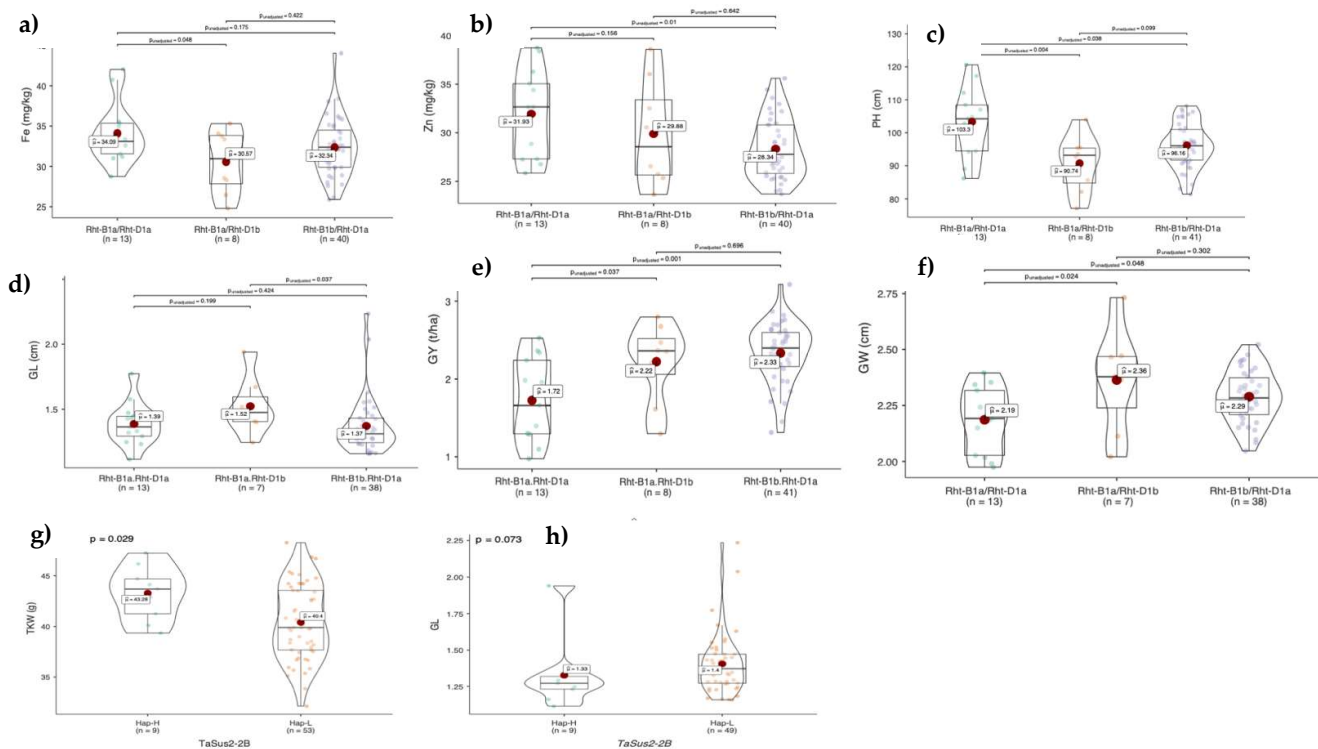


Figure 4. Allelic effects of *Rht-1* haplotypes (a-f) and *TaSus2-2B* gene (g-h) on important morphological traits in historical wheat cultivars.

4. Discussion

A collection of historical wheat cultivars released over a period of 105 years were evaluated for yield related traits and grain micronutrients. There were significant variations observed for all of the traits which indicated the progress in improving yield metrics over the course of selection breeding. The improvement of grain yield at the rate

of 0.4% per year is relatively low compared to genetic gain in yield in other parts of the world [16, 17]. The improvement in GY in Siberia over the period between 1900 to 2010 was 0.59% [17], 0.58-1.25% in Great Plains hard winter wheat, while Gao et al [18] reported a 57.5 kg ha⁻¹ yr⁻¹ gain in GY in Chinese bread wheat cultivars from 1950 to 2012 in the irrigated plain of China. The reason for the slightly slower rate of genetic gain is that present comparison involved a relatively longer duration of 105 years, and the genetic improvement in yield in Pakistan was not temporally smooth. The current data supported this hypothesis showed that CV (%) of the cultivars released after 2000 was half compared to the CV (%) of the cultivars released in previous periods. This indicated the consistent progress made for improving GY and high stability of yield in modern cultivars.

Although significant progress has been made in improving productivity, there was limited progress in improving micronutrients in Pakistan and elsewhere. The cultivars released before so-called Green Revolution in 1965 had relatively higher levels of GFe and GZn. It has been well-established in previous studies that micronutrient concentrations decreased over time in modern wheat cultivars, and this has been validated in US hard winter wheat [19], historical and modern soft white wheat cultivars from US [9], and in BroadBalk wheat experiment in UK [7]. It was concluded that grain mineral concentration remained stable in wheat cultivars from 1845 to 1960s, while significantly decreased in cultivars afterwards. However, some reports contradict this trend, as observed in the Siberian wheat cultivars where no significant change in mineral concentrations was observed over the period of 110 years [17]. Our results were in complete agreement with these studies, and partially supported by the allelic effect of *Rht-1* haplotypes on GFe and GZn. Previously, analysis of GFe and GZn in several bread wheat and durum wheat near-isogenic lines of *Rht-1* genes revealed that semi-dwarf lines reduced GFe by 3.2 ppm and GZn by 3.9 ppm [8]. In another set of near-isogenic lines of *Rht-1* genes, semi-dwarf lines showed decreased level of GFe and GZn, while K and Ca were increased [20]. However, no confounding effect of *Rht*, *Ppd* and *Vrn* genes was found affecting GZn and GFe concentrations in the association mapping panel of HarvestPlus [21], which supports our results that such effects could not be significant in natural germplasm.

In this study, the correlation between GFe and GZn was positive, which corroborates with most of the previous findings [22-24]. However, the extent of correlation is highly variable in most of the studies. The positive correlation between GFe and GZn is very useful to identify the common genetic basis to breed for both traits. It was important to observe that grain P was highly positively correlated with most of the micronutrients including GZn, Ca, Mg, Mn and Se. In wheat grain, P is stored as phytic acid as aleurone, and significantly inhibits the bioavailability of divalent mineral cations [25]. Therefore, it is very important to devise biofortification breeding strategies to modify the distribution of P between phytic acid and inorganic P [24]. The principal component analysis suggested that most of the variation was explained by first two principal component, and PC1 weighted towards the micronutrients with highest loadings. Therefore, wheat cultivars with high scores for PC1 are likely to have high mineral concentrations, and most of the old cultivars are included in this category. The correlation of GZn with GY negative but was not significant, but Cu and Al had significant negative correlations with GY. This contradicts with most of the studies where negative correlation was observed between GY and micronutrients [22, 24, 26]. However, the insignificant correlation of micronutrients with TKW has been reported elsewhere [22, 27], which corroborates our results. It is important to carefully assemble the diversity collection for such relationships, as in our case the cultivar collection was from irrigated and rainfed areas from South and Central Pakistan, which have different yield related attributes like TPP and TKW. Historically, the wheat cultivars from South Pakistan have more TKW on an average compared to cultivars from other parts of the country.

The target to increase of GZn concentration by 12 mg/kg, and similar increase in GFe specifically for Pakistan is very challenging. The approaches need to enhance the GZn, GFe and other micronutrients will include to introduce diversity from other genetic resources or wild species of Triticeae [28, 29]. In most cases, the landraces, synthetic

hexaploidy wheat and wild relatives of wheat were identified with higher levels of grain micronutrients [30-32]. The conventional breeding approaches have been successfully used to incorporate such diversity in to elite germplasm. CIMMYT's biofortification breeding program has developed elite cultivars by targeted crosses between high yielding germplasm and high micronutrient germplasm, and selecting the desired traits in large population sizes [4]. This strategy has resulted in the development and released of several cultivars like 'Zinc Shakti (Chitra)' in India, WB-02, HPBW 01 (PBW 1 Zn), Zincol-2016 in Pakistan, and BARI-Gom 33 in Bangladesh, having 33-40% more GZn compared to check cultivars [33].

Apart from the GFe and GZn, other micronutrients like Ca, Se and Mn are also important for human health and breeding for such micronutrients has been largely ignored. Our data showed significant variations of other micronutrients. Selenium is essential micronutrient with antioxidant, anti-cancer and anti-viral effects. In this study, grain Se concentration was positively correlated with GFe, GZn and TKW, and a two-fold increase was observed in some cultivars. Previously, significant variation was observed for Se concentration in bread wheat and related species, and like other minerals, Se variation was associated with spatial variation in soil Se [34]. Similarly, the decrease in Ca concentration in modern wheat cultivars might have adverse health consequences, and biofortification for grain Ca is largely ignored [35]. The correlation was positive between Ca and GFe and GZn and other micronutrients which suggested common breeding strategies could be devised for simultaneous improvement of these micronutrients in wheat.

5. Conclusions

Conclusively, this study provided insight into the mineral status and yield of wheat cultivars historically deployed in Pakistan. Overall, with the improvement of GY was not translated with the improvement in micro- and macronutrients. Although GFe (0.06 mg/kg/year) and GZn (0.15% year) slightly declined in modern wheat cultivars compared to old cultivars, there are some high yielding cultivars like Zincol-2016 and AAS-2011 which have high levels of micronutrients. Elucidating the genetic basis of GY and micronutrient concentrations could help to develop cultivars with both improved yield and biofortification status.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1. Genetic gain analysis for micronutrients and morphological traits in historical wheat cultivars of Pakistan. Table S2. BLUEs for all phenotypes and the genotypic data used in this study

Author Contributions: AR, ZH, XX and TM: designed the experiment. MS, MS, SuR, MA: performed the experiment. AR and ZM wrote the manuscript. SN and SM: performed the genetic studies. YH, AR and ZH: conceived the idea and edited the manuscript.

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