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Increasing Iron, Zinc, Calcium, Magnesium and Potassium Concentration and Their Bioavailability in Spring Wheat Mutant Lines for Improving Health

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Abstract: Metal, primarily Fe and Zn, deficiencies affect over half of the world's population. Human diets with prevalent cereal products cause micronutrient malnutrition. Biofortification is one of the most effective approaches to alleviate malnutrition. Spring wheat genetically stable (M_7) mutant lines developed with 100 and 200 Gy gamma treatments to broaden genetic variation and search for new resources were analyzed for nutritionally important minerals (Ca, Mg, K, Fe, and Zn), their bioavailability, and grain protein content (GPC). The variation was 172.3–883.0 mg/kg for Ca, 472.9–1088 mg/kg for Mg, 3128.6–5487.5 mg/kg for K, 40.9–89.0 mg/kg for Fe, and 22.2–89.6 mg/kg for Zn. In mutant lines, among the investigated minerals, the highest increases in concentrations were observed in Fe, Zn, and Ca when compared to the parent. Some mutant lines, mostly in the 100 Gy-derived germplasm, had two to three times higher Fe, Zn, and Ca concentrations, lower phytic acid concentration (1.4–2.1 times), and 6.5–7% higher GPC compare to the parent. Variation was detected for the Ca:Phy, Mg:Phy, Phy:K, Phy:Fe, and Phy:Zn molar ratios (1.27–10.41, 5.05–18.68, 1.66–4.87, 1.40–5.32 and 1.78–11.78, respectively). The results showed how the genetic variation could be generated through radiation and be useful to develop biofortification by micronutrient varieties with their appropriate bioavailability to overcome malnutrition.

Keywords: bread wheat; correlations, gamma, grain nutrients concentrations; mutation, phytic acid; metals bioavailability

1. Introduction

Nutrient malnutrition represents one of the major challenges worldwide characterized by an increasing number of people with different forms of its manifestation [1–3]. Nearly 30% of humanity—infants, children, adolescents, adults, and older persons in the developing world—are currently suffering from one or more of the multiple forms of malnutrition [2]. Malnutrition and diet are by far the biggest risk factors for this global disease: every country is facing a serious public health challenge from malnutrition [3]. There are 49 known essential nutrients for sustaining human life [4]. Insufficient intake of even one of these nutrients resulting from diets can cause deleterious metabolic contraventions leading to diseases, reduction of human health and shortened life expectancy, the

impairment of the quality of life cognitive functions, delayed development in children, and large economic costs to society [4,5]. Malnutrition, widely recognized as a major health problem, is especially caused by cereal-based diets that are primarily deficient in micronutrients (Zn and Fe) and is predominant in low-income and middle-income countries [6]. The consequences of a majority of malnutrition and nutrition-related diseases including intrauterine growth, which is 23.8% of all births per year; protein-energy malnutrition (underweight) is in 26.7% of children under-five worldwide; and over 60% of the world's 6 billion people are Fe deficient and over 25% are Zn deficient [7].

In human nutrition, Fe and Zn are frequently evaluated together [8] as these minerals share common dietary sources, the consumption of both metals from food is enhanced and inhibited by similar compounds [5,9], and as a result, a deficiency of both nutrients is thought to occur simultaneously. In addition, calcium (Ca), magnesium (Mg), and copper (Cu) deficiencies are common in many developed and developing countries [10–14].

The 22 developing countries listed are those whose populations receive 43–78% of dietary Fe and 56–88% Zn from C_3 grains and legumes [6]. Wheat (*Triticum aestivum* L.) is consumed as one of the major human foods with increasing demand due to growing population and as a source of essential nutrients and protein for nutrition [2]. Wheat provides 28% of the world's edible dry matter and up to 60% of the daily calorie intake in developing countries [1,15,16]. However, genetic gains in grain yield have not changed over the years or decreased wheat nutritional value, particularly in the Fe and Zn grain protein content [17–19]. Such a trend will worsen since as it has recently been shown that the edible portions of food crops grown in open field conditions under elevated atmospheric CO_2 of 550 parts per million have significantly decreased iron contents by 4–10% [20]. In addition, over the years, wheat breeding has reduced genetic diversity through the replacement of cultivars by modern higher yielding varieties[5], which can result in decreased nutritional quality. The increase in yield was accompanied by the dilution effect of micronutrient concentrations due to increased kernel weight [21].

Biofortification, i.e., the genetic enhancement of nutrient concentration in the grains of stable food crops through breeding, is one of the most cost-effective and environmentally safe ways to prevent and alleviate nutrient malnutrition in humans [4,22,23]. It was demonstrated, for example, under biofortified wheat in the diet, Zn human consumption was substantially higher relative to the non-biofortified one [24]. Moreover, the biofortification of crops through breeding has multiplicative advantages [22].

Along with increasing the concentration of nutrients in food crops, it is importance to reach a high bioavailability for human nutrition [25]. Wheat foods are rich in antinutrients, first of all, phytic acid (Phy), which interferes with the absorption or utilization of nutrients in humans [1,5]. In general, staple food crop grains contain very low bioavailable Fe and Zn (i.e., about 5% of the total grain Fe and about 25% of the total Zn are bioavailable). To increase the Fe bioavailability from 5 to 20%, it would be equivalent to increasing the total Fe fourfold [26]. It has been noted that it is genetically much easier to significantly improve the bioavailability of Fe and Zn in comparison to increasing their total concentration by this magnitude based on conventional breeding [26,27]. To measure mineral bioavailability in the human diet, their molar ratio with Phy has been widely used [4]. The regulation of Fe status in the human body is carried out through absorption rather than secretion and at the same time, its Zn homeostasis in addition to absorption is also regulated through the gastrointestinal secretion and excretion of endogenous Zn [28].

Breeding for low Phy concentration is considered to be a reasonable objective to enhance the nutrient bioavailability of crops. For the decline of Phy, essential efforts have been made to mutagenize crops. Low phytate mutants (*lpa*) have been reported for several cereals using chemical and physical mutagenesis [29–33]. Sussessful breeding for associated increases in the nutritional value of cereal foods and yield-associated traits requires genetic variation, which has to be achieved without negative effects on valuable ones. The genetic diversity of crops has decreased primarily as a consequence of breeding, including the repeated use of local germplasm and the adoption of breeding schemes that do not favor genetic recombination [34]. Mutagenesis is a powerful instrument to broaden genetic variation and has been used for yield increase, but has been studied less for the improvement of grain nutritional value [35,36]. The FAO/IAEA Mutant Variety Database in 2014 reported 3220 worldwide mutant varieties of 214 plant species [37]. Mutagenesis is especially valuable for inducing novel genetic variations in major crops that have restricted genetic variability [35].

Macro elements such as Ca, Mg, and K, etc. as abundant minerals in the body are the most important components required by humans in their daily food [4,14,38]. All body processes depend upon the action of minerals to activate diverse enzymes performing metabolic functions. An adult body contains approximately 140 g K, the majority of which resides intracellularly with a small amount in extracellular fluid [39]; 25 g Mg with 50% to 60% present in the bones and the rest mostly in the soft tissues [38]; and less than 1% of total body calcium is needed to support these critical metabolic functions [40]. A low level of these essential nutrients have been linked to metabolic syndrome, insulin resistance, heart disease, hypertension, and some forms of cancer. The relationship between Ca and Mg, adequacy of both Ca and Mg as well as the Ca:Mg ratio are important for health [41]. Health consequences should be considered for the 64-67% of adults in the USA who do not meeting their Mg requirement from foods, many of whom also consume below their Ca requirements and their increasing Ca:Mg ratio from foods [42].

Human studies indicated that Phy inhibits Ca absorption, but its effect on Ca bioavailability seems to be less pronounced when compared to that on the bioavailability of Fe and particularly Zn [43]. This is possibly related to the relatively high Ca concentration of cereals foods, the capability of the bacterial flora in the colon to dephosphorylate Phy, and the intake of Ca from the colon [44]. Relatively few studies have described the effects of Phy on dietary Mg utilization. Phytate has been shown to decrease its bioavailability in in vivo studies, but it appears that the effect of phytate on Mg availability is less marked than those for some other essential elements [45].

The nutritional value of a crop is dependent on grain protein content (GPC) having a great impact on the end products [46]. Breeding for improvement of GPC is difficult due to a restriction of the range of GPC variation in cultivars.

The objectives of this study were (1) to evaluate the variability in Ca (CaC), Mg (MgC), K (KC), Fe (FeC), Zn (ZnC), and Phy (PhyC) concentrations, and grain protein content (GPC) in spring wheat grain of parent (cv. Zhenis), advanced mutant lines (M_7) which are derived 100- and 200 Gy-gamma doses treatment; (2) to estimate the bioavailability of metallic nutrients, and (3) to evaluate the square correlation between thousand grain weight (TGW) and quality parameters. The comparison of mineral concentration in cv. Zhenis, 100, and 200 Gy-derived mutant lines with recommended uptake and percentage of recommended uptake from flour consumption was also determined.

2. Materials and Methods

2.1. Plant Material

Grains of the spring bread wheat awn-less variety Zhenis (*Triticum aestivum L.)* were irradiated with 100 Gy and 200 Gy doses from a ^{60}Co source at the Kazakh Nuclear Centre. After irradiation, seeds were planted to raise M_1 plants [47]. The M_1 generation was grown in the experimental field of the Kazakh Institute of Agricultural and Breeding near Almaty (43°15′N, 76°54′E, elevation 550 m above mean sea level). Single spikes from each plant for the M_2 generation were harvested, and selection of the best lines based on the yield of individual plants continued to the M_7 generation. Seed was gathered from the main spike; although tiller number and size varied, each plant produced only a single main spike. Seeds from the best yielding mutant lines were individually selected in each generation. The selection criteria for these lines were grain weight per main spike (GWS) and per plant (GWP) and applied in the M_3 and M_4 generations (2011 and 2012) based on the values for the parent cv. Zhenis grown in the same trial conditions. In 2011, the parent line had mean GWS of 1.20 ± 0.51 g and GWP of 1.85 ± 0.61 g. The threshold criteria for selection in the M_4 generation were GWS > 1.4 g and GWP > 2.3 g for the mutant lines. The initial number of lines in the M_1 generation was 2000 each for the 100 Gy and 200 Gy radiation doses. In the M_3 generation, 61 lines (20%) were selected from the 100 Gy irradiation dose population and 48 lines (16%) were selected from the 200 Gy dose. The same numbers of lines for each radiation dose were selected for the M_4–M_6 generation. After harvesting the M_7 plants, 23 lines and 8 lines from the original 100 and 200 Gy-treated germplasm were selected. The 100 Gy lines were numbered as follows: 6(9), 10(15), 11(6), 13(9), 15(1), 16(4), 17(7), 18(2), 20(10), 24(7), 26(5), 29(8), 36(13), 37(4), 39(2), 42(4), 43(43), 45(1), 47(2), 49(2), 52(10), 53(5), and 55(10); and 200 Gy lines were numbered: 57(4), 58(8), 59(2), 61(2), 62(2), 63(2), 64(2), and 65(3). These mutant lines, selected from the two different levels of irradiation doses, were then used for further analysis for nutritional qulaity. Grain samples from each mutant line and parent Zhenis were planted together in a field trial and were grown in three replicates of three row plots, 2 m long x 1.20 m width, and 20 cm between rows with 30 seeds planted per row for further evaluation. The trial was managed according to locally recommended agronomic practices. Applied fertilizers, time of their use, and soil were described [47]. Ten randomly selected lines were taken for analysis (five samples per row). To record yield associated traits, thousand grain weight (TGW) were measured as the mean weight of three sets of 100 grains per line multiplied by 10.

2.1. Determination of Grain Protein Content

Grain protein content was determined with near-infra red reflectance (NIR) spectroscopy on whole grains (ZX50 Portable Grain Analyzer, USA) using proprietary calibration software provided (Zeltex Hagerstown, Ma USA). Three repetitions were done using 25 grains per line.

2.2. Analysis of Grain Iron and Zinc Concentrations

Grain samples (advanced M_7 mutant lines and parent, cv. Zhenis) were washed with sodium dodecyl sulfate (0.1%), rinsed in deionized water, dried to a constant weight at 65–70 °C, and then ground with a mixer mill (Retsch MM400 GmbH). The digestion and extraction of the sample (0.2 g) are described in [47]. Magnesium (MgC), calcium (CaC), potassium (KC), iron (FeC), and zinc (ZnC)

concentrations were measured using flame atomic absorption spectroscopies Model NovAA350, AnalytikJena, Jena, Germany). Measurements of all minerals were checked against the certified reference values from the state standard samples LLC "HromLab", K-7473-98, Ca-7475-98, Mg-7190-95, Fe-7254-96, and Zn-7256-96 diluted by 0.3% HNO_3. Three extract and analysis repetitions were performed.

2.3. Phytic Acid Extraction and Determination, and Molar Ratios of Phy:Metals and Metals:Phy

The extraction of Phy from the milled grain samples (0.3 g) was performed as described in [48] and 2.5 mL of the supernatant was treated with 2 mL 0.2% $FeCl_3$, and the mixture was boiled for 30 min with further centrifugation after cooling. The residue was washed twice with deionized water. A total of 1.5 M NaOH was added to the precipitation, shaken, and the solution centrifuged. Three mL of 0.5 M HCl was added to the precipitation, then shaken until the precipitation dissolved. The solution was diluted to 25 mL to measure Fe remaining in the solution by atomic absorption spectrophotometer (AAS, Shimadzu AA6300, Japan). Phy sodium (Sigma St Louis, Missouri, USA) was used to test the Phy recovery rate. The Phy test results suggested that the recoveries were between 96 and 102%. The determination of Phy was based on the precipitation of ferric phytate and measurement of Fe remaining in the supernatant. The grain Phy was calculated by multiplying Fe content by a factor of 4.2. To calculate the molar ratios of Ca:Phy and Mg:Phy and Phy:K, Fe and Zn, the concentrations of Phy and the metals were converted into moles by dividing by their respective molar masses and atomic weights. The [Ca][Phy]/[Zn] (mol/kg) was also calculated.

2.4. Statistical Analysis

One-way ANOVAs were used for comparisons; all data were evaluated in R 3.0.2 (R Core Development Team 2013). Simultaneous tests of general linear hypotheses and Dunnett's contrasts were used for multiple comparisons of the means. Summary data are reported as mean values ± standard deviations. Square correlation coefficients between TGW and grain quality parameters and p values were calculated using GenStat software (10th edition). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Variability in Ca, K, Mg, Fe and Zn Concentrations in Grain of Spring Wheat M_7 Mutant Lines and Parent

Significant differences in Ca, Fe, Zn, K, and Mg concentrations were found among the new spring wheat M_7 mutant lines developed using dose radiation of 100 Gy and 200 Gy and parent cv. Zhenis. Table 1 shows the means and ranges of the parameters. The CaC varied from 172.3–883.0 mg/kg in mutant lines (n = 93). Significantly enhanced CaC exceeded the parent by 1.23 to 2.47 times with its highest mean in 100 Gy-treated lines identified in 15 M_7 lines (48.4%).

Table 1.	cv. Zhenis	100 Gy-lines	200 Gy-lines
Comparison			
between trait			
means and			

ranges for spring wheat cv. Zhenis (parent), M$_7$ derived from 100 Gy- and 200 Gy- irradiated mutant lines**Trait**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**
TGW (g)	40.75	40.35–41.45	42.46	36.25–57.4	41.15	33.25–52.15
GPC, %	13.00	13.0–13.1	13.85	13.6–14.3	13.34	13.2–13.7
CaC (mg/kg)	357.07	350.6–365.1	568.13	172.30–883.0	530.80	203.40–856.5
KC (mg/kg)	3173.33	3156.8–3187.5	4140.18	3128.60–5284.20	4335.7	3256.5–5487.5
MgC (mg/kg)	686.53	677.2–698.9	871.83	629.80–1083.30	880.10	472.90–1088.4
FeC (mg/kg)	33.20	31.10–35.10	57.20	40.95–88.83	65.09	44.57–89.03
ZnC (mg/kg)	36.1	33.1–39.3	56.03	22.2–89.60	56.40	22.2–88.10
PhyC (mg/g)	2.59	2.58–2.63	2.23	1.17–2.66	2.47	2.02–2.65
Ca:Phy	2.26	2.24–2.28	4.34	1.39–10.41	3.57	1.27–5.95
Mg:Phy	7.27	7.12–7.47	11.32	6.79–18.68	9.81	5.05–13.03
Phy:K	4.84	4.78–4.92	3.273	1.66–4.87	3.50	2.42–4.80
Phy:Fe	6.65	6.21–7.19	3.49	1.40–5.32	3.39	2.18–4.90
Phy:Zn	7.16	6.66–7.71	4.48	1.78–11.78	5.59	2.60–11.19
[Ca][Phy]/[Zn] (mol/kg)	0.97	0.92–1.05	1.01	0.21–3.01	1.15	0.30–3.32

The KC ranged from 3128.6–5487.5 mg kg^{-1} with a mean 4190.6 ± 680.7. The four genotypes (12.9%) had significantly 1.65 to 1.73 times higher KC than the parent. Variation for Mg was 472.9–1088 mg/kg with 877.9 ± 128.4. In 27 lines (87.1%), significantly higher MgC relative to the parent by 1.14 to 1.56 times was revealed. Significant variation was also found for the microelements, Fe and Zn, between mutant lines which were derived 100 and 200 Gy-irradiated lines with means of 40.95–89.03 mg/kg and 22.2–89.6 mg/kg (n = 93), respectively. Significantly higher FeC and ZnC than the parent by 1.23 to 2.66 times and 1.45–2.42, respectively, were identified in 16 (51.6%) and 17 (54.8%) M$_7$ lines. The GPC varied from 13.1 to 14.3% with a mean of 13.72 ± 0.26% (n = 93). The 17 genotypes (54.8%), all of the 100 Gy-dosed lines, had a significantly 6.5 to 7.6% higher GPC relative to the parent. The mutant lines exhibited wide variations in PhyC from 1.17 to 2.66 mg/g (Table 1). When compared to the parent, significantly lower PAC by 1.25 to 2.02 times was detected in nine mutant lines (29%).

Analysis of variance (ANOVA) with differences in all nutrient concentrations among cv. Zhenis and mutant lines are shown in Table 2. These results revealed significant differences between the cv.

Zhenis and derived 100 Gy- and 200 Gy-mutant lines for all traits except that of Phy:Zn for cv. Zhenis and the 200 Gy-treated lines (Table 2). However, the interactions between the 100 Gy- and 200 Gy-dosed lines were significant in terms of nutrient concentrations of FeC, GPC, and PhyC. For metals bioavailability, such associations were in the Ca:Phy and Mg:Phy molar rations. The radiation effect of 100 Gy was highest in GPC, indicating its increased efficiency to generate mutations in the genome associated with this trait. Traits such as KC and FeC, and therefore in Phy:K and Phy:Fe, explained that greater variation of 200 Gy than 100 Gy treatment showed the most influence for their improvement under a high level of radiation.

Significant variations in the molar rations of Phy:metals (Ca, Mg, K, Fe and Zn) were also noted between the parent and mutant lines (Table 2). Variation was detected for the Ca:Phy, Mg :Phy, Phy:K, Phy:Fe, and Phy:Zn molar ratios, (1.27–10.41, 5.05–18.68, 1.66–4.87, 1.40–5.32 and 1.78–11.78, respectively) (Table 1).The most noticeable variation in metal bioavailability between the parent and mutant lines was found for the Phy:Fe molar ratios, followed by Ca:Phy and Phy:Zn. The lowest means of these characteristics and therefore their highest bioavailabilities presented in the 100-Gy-derived mutant lines were in intervals of 4.02–4.75 fold lower. It was also observed that the 100 Gy-treated lines significantly differed from the ones developed by the 200 Gy treatment in the Ca:Phy and Mg :Phy molar ratios (Table 2).

Table 2. Comparison of Ca, Mg, K, Fe, Zn, and phytic acid concentrations and Phy:nutrients molar rations of advanced M$_7$ mutant lines and parent grain of spring wheat cv. Zhenis (ANOVA analysis).

Source of variation	Df	CaC	MgC	KC	FeC	ZnC	GPC	PhyC	Ca: Phy	Mg:Phy	Phy:K	PhyFe	Phy:Zn
cv. Zhenis x 100 Gy-dosed lines	83	23.13 ***	58.33***	44.75 ***	64.97***	21.87 ***	647.78 ***	16.20 ***	27.18***	41.80***	84.32***	169.05 ***	29.50 ***
cv. Zhenis x 200 Gy-dosed lines	38	28.89***	46.45***	76.93 ***	203.83 ***	26.98 ***	149.29 ***	19.62 *	36.50***	51.27**	134.82 ***	468.55 ***	10.08

100 Gy-x 200 Gy-dosed lines	92	0.74	0.008	1.62	7.15*	0.01	244.41***	9.79***	4.49*	6.79*	1.72	0.20	4.29

The lines were significantly different from the parent line. Each line was analyzed by three replicates. *, **, and *** denote significance at the 0.05, 0.01, and 0.001 probability level, respectively.

3.2. Correlations between Nutrient Concentration, Phytic Acid and Thousand-Gain Weight

To examine the relations among the macro- and microelement concentrations with phytic acid and yield, R squared correlation analysis was conducted (Table 3). In the parent, several traits showed consistent correlations between them. For instance, among the macroelements and macroelements with microelements, a highly significant relationship was detected between MgC and KC, and FeC and CaC, respectively. The correlations between PhyC and the metal concentrations were highly significant in the order of ZnC, CaC, and FeC.

Table 3. R^2 correlation coefficients with p values between nutrient concentration, phytic acid, and thousand grains weight.

	TGW	GPC	FeC	ZnC	CaC	KC	MgC
cv. Zhenis							
GPC, %	0.024						
FeC (mg/kg)	0.002	0.000					
ZnC (mg/kg)	0.622***	0.017	0.34*				
CaC (mg/kg)	0.011	0.000	0.98***	0.052			
KC (mg/kg)	0.908***	0.02	0.12	0.32*	0.042*		
MgC (mg/kg)	0.606***	0.0125	0.44**	0.48**	0.297*	0.869***	
PhyC (mg/g)	0.454**	0.0128	0.504**	0.972***	0.646***	0.175	0.004
100 Gy-dosed lines							
GPC, %	0.01						
FeC (mg/kg)	0.081*	0.014					
ZnC (mg/kg)	0.084*	0.000	0.11**				
CaC (mg/kg)	0.005	0.025	0.045	0.063*			
KC (mg/kg)	0.028	0.009	0.009	0.006	0.048		
MgC (mg/kg)	0.0037	0.022	0.022	0.000	0.011	0.019	
PhyC (mg/g)	0.092**	0.012	0.080*	0.043	0.021	0.022	0.000
200 Gy-dosed lines							
GPC, %	0.005						
FeC (mg/kg)	0.220*	0.011					

ZnC (mg/kg)	0.009	0.012	0.35**			
CaC (mg/kg)	0.03	0.014	0.036	0.004		
KC (mg/kg)	0.225*	0.27**	0.442*	0.28**	0.000	
MgC (mg/kg)	0.037	0.164*	0.052	0.105	0.511***	0.052
PhyC (mg/g)	0.323**	0.254*	0.273**	0.027	0.011	0.175	0.13

The lines were significantly different from the parent line. Each line was analyzed by three replicates. *, **, and *** denote significance at the 0.05, 0.01, and 0.001 probability level, respectively.

Generally, the correlations between the investigated traits were found to be essentially lower than the cv. Zhenis and 200 Gy-derived lines, for the 100 Gy-derived lines. There was a significant and positive association between FeC and ZnC (r^2 = 0.11, p < 0.01) (Table 3). As we revealed for the 100 Gy- and 200 Gy-derived lines, but not for the parent, there was a significant and positive association between FeC and TGW that was stronger in the 200 Gy-derived lines. Among the macroelements and macroelements with microelements, the 200 Gy-dosed lines showed significant relations of MgC, unlike the parent and 100 Gy-derived lines, with CaC and also with GPC. Another distinctive significant feature in the 200 Gy-dosed lines was the correlation between KC with GPC, FeC, and ZnC. A significant relation was also revealed between Phy and FeC, and also GPC, but not with ZnC.

The relations between Phy:nutrients molar rations and TGW and GPC were also analyzed (Table 4). In the parent cv. Zhenis, there was only a significant relation between TGW and Phy:Zn. The 100 Gy- and 200 Gy-dosed lines presented significant correlations between TGW and Phy:Fe and Phy:K molar rations with their means more two times higher in 200 Gy-dosed lines. These results indicate highly possible simultaneous improvement of both Fe and Mg bio-availabilities with the spring wheat productivity component.

Table 4. Association between phytic acid:nutrients molar rations and thousand grains weight and grain protein content for cv. Zhenis, spring wheat and advanced mutant lines (100 Gy- and 200 Gy- derived).

	Phy:Fe	Phy:Zn	Ca:Phy	Phy:К	Mg: Phy
cv. Zhenis					
TGW, g	0.007	0.759***	0.126	0.0065	0.067
GPC, %	0.00	0.013	0.002	0.001	0.000
100 Gy-derived lines					
TGW, g	0.086*	0.02	0.03	0.112**	0.034
GPC, %	0.025	0.005	0.00	0.133**	0.004
200 Gy-derived lines					
TGW, g	0.222*	0.033	0.003	0.292**	0.14
GPC, %	0.0012	0.141*	0.014	0.106	0.26*

The lines were significantly different from the parent line. Each line was analyzed by three replicates. *, **, and *** denote significance at the 0.05, 0.01, and 0.001 probability level, respectively.

A significant relation between the Phy:K molar rations and GPC was only detected in the 100 Gy-treated mutant lines. In the case of the 200 Gy-treated lines, GPC showed a significant correlation with the Phy:Zn and Phy:Mg molar rations.

3.3. Estimated Nutrients Bioavailability in Wheat Flours

To estimate how the highest means of mineral concentrations from the parent and mutant lines provided the daily intake of Fe, Zn, Mg, Ca, and K, we calculated the ratio of grain minerals concentration from 200 g flour consumption to the percentage of the recommended uptake (Table 5). These calculations were based on the statistics from the FAO [49], where the mean consumption of wheat flour is about 200 g per person per day, and on the values for the recommended intake for adults according to the DGE (German Nutrition Society) [50]. As indicated by these results, in the case of the parent, Fe, Zn, and Mg deficiencies were strongly manifested in decreasing order. The highest concentration of these minerals from the mutant lines that produced whole grain flour was revealed to provide 1.28–1.32 times more than that of the daily intake of Fe, Zn, and Mg. The K concentration and particularly Ca in the grain of the parent and mutant lines supplied were 5.05–12.5 and 1.46–2.44 times higher, respectively, when compared to the required daily consumption of these minerals.

Table 5. Comparison of mineral concentration (mg/kg) in cv. Zhenis, 100 Gy- and 200 Gy-derived lines with the recommended uptake and percentage of recommended uptake from flour consumption 200 g/person/day (mg/day).

Mineral concentration (mg/kg)	Genotypes		Recommended uptake of minerals (mg/day) according to DCE	Percentage of recommended uptake from flour consumption and 200 g/person/day	mg minerals from 200 g flour consumption obtained from max values of genotypes			Minerals mg from percentage of recommended uptake	Ratio of grain minerals concentration from percentage of recommended uptake		
	cv. Zhenis	100 G			cv. Zhenis	100 Gy-dosed lines	200 Gy-dosed lines		cv. Zhenis	100 Gy-	200 Gy-

		γ-dosed lines								dosed lines	dosed lines
Fe	31.30	88.83	10	76	6.26	17.77	17.81	13.50	0.35	1.32	1.32
Zn	36.1	89.60	10	78	7.22	17.92	17.62	13.98	0.40	1.28	1.26
Mg	686.53	1083.30	350	78	137.31	216.66	217.68	168.99	0.63	1.28	1.29
Ca	357.07	883.0	1000	8	71.41	176.6	171.30	14.13	5.05	12.50	12.12
K	3173.33	5284.2	2000	41	634.67	1056.84	1097.50	433.30	1.46	2.44	2.53

4. Discussion

This study clearly showed the potential of spring wheat genetically stable advanced (M_7) mutant lines which were derived the 100 Gy and 200 Gy irradiation exceptionally high concentrations of nutritionally important nutrients (Ca, Mg, K, Fe, Zn, and GPC) and their bioavailability (Tables 1 and 3). The variation was 172.3–883.0 mg/kg for CaC; 472.9–1088 mg/kg for MgC; 3145–5485 mg/kg for KC; 31.1–89.0 mg/kg for FeC; and 22.2–89.6 mg/kg for ZnC. Among investigated minerals, the greatest increase in their concentrations in the mutant lines when compared to the parent was found in FeC, followed by CaC and ZnC with means of 2.66, 2,47, and 2.42, respectively. The 17 genotypes (54.8%), all of the 100 Gy-derived lines, had a significantly 6.5 to 7.6% higher GPC relative to the parent.

The concentrations of minerals in the wheat mutant lines exceeded those already reported for hexaploid wheat by 2.35–2.96-fold for FeC [17,18,51–54]. Although environmental factors can influence grain metal concentrations, in this work, all the mutant lines and the parent were grown under the same field conditions treated same, with no specific fertilizer supplementation or other inputs of these metals. The study that used 63 historical and modern wheat cultivars for the evaluation of grain yield and concentration of Ca, Cu, Fe, Zn, Mg, Mn, P, and Se reported that over a long time, the concentrations of all minerals, except Ca, decreased, while grain yield increased [17,18]. Therefore the situations shows that a greater consumption of wheat bread from modern cultivars is required to achieve the same percentage of recommended dietary allowance levels contributed by other genetic resources or older cultivars with low yield.

Micronutrient intake from wheat is essentially determined by the amount of their availability for human absorption. High micronutrient bioavailability can be achieved by the reduction of antinutritional agents, particularly Phy, considered to be the most important causative factor [4,5,14]. The main functions of Phy is as a phosphorus and energy store, and a source of cations and myoinositol as well as combating environmental issues related to seed PA-P could be improved by decreasing PhyC.

Breeding for low PAC is a reasonable objective to enhance nutrient bioavailability in grain. For the decline of PhyC, essential efforts have been made to mutagenize crops. Low phytate cereal mutants (*lpa*) have been reported using chemical and physical mutagenesis [30–32,33,55]. In wheat, the *lpa* mutant was isolated by chemical mutagenesis [32]. The *lpa* maize mutants have been used for the breeding of high inorganic phosphate (HIP) phenotypes [29]. At the same time, the *lpa* mutations found in several crops usually lead to pleiotropic effects on plant and seed performance such as reduced germination and emergence rate, lower seed filling, and susceptibility to stress [29]. Our experiments showed that low Phy wheat lines generated on cv. Zhenis and the 100 Gy and 200 Gy radiation did not differ when compared with the parent in seed viability, shoots and roots growth (data not shown).

The variation found for PhyC ranged from 1.17 to 2.66 mg/g (2.3-fold variation) in all of the mutant lines (Table 1). There were significant differences between the 100 Gy- and 200 Gy-derived mutant lines (Table 2). The lowest PhyC presented in the 100 Gy-dosed lines and numbed by (24(7) was more than two times lower, relative to that of the parent. It is an interesting fact that searching for low Phy lines among the M_7 mutant lines generated by the 100 Gy on a genetic background of cv. Eritrospermum-35, allowed us to identify the lowest PhyC, which was lower relative to the parent by 3.5 times [57].

Studies of natural wheat variation revealed the huge difference for PhyC. Similar PhyC to the means of the current study were reported in Liu et al. (2017) [58]. Higher PhyC was found in 93 wheat genotypes (5.9 to 20.1 mg/g) [59], in modern varieties (12.5 to 34.2 mg/g) [60], Indian bread wheat (23.9 mg/g) [61], from 6.7 to 10.5 mg/g [44], from 7.65 to 8.8 mg/g [62], and ranged from 7 to 13 mg/g depending on Zn fertilization [63]. The highest PhyC was also reported for whole-wheat flour (44.91 mg/g) [64] and Spanish wheat (24.6–45.4 mg/ g) [65]. Possible explanations for the inconsistent finding might be to do with the methodology of PA determination also indicared by Gibson et l., 2010 [66]. that the selection of the most appropriate method for the PA analysis is critical.

The potential bioavailability of the nutrients for human consumption is estimated by the Phy:micronutrients molar ratios or vice versa for the microelements. In general, low molar ratio means high mineral bioavailability and the same conversely. In the current study, significant variability for the Ca:Phy, Mg:Phy, Phy:K, Phy:Fe, and Phy:Zn molar ratios between the parent and mutant lines was detected (Table 1). Among them, the most pronounced variation when compared to the others were for Ca:Phy (more than 7-fold) and for Phy:Zn (around 7-fold) in the 100 Gy-dosed mutant lines. A high variation for Phy:Fe (1.40–5.32) with its mean of about a 4-fold difference was also detected. To significantly increase Fe and Zn absorption Phy:Fe molar ratios was estimated at <1 or preferably <0.4 [67] and for the Phy:Zn molar ratio, <5 was considered as high Zn bioavailability, corresponding to approximately 50% of the total Zn [68]. A similar study with a Phy:Fe range of 1.96–3.86 in 12 bread wheat varieties was reported in [69]. There were higher Phy:Fe molar ratios in the literature such as means of around 12 in two bread wheat cultivars [70], 10–12 in wheat whole flour

[57], and of 15.5–31.3 in a set of nine bread wheat varieties [71]. For the Phy:Zn molar ratio, means of 23.9–41.4 in 65 bread wheat varieties of Pakistan [72], and ones of 29–178 in bread wheat [73] have been reported. In human studies, the molar ratio [Ca][Phy]/[Zn] is a better forecaster of Zn bioavailability as Ca strengthens the effect of Phy on Zn absorption due to the existence of kinetic synergism between the Ca and Zn ions resulting in the formation of a more insoluble Ca:Zn:Phy complex when compared to the Phy complexes formed by either alone. A [Ca][Phy]/[Zn] molar ratio greater than 0.5 mol/kg may reduce the Zn bioavailability [74]. The current study showed considerable variation for this character (0.21–3.01 and 0.30–3.32) in the 100 Gy- and 200 Gy-derived mutant lines, respectively (Table 1). This means that there was a 11.0–14.3-fold variation in a set of lines and the ones with the lowest values for [Ca][Phy]/[Zn] fall in the category of high Zn bioavailability according to [74].

5. Conclusions

In our study, to search for biofortification by nutrients (Ca, Mg, K, Fe, Zn, and GPC) and high bioavailability, spring wheat new genetically stable (M_7) mutant lines which were 100 and 200 Gy treatments and the parent cv. Zhenis were investigated. The results indicate that these mutant resources of spring wheat can substantially increase the metals bioavailability and health impact of wheat end products. There was only a correlation between TKW and concentrations of Fe in the mutant lines; in addition, such relations were observed for Ca and Phy, and a significant correlation existed between GPC and the concentrations of K, Mg, and Phy. Thus, a greater consumption of whole wheat bread from new mutant lines could contribute to achieving the percentage of recommended dietary allowance levels. Using the abundant variation present in wheat mutant lines, it should be possible to improve mineral concentrations and their bioavailability in modern cultivars without negatively affecting yield.

Author Contributions: S.K., F.S., and G.Zh. conceived and designed the experiments; G.D., A.A., N.O., S.T. .and G.E. performed the experiments; S.K., F.S., and S.A. analyzed the data and wrote the paper.

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