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New Spring Wheat Mutation Resources with Improved Grain Quality, Metals Bioavailability and Yield Components

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Abstract: Genetic variability in micronutrients is limited in wheat varieties. To broaden variation, seeds of cv. Eritrospermum-35 were irradiated with 100 and 200 Gy using ^{60}Co, the treated seeds were grown to M_7 generation with successive selection of the highest yield lines in each generation. Selected lines were evaluated for yield and grain quality traits such as iron, zinc and phytic acid concentrations, calculation of molar ratios of PA:Fe and PA:Zn. A number of mutant lines had 2 to 3 times more iron and zinc concentrations and lower phytic acid concentration (1.1-3.5 times) and higher protein content (11.2-12.4%) relative to the parent were identified. Some M_7 lines showed significantly larger grain weight (1.3-1.5 times) and number per spike (2.0-2.1 times) than the parent. There were significant correlations between zinc and iron concentrations, between grain protein content, zinc, iron and phytic acid concentrations and grain weight per spike, mainly in 100 Gy-dosed lines. The *TaPhyllc* cDNAs nucleotide and protein sequence similarities between parent and these M_7 lines varied from 28.2% and 30.2% to 45.7% and 56.5%, respectively. The results clearly indicate that variability in grain nutrients and metals bioavailability in mutation resource is potential in wheat improving nutritional outcomes and overcome malnutrition.

Keywords: *Triticum aestivum*, Correlation, Grain Nutrients Concentrations, Yield Components, *TaPhyllc* Multiple Inositol Phosphate Phosphatases cDNA Sequence

Introduction

Iron and zinc deficiencies affects over half of the world's population (Borrill *et al*., 2014). Wheat (*Triticum aestivum* L.) is a major cereal source of essential nutrients for human and animal nutrition (Borrill *et al*., 2014; Balyan *et al*., 2013; Lafiandra *et al*., 2014). Thus genetic enhancement of wheat with more of these micronutrients is required to become as one of the most cost-effective and environmentally safe way to alleviate malnutrition (Welch and Graham, 2004). Biofortification of crops through breeding has multiplicative advantages (Borrill *et al*., 2014). Over the years wheat breeding has reduced genetic diversity with the replacement of cultivars by modern higher yielding varieties (FAO, 2014)

and this can result in decreased nutritional quality. High yielding varieties led to the dilution effect of micronutrient concentrations due to increased kernel weight.

Phytic Acid (PA, myo-inositol 1,2,3,4,5,6-hexakisphosphate) as the major storage form of seeds phosphorus (P) (Shahzad *et al*., 2014; Madsen *et al*., 2013) affects the bioavailability of metals due to the strong chelation between the two, forming phytate salt (Shahzad *et al*., 2014). It was shown that the phytate levels affected more on Fe bioavailability than total Fe Zn content (Eagling *et al*., 2014). There was the relationship between phytate and Zn bioavailability (Frontela *et al*., 2009). Therefore, breeding for low phytate content considered to be reasonable objective to enhance nutritional quality of any crop. During seeds

germination, PA is sequentially hydrolyzed by phytases (myo-inositol hexakisphosphate phosphohydrolases, MINPP) (EC 3.1.3.26 and EC 3.1.3.8) by the removal of one or more phosphate groups (Dai *et al*., 2007; Dionisio *et al*., 2007). For the decline of PA biosynthesis essential efforts have been made to mutagenize crops. Low phytate mutants (*lpa*) have been reported for several cereals using chemical and physical mutagenesis (Raboy *et al*., 2000; Liu *et al*., 2007; Badone *et al*., 2012). In wheat, *lpa* mutant was isolated by chemical mutagenesis (Guttieri *et al*., 2004). The *lpa* maize mutants have been used for the breeding of High Inorganic Phosphate (HIP) phenotypes (Raboy *et al*., 2000; Pilu *et al*., 2003). At the same time, *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, susceptible to stress (Raboy *et al*., 2000; Guttieri *et al*., 2004; Doria *et al*., 2009).

The nutritional value of a crop is dependent on grain protein content (GPC) having great impact on end products (Balyan *et al*., 2013). Breeding for improvement of GPC is difficult due to restriction of the range of GPC variation in cultivars. There is a negative correlation between GPC and grain yield (Brevis *et al*., 2010).

Successful breeding for grain quality and yield-associated traits requires genetic variation which has to be distinguishable from environmental effects. Advances in desired traits must be achieved without negative effects on valuable others. 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 favour genetic recombination (Akfirat and Uncuoglu, 2013). Mutagenesis is a powerful instrument to broaden genetic variation and it has been used for yield increase, but for improvement of grain nutritional quality have been much less studied. The FAO/IAEA Mutant Variety Database in 2014 reported 3220 worldwide mutant varieties of 214 plant species (http://mvgs.iaea.org/). To generate genetic variability with further selection of useful traits for crop improvements are achieved using radiation and chemical mutagens (Shu and Lagoda, 2007; Oladosu *et al*., 2015). The major advantage of physical mutagenesis compared to chemical treatments is the measure of accuracy and sufficient reproducibility, particularly for gamma rays, which uniformly penetrate in the tissue (Yamaguchi *et al*., 2003). As it has been shown gamma-irradiation generates a mix of small (1-16 bp) and large (up to 130 kbp) deletion mutations in the genomes of Arabidopsis and rice (Cecchini *et al*., 1998; Morita *et al*., 2009). Mutagenesis is especially valuable for inducing novel genetic variation in major crops that have restricted genetic variability (Parry *et al*., 2009).

This study describes the variability in grain iron (GIC) and zinc (GZnC) and PA (GPA) concentrations and GPC in spring wheat 100- and 200 Gy-dosed mutation resource; the bioavailability of metals, square correlation between on yield-associated and quality parameters; the nucleotide and protein sequence similarities of the *TaPhyIlc* cDNAs MINPP between parent and M_7 mutant lines differing in GPAC.

Materials and Methods

Plant Material

Seeds of the spring bread wheat awn variety Eritrospermum-35 (*Triticum aestivum* L.) were irradiated with 100 and 200 Gy doses from a ^{60}Co source at the Kazakh Nuclear Centre. After irradiation seeds were planted to raise M_1 plants (Kenzhebayeva *et al*., 2017). The M_1 generation was grown in the experimental field of the Kazakh Institute of Agricultural and Breeding in 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 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 selected individually in each generation. The selection criteria for these lines was grain weight per main spike (GWS) and per plant (GWP) and it was applied in the M_3 and M_4 generations (2011 and 2012) and based on the values for the parent cv. Eritrospermum-35 grown in the same trial conditions. In 2011 the parent line had mean GWS of 0.79±0.24 g and GWP of 2.02±0.6 g yield values. The threshold criteria for selection in the M_4 generation were GWS >1.1 g and GWP >2.2 g for mutant lines. The initial number of lines in the M_1 generation was 300 each for the 100 and 200 Gy radiation doses. In the M_3 generation, 61 lines (20%) were selected from the 100 Gy radiation 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, 14 lines and 24 lines from the original 100 and 200 Gy-treated germplasm were selected. The 100 Gy lines were numbered as follows: 145(12), 147(25), 148(1), 149(2), 151(2), 153(4), 155(2), 159(2), 161(7), 165(2), 166(10), 167(2), 169(14) and 171(1) and 200 Gy lines were numbered: 5(43), 6(4), 7(4), 8(26), 11(5), 11(14), 13(3), 14(3), 16(12), 20(4), 22(46), 26(2), 29(15), 30(4), 31(3), 32(3), 33(1), 34(12), 35(1), 36(5), 37(4), 38(1), 41(1) and 172(1). These mutated populations, selected from the two different levels of radiation, were then used for

further analysis. Grain samples from each mutant line and parent Eritrospermum-35 were planted together in a field trial and were gown in three replicates of three row plots, 2 m long, 1.20 m width and 20 cm between rows with planting 30 seeds 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 (Kenzhebayeva *et al.*, 2017). Ten randomly selected spikes from each line were taken for analysis (5 samples per row). To record yield associated traits, the following plant parameters were measured: Grain number and weight per spike (GNS, GWS) and thousand grain weight (TGW) calculated as the mean weight of three sets of 100 grains per line multiplied by 10.

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.

Analysis of Grain Iron and Zinc Concentrations

Grain samples (100 Gy and 200 Gy-dosed M_7 mutant lines and cv. Eritrospermum-35) 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 sample (0.2 g) were described (Kenzhebayeva *et al.*, 2017). Iron and zinc concentrations were measured using atomic absorption spectrophotometer (Shimadzu AA6300, Japan) and flame atomic absorption spectroscopies Model NovAA350, AnalytikJena, Jena, Germany), respectively. Measurements of Zn was checked against the certified reference values from the state standard samples LLC "HromLab", Zn 7837-2000 diluted by 0.3% HNO_3. Three extract and analysis repetitions were performed.

Phytic Acid Extraction and Determination

The extraction of PA from milled grain samples (0.3 g) was performed as described by (Dai *et al.*, 2007) and the 2.5 mL of supernatant was treated with 2 mL 0.2% $FeCl_3$ and the mixturewas boiled for 30 min with further centrifugation after cooling. The residuewas washed twice with deionized water. The 1.5 MNaOH were added to the precipitation, shaken and the solution was centrifuged. The 3 mL of 0.5 MHCl was added to the precipitation and 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). PA sodium (Sigma St Louis, Missouri, USA) was used to test

the PA recovery rate. The PA test results suggested that the recoveries were between 96 and 102%. The determination of PA was based on precipitation of ferric phytate and measurement of Fe remaining in the supernatant (Dai *et al.*, 2007). The GPAC was calculated by multiplying Fe content by a factor of 4.2.

Molar Ratios of PA:Fe and PA:Zn Calculations

The concentrations of PA, Fe and Zn, were converted into moles by dividing by their respective molar mass and atomic weight (660.04, 55.85 and 65.4 g mol^{-1}, respectively). The molar ratios of PA:Fe and PA:Zn were then calculated.

RNA Extraction, PCR Amplification and Sequence Analysis

Total RNA was isolated from leaves of 7-10-day-old seedlings using the TaKaRaUniversal Genomic RNA Extraction Kit (Takaba Bio Inc. Tokyo, Japan). The first strand cDNA was synthesized at 65°C for 5 min using oligo d(T) primer (50 µM, 1 µL), random 6 mers 50 µM, 1 µL, dNTP mixture (10 mM each) 1 µL, RNA 5 µL and RNase free dH_2O, 2 µL. Then after cooling the reaction mixture by combining the following reagents to total volume of 10 µL 5 x Prime Script II Buffer 4 µL, RNase Inhibitor (40 U $µL^{-1}$) 0.5 µL, Prime Script II RTase 1 µL and RNase free dH_2O up to 10 µL was incubated at 65°C for 1 h. The inactivation of enzyme was done by incubating the reaction mixture at 95°C for 5 min.

Primers for cloning wheat mutants MINPP cDNA were designed on the basis of the sequences presented by (Dionisio *et al.*, 2007) (Suppl. Table 1). PCR cloning from cDNA was performed using the high-fidelity DNA polymerases. PCR mixture (25 µL) consisted of 2.5 µL 10 x Trans TaqHiFi Buffer II ($+Mg^{2+}$), 2 µL 2.5 Mm dNTPs, 1 µL 10 µM primers, 0.2 µL 5 UNIT $µL^{-1}$ of Taq polymerase High Fidelity (Beijing TransGen Biotech, Beijing, China), 18.3 µL ddH_2O and 1 µL 50 ng of cDNA. Thermo cycling conditions were as: 1 min at 95°C, 45 s at 58°C and 2 min at 72°C, total 45 cycles. Amplification was performed on a cDNA Engine Dyad thermal cycler (Bio-Rad Labs, USA). Amplified PCR products were separated on 1% agarose gel at 30 min at 130 V and stained with ethidium bromide and then visualized with gel documentation system Bio-Imaging Systems 910 Ұ. DNA sequencing with purified PCR product was performed on an ABI 3730XL sequencer following the manufacturer's instructions (Applied Biosystems, Framingham, MA, USA). All results were conducted with two independent PCR products.

The alignment analysis of nucleotide's and protein's sequences between *TaPhyIIc* MINPP cDNA from parent and mutant lines was performed based on NCBI Blast 2.6.0+, Java Script DNA translator and EMBOSS Stretcher alignments.

Table 1: The square R correlation coefficients with *p* values between yield-associated traits (thousand grain weight, TWG, grain number per spike, GNS, grain weight per spike, GWS) and grain quality parameters (grain protein content, GPC, grain iron concentration, GIC, grain zinc concentration, GZnC and grain phytic acid content, GPAC in parent, cv. Eritrospermum-35 and spring wheat 100 Gy- and 200-Gy dosed M_7 mutant lines

Traits	GWS [g]	TGW [g]	GPC [%]	GIC [mgkg^{-1}]	GZnC [mg kg^{-1}]	GPAC [mgg^{-1}]
cv.Eritrospermum-35						
GNS	0.566**	0.261	0.000	0.118	0.018	0.379*
GWS [g]		0.118	0.250	0.330*	0.273	0.176
TGW [g]			0.008	0.021	0.016	0.200
GPC [%]				0.014	0.063	0.000
GIC [mgkg^{-1}]					0.872***	0.018
GZnC [mgkg^{-1}]						0.002
100 Gy-dosedlines						
GNS	0.087	0.047	0.141*	0.011	0.007	0.01
GWS [g]		0.087*	0.013	0.123*	0.181**	0.313***
TGW[g]			0.001	0.014	0.005	0.001
GPC [%]				0.090*	0.050	0.110*
GIC [mgkg^{-1}]					0.590***	0.607***
GZnC [mgkg^{-1}]						0.711***
200 Gy-dosedlines						
GNS	0.304***	0.001	0.014	0.082*	0.081*	0.003
GWS [g]		0.201***	0.068*	0.041	0.027	0.042
TGW [g]			0.030	0.034	0.006	0.008
GPC [%]				0.047	0.276***	0.000
GIC [mgkg^{-1}]					0.004	0.198***
GZnC [mgkg^{-1}]						0.120**

*, ** and *** denote significance at < 0.05, <0.01 and <0.001 probability level, respectively.

Statistical Analysis

All data were evaluated in R 3.0.2 (R Core Team, 2014). The simultaneous tests of general linear hypotheses, Dunnett Contrasts, were used for multiple comparisons of the means. Summary data are reported as mean values ± standard deviations. Data with box plots and Bonferroni tests, square correlation coefficients between productivity components and grain quality parameters and *p* values were calculated using the GenStat software (10th edition). A *p*-value of less than 0.05 was considered statistically significant.
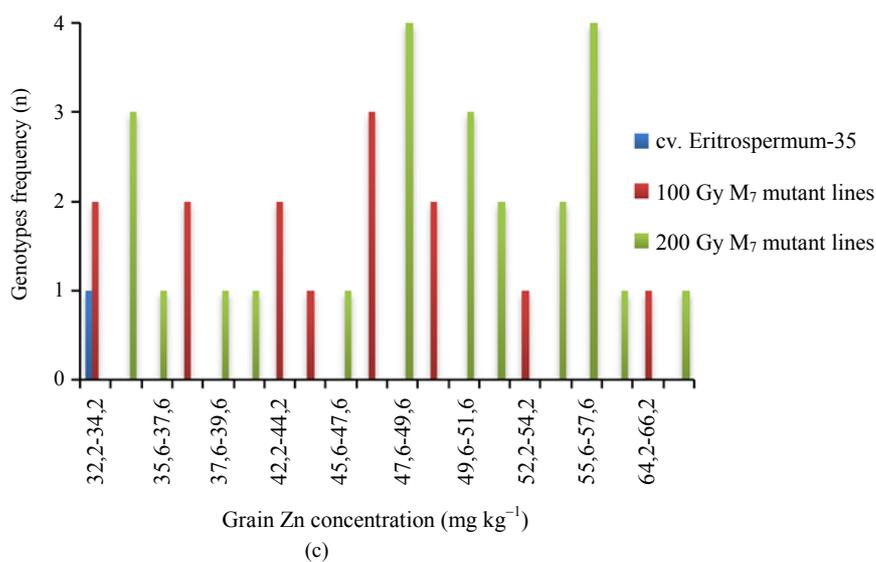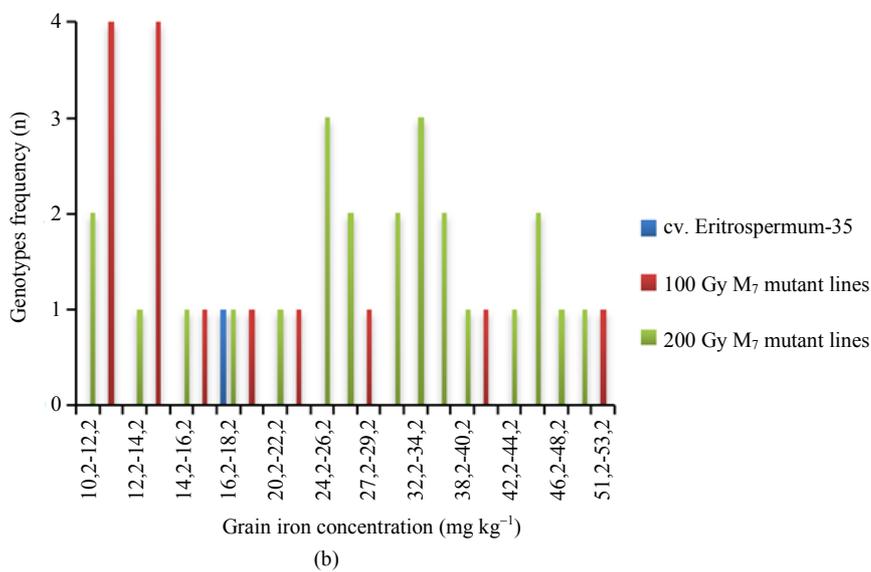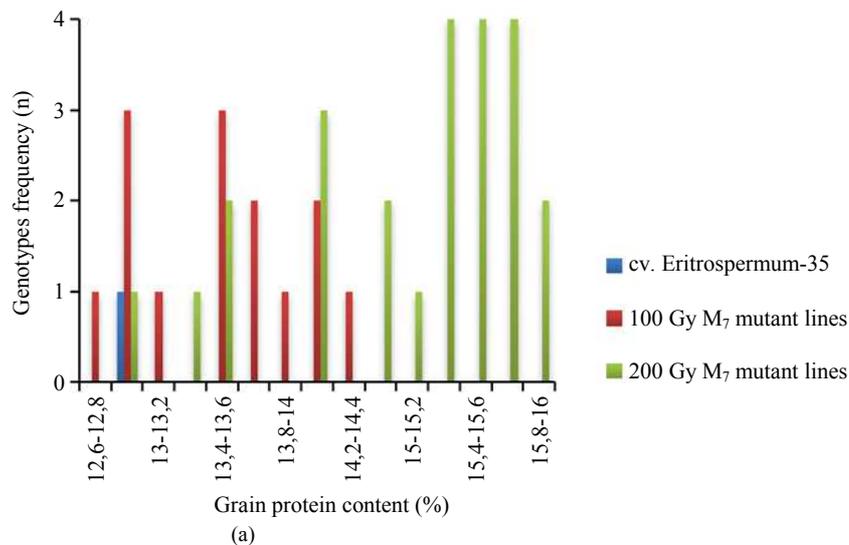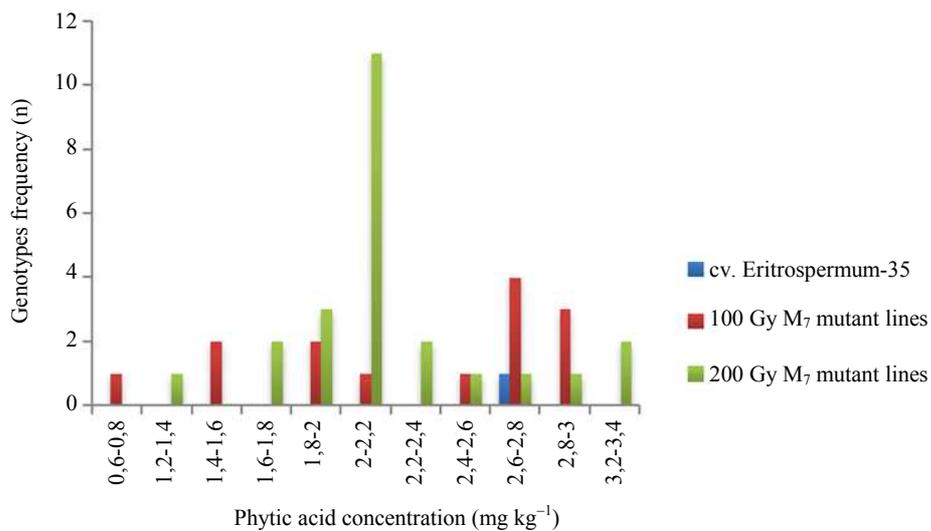
Results and Discussion

New spring wheat M_7 mutation resource developed using dose radiation of 100 and 200 Gy and parent cv. Eritrospermum-35 was used to measure productivity components including GNS, GWS, TGW and some grain quality parameters, namely GPC, Fe and Zn concentrations and GPAC. To show the range of values generated by the irradiation treatments the pooled grain chemical analysis data were plotted. Frequency distributions of GPC, GIC, GZnC and GPAC are represented in Fig. 1. The GPC varied from 12.5 to 16.0% with a mean of 14.35±1.05% (n = 114) (Fig. 1a and 3a). The 29 genotypes (76.3%), mostly 200 Gy-dosed populations (20 lines), had significantly 10.83 to 12.23% higher GPC than parent.

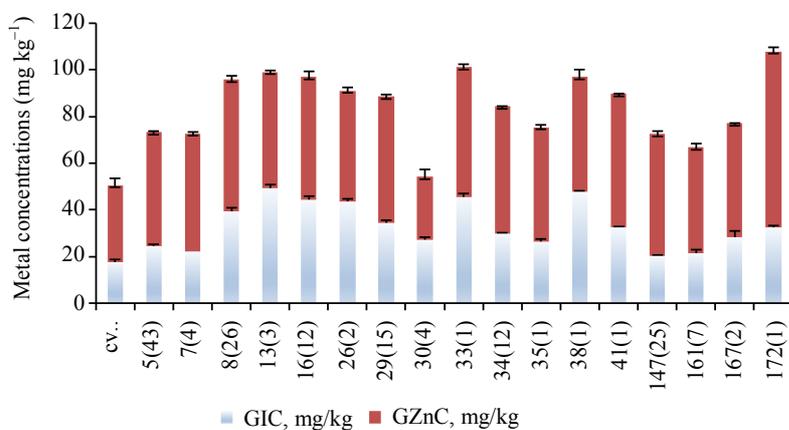The irradiated mutant lines exhibited wide variations in grain metals concentrations with significantly higher GIC and GZnC relative to parent. The GIC varied from 10.36 to 52.25 mg kg^{-1} with a mean of 26.30±12.41 mg kg^{-1} (n = 114) (Fig. 1b). Significantly enhanced GIC exceeded the parent by 1.2 to 3.0 times with the highest GICin 100 Gy-treated line were identified in 24 M_7 lines (63.2%). The GZnC ranged from 32.25-75.20 mg kg^{-1} with a mean of 47.86±9.15 mg kg^{-1} (n = 114) (Fig. 2c). The 32 genotypes of which 24 numbers of 200 Gy-dosed lines showed significantly by 1.2-2.3 times higher GZnC than that parent. Comparing GIC and GZnC showed their significant enhancement with mean of 33.39±9.76 mg kg^{-1} and 51.41±9.13 mg kg^{-1}, respectively, in 17 lines (44.7%), mainly, the 200 Gy-treated lines (Fig. 1e). The wide variation in GPC, GIC and GZnC among M_7 mutation resource suggests that it is possible to broaden and identify genotypes with high grain metals concentrations. Crops useful variability contributes to a sustainable production and is prerequisite in biofortification (Welch and Graham, 2004; Borrill *et al.*, 2014; Shahzad *et al.*, 2014).

As indicated studies undertaken for the germplasm evaluation on quality, the variability for micronutrients is limited in the modern wheat varieties (Monasterio and Graham, 2000; Chhuneja *et al.*, 2006; Rawat *et al.*, 2009; Mallick *et al.*, 2013). The advantage of wild emmer over cultivated wheat for higher nutrient concentrations previously demonstrated (Rawat *et al.*, 2009; Chatzav *et al.*, 2010) can be belittled because of the need to eliminate deleterious genes from these relatives that are not related to helpful traits by an extensive program of backcrossing.
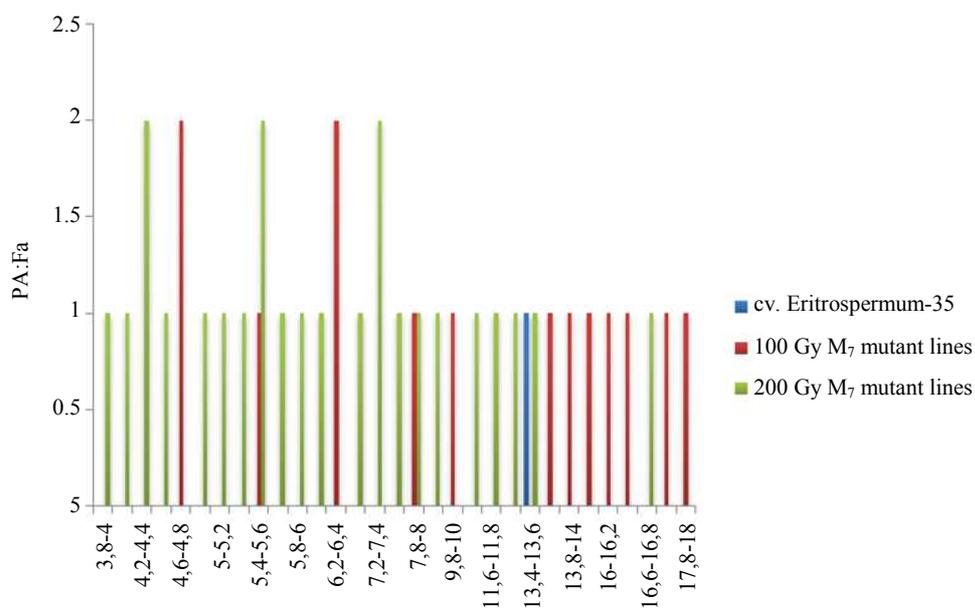
(a)

(b)

(c)

171
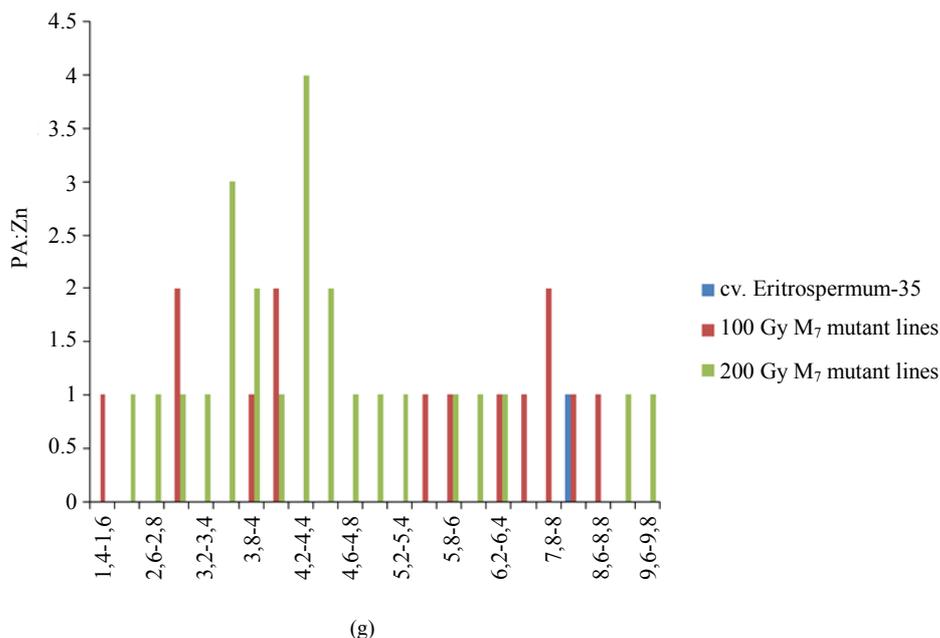
(d)

(e)

(f)

(g)

Fig. 1: Frequency distribution for some pooled grain parameters to show the range of values. Data include: (a) GPC, (b) GIC, (c) GZnC, (d) GPAC and variance for GIC and (e) GZnC in 100-Gy and 200 Gy-treated M_7 wheat mutant lines and parent (cv. Eritrospermum-35). (f) The molar rations of PA:Fe and (g) PA:Zn in the parent line, M_7 100-Gy and 200-Gy dosed lines

Also, it may be required to do an embryo rescue or use a tissue culture to recover fertile embryos (Lafiandra *et al*., 2014). The high increase in GIC and GZnC in the spring wheat mutagenized populations suggests their greater capacity for grain micronutrients accumulation affecting all plant biochemical and physiological functions and can also indicate an efficiency of induced mutation in genes involved in mineral homeostasis processes. The values reported for GIC in hexaploid wheat and landraces have been reported as ranging from 15.0 to 67.6 mg kg^{-1} (Graham *et al*., 1999; Rawat *et al*., 2009; Liu *et al*., 2006; Velu *et al*., 2011; Salunke *et al*., 2014). The GZnC concentrations were 15.0-51.0 mg kg^{-1} (Liu *et al*., 2006; Rawat *et al*., 2009; Mallick *et al*., 2013; Velu *et al*., 2011; Oury *et al*., 2006). The highest GZnC in spring wheat mutation resource exceed these upper limits in previously published data for metal (Fig. 4b and 4c). Although environment can influence grain metal concentrations, in this work all the mutant lines and the parent were grown under the same field conditions with no fertilizer supplementation of these metals.

Cereals iron and zinc biofortification points at either increasing the accumulation of these metals in grains or improving their accessibility to fulfill both aims. High metals bioavailability can be achieved by the reductions of anti-nutritional agents, particularly PA considering to be the most important causative factor (Bouis, 2000; Shahzad *et al*., 2014; Fredlund *et al*., 2006; Gibson *et al*., 2010; Zhao *et al*., 2008). For the past years, it is being propagandized that Fe and Zn malnutrition as well combat

of environmental issues related to seed PA-P could be improved by decreasing PAC.

Our results demonstrate that relative to the parent, GPAC were significantly decreased and increased in the irradiated lines of wheat (Fig. 1d and 3d). The ranges of GPAC were 0.78-3.96 mg g^{-1} with a mean of 2.22±0.54 mg g^{-1} (*n* = 114) (Fig. 1e). These ranges of values define the genetic variability that exists in the pooled parent and gamma-irradiated lines under one set of environmental conditions. The 23 mutant genotypes (60.5%) of which 17 lines are 200 Gy-treated had significantly lower GPAC by 1.1- to 3.5-times than that parent (2.74±0.09 mg g^{-1}), with the lowest mean of 0.78±0.02 mg g^{-1} in 100 Gy-dosed line (Fig. 1d).

Studies of natural variation revealed the huge difference for GPAC wheat germplasm. The PA content in the 93 wheat germpasms ranged from 0.59 to 2.08% (Zhao *et al*., 2008), in modern varieties from 1.25 to 3.42% (Ahmad *et al*., 2013), from 6.7 to 10.5 mg g^{-1} (Hídvégi and Lásztity, 2002), from 7.65 to 8.8 g kg^{-1} (Tavajjoh *et al*., 2011) and the range from 7 to 13 mg g^{-1} depending on Zn fertilization (Erdal *et al*., 2002). High PAC was reported for whole-wheat flour (44.91 mg g^{-1}) (Frontela *et al*., 2011), indian bread wheat (23.9 mg g^{-1}) (Yenagi and Basarka, 2008) and spain wheat (24.6-45.4 mg g^{-1}) (García-Estepa *et al*., 1999). Possible explanations for these inconsistent finding might be deal with a methodology used for PA determination. The selection of the most appropriate method for the PA analysis is critical (Gibson *et al*., 2010).
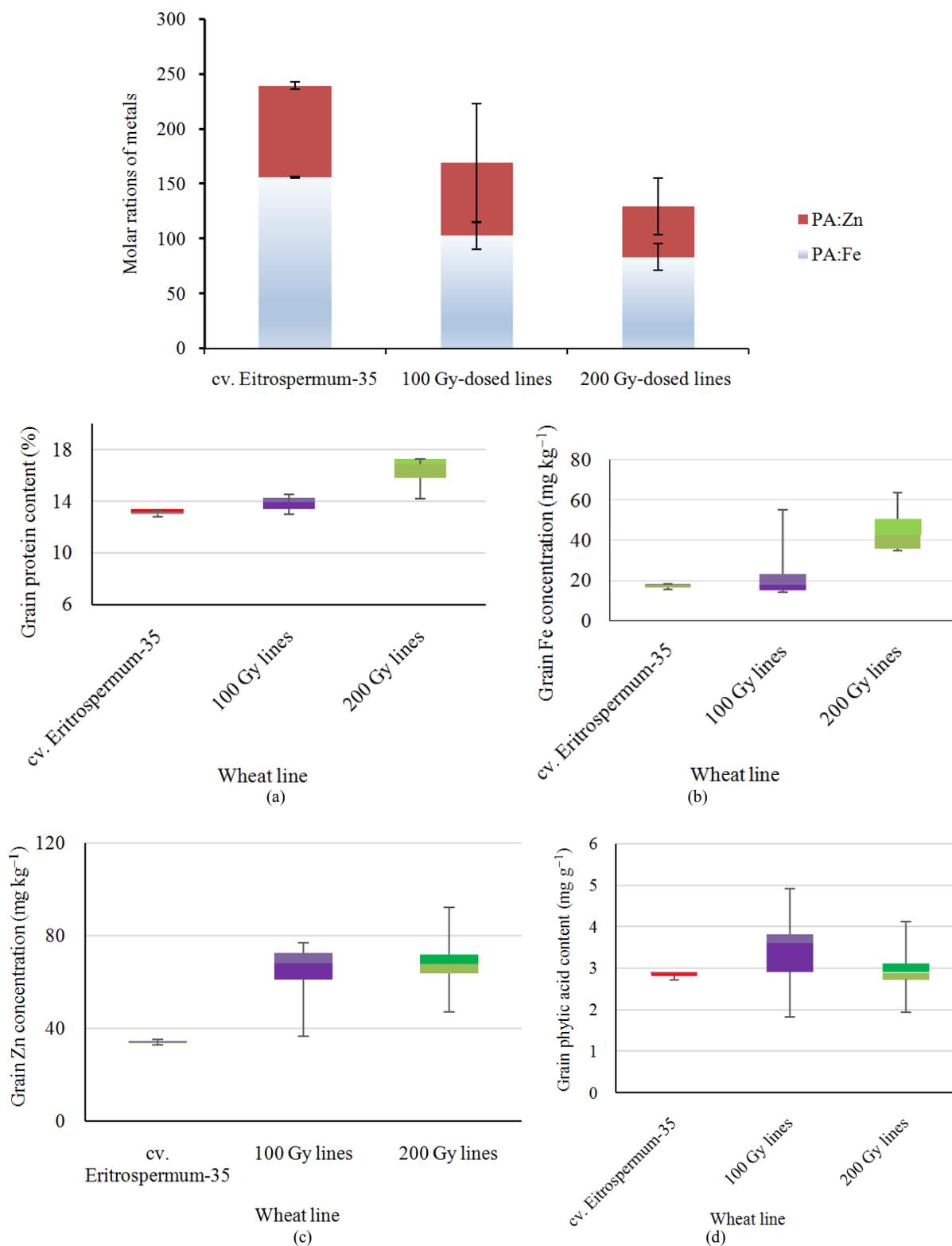
Fig. 2: Box plots showing the statistical testing for the relationships between grain analysis parameters for the parent, 100 and 200 Gy irradiated wheat. Parameters are tested compared to the parental line for the M_7 generation after two levels of gamma-irradiation dose (100 and 200 Gy) and yield selection. (a) Grain protein content (%), (b) Grain Fe concentration, (mg kg^{-1}), (c) Grain Zn concentration (mg kg^{-1}), (d) Grain phytic acid content (mg g^{-1})
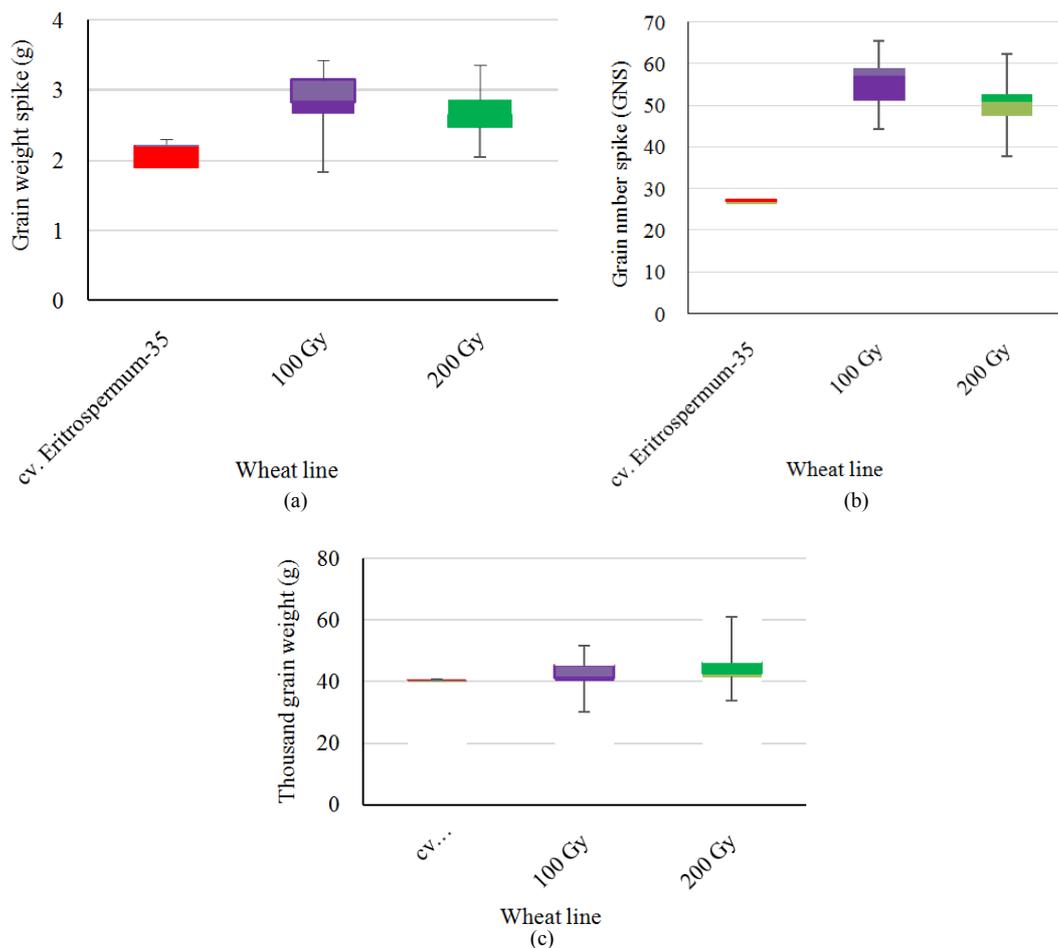
Fig. 3: Box plots showing the statistical testing for the relationships between grain yield parameters for the parent, 100 and 200 Gy irradiated wheat. Parameters are tested compared to the parental line for the M_7 generation after two levels of gamma-irradiation dose (100 and 200 Gy) and yield selection (a) Grain weight per spike g (b) Grain number per spike (c) Thousand grain weight
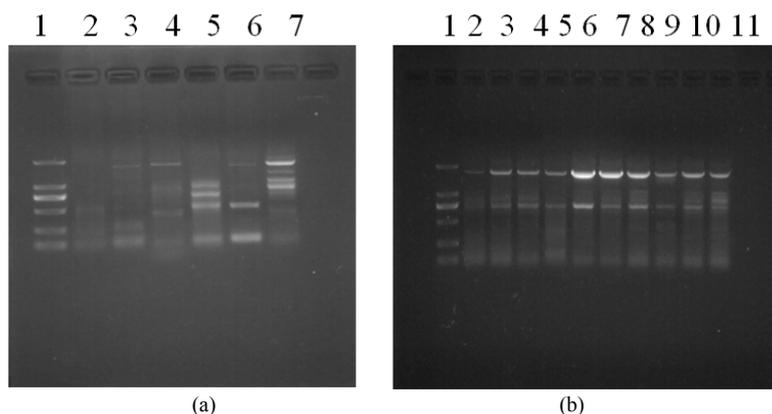

Fig. 4: Amplification product obtained from the cDNAs of 200 Gy-treated 35(1) M_7 mutant linein PCR cloning with three oligonucleotides. (a) *TaPhylla1, TaPhylla2*(2, 3), *TaPhyllb*(4, 5) and *TaPhyllc*(6, 7). (b) Amplification product obtained from parent variety and some 100 and 200 Gy-treated mutant lines in PCR with the *TaPhyllc* primer pair (a) 1-DL2000 DNA Marker, 2, 3-*TaPhylla1, TaPhylla2* primer,4, 5-*TaPhyllb* primer,6, 7*TaPhyllc* primer. (b) 1 is DL2000 DNA Marker; The lines with *TaPhyllc* primer: 2-35(1), 3-145(12), 4-166(10), 5-7(4), 9-cv. Eritrospermum-35, 10-171(1), 11-147(25)

Significant variation was detected among M_7 mutation resource for PA: Fe and PA:Zn molar ratios (3.2-21.1 and 2.2-10.7, respectively) with their means of 8.74±4.5 and 4.92±1.8 (*n* = 114) (Fig. 1f and 1g). This indicates that there were almost 7- and 5-fold variation for PA:Fe and PA:Zn, respectively. Higher averages of PA:Fe in range of 7.3-13.3 compared with PA:Zn (4.5-8.2) imply less bioavailable GIC relative to GZnC. For these parameters no significant difference could be obtained between grain of 100 and 200 Gy irradiation doses. The lowest means of PA:Fe and PA:Zn found in 200 Gy-dosed lines 38(1) and 172(1), respectively, which was 3.5- and 3.2-times lower than that parent (Fig. 1f and 1g). That could be considered them as to fall in the category of sufficient bioavailability for both Fe and Zn according to these researches (Gibson, 2006; Hurrel and Egli, 2010). The results implied that the negative effect of PA on metals bioavailability might be reduced or minimized through use of low PA wheat mutated lines.

The scarce data for PA:Fe range in wheat varieties as 15.5-31.3 (Salunke *et al*., 2014), mean of 12 (Eagling *et al*., 2014), similar to this study for parent and 100 Gy-dosed lines, as well as low range of 1.96-3.86 (Akhter *et al*., 2012) and very high value of 265.6 (Frontela *et al*., 2011) were reported. Available evidences for PA:Zn including a ranges of 29-178 (Erdal *et al*., 2011), of 5.11-20.5 (Akhter *et al*., 2012) and high mean of 265.6 (Frontela *et al*., 2011), exist in literature.

Different strategies that can be addressed to develop varieties with LPA were discussed (Raboy, 2009; Gupta *et al*., 2015b). In cereals, several viable *lpa* mutants with a substantial decrease in PA-P, but not in total P and mineral content (Raboy *et al*., 2000) were obtained. The *lpa* plants have been generated by chemical mutagenesis in wheat (Guttieri *et al*., 2004), rice (Liu *et al*., 2007; Zhao *et al*., 2008), maize (Raboy *et al*., 2000), barley (Rasmussen and Hatzack, 1998) and used in breeding (Raboy *et al*., 2001). The wheat *12-lpa* mutants had grains PA-P represented 48.2% of P, in contrast to 74.7% of control P (Guttieri *et al*., 2004). A few negative pleiotropic effects, the most relevant of which is a decrease in germination were observed in wheat (Guttieri *et al*., 2004) and *lpa1-241* maize mutant Pilu *et al*. (2005). The spring wheat *LPA* genotypes developed earlier and their grains were heavier and larger than that parent, but grain yield was reduced 22% (Guttieri *et al*., 2006). The deleterious effects of *lpa* mutations on grain yield might be mitigated by breeding (Zhao *et al*., 2008; Guttieri *et al*., 2006). Reducing PA content through development of *lpa* mutants connected with knocking out genes involved in PA biosynthesis pathway (Gupta *et al*., 2015a).

Scatter plots of data for grain quality were compared with yield associated parameters; some example plots are shown in Supp. Figure 1. These plots illustrate the spread of values and a fitted correlation line is used to test if there is any evidence for a relationship between the parameters. These data showed that for some parameters the radiation level (100 or 200 Gy) gave similar populations of data that are different from the parent (Supp. Fig. 1a, GNS Vs GWS, Supp. Fig. 1b, GWS Vs TGW, Supp. Fig. 1c, GIC Vs GZnC, Supp. Fig. 1d, GPAC Vs GZnC). The other parameters revealed radiation-dose dependence of two populations (Supp. Fig. 1e, GPC Vs GIC, Supp. Fig. 1f, GPC Vs GZnC, Supp. Fig. 1g, GIC Vs GPAC). The distribution of points in Supp. Figure 1e-g for each of the populations shows how gamma-irradiation had generated a much higher level of variation in the GPC, metal concentrations and GPAC than that parent. We used statistical testing to compare the relationships between pairs of yield and grain chemical parameters to identify when significant correlations could be observed. The results of the testing with statistically significant correlations (*p*<0.05) for the GPC, metal concentrations and GPAC relationships (Fig. 3) and yield parameters (Fig. 4) are shown as box plots. Grain analysis of GPC, GIC and GZnC indicated significantly higher mean values in both irradiated lines when compared with parent (Fig. 2a-c). In the case of GPAC, we observed lower means than parent in 200 Gy-treated lines (Fig. 2d). Comparing all four analysis showed there were significant differences between the 100- and 200 Gy-treated lines for GPC (Fig. 2a), GIC and GZnC (Fig. 2b and 2c).

The GNS as statistical testing indicated significantly increased in the irradiated lines relative to parent and the 100 Gy dose is larger than 200 Gy level (Fig. 3b). For this parameter significant difference could be obtained between grain of 100- and 200 Gy irradiation doses. The data shows that the treatments of 100 and 200 Gy have generated mutated lines with the highest variation in GNS when compared with parent (Supp. Table 1, Fig. 3b). The GNS ranged from 25.8 to 56.9 with a mean of 44.2±5.39 (n = 114). Twenty-eight genotypes (73.37%), majority of 200 Gy-dosed lines, had more significant GNS than other mutant lines and parent (Supp. Table 1). There were no significant differences for GWS between M_7 lines and cv. Eritrospermum-35. The TGW varied from 30.2 to 60.9 g with a mean of 43.6±5.7 g (n = 114). Five lines (13.2%) of them, as in case of GNS, 200 Gy M_7 lines, were characterized by significantly higher TGW than parent. Three 26(2), 30(4) and 34(12) lines (7.9%) had significantly higher GNS and TGW than parent.

It is important to consider the relation between grain nutrients and yield associated parameters.

Understanding the associations between micronutrients and grain yield, plant height, grain size and end-use quality parameters would facilitate the selection of mineral-dense progenies by breeding with desired traits (Velu *et al.*, 2013). The existence of one or more common genetic-physiological mechanisms underlying in plant mineral homeostasis may be dictated by correlations between grain concentrations of micronutrients (Chatzav *et al.*, 2010).

The parent GWS showed significant and positive correlation with GNS ($r^2 = 0.566$, $p<0.01$) (Table 1). For grain chemical analysis, GIC significantly positive correlated with GWS ($r^2 = 0.330$, $p<0.01$) and GZnC ($r^2 = 0.872$, $p<0.001$). The positive correlation was observed between GPAC and GNS ($r^2 = 0.379$, $p<0.05$).

As we only revealed for 100 Gy-treated populations, significant and positive association was between GPC and GNS ($r^2 = 0.141$, $p<0.05$) (Table 1). An inverse relationship between yield and GPC was addressed in wheat (Murphy *et al.*, 2008; Blanco *et al.*, 2012).

There was the significant relationship of GWS with GIC ($r^2 = 0.123$, $p<0.05$), with GZnG ($r^2 = 0.181$, $p<0.01$) and with GPAC ($r^2 = 0.313$, $p<0.001$), but only in 100 Gy population (Table 1). In this dose-treated lines strong significant correlation was also found between GIC and GZnC ($r^2 = 0.590$, $p<0.001$) (Table 1), perhaps indicating that their accumulation may be controlled by the same loci. Importantly to note that the 100 Gy-dosed mutant lines were also characterized by a highly significant correlation of GPAC with both metals (GIC, $r^2 = 0.607$, $p<0.001$, GZnC, $r^2 = 0.711$, $p<0.001$) and in addition, with GPC ($r^2 = 0.110$, $p<0.05$). These findings suggest there is scope to increase wheat GPC, metals and decrease PA without compromising yield breeding and radiation dose dependent mutation resource may be a useful tool for improving grain quality and metals bio-availabilities. Previous studies showed the negative correlation between GIC and PA in wheat (Gupta *et al.*, 2015a). Earlier in studies with M_5 lines developed on background of cv. Almaken, we found that in 100 Gy-dosed mutants GIC correlated with GWP and TGW ($r^2 = 0.302$, $p<0.001$ and $r^2 = 0.153$, $p<0.01$, respectively) (Kenzhebayeva *et al.*, 2017).

Generally, in 200 Gy-dosed lines, lower correlation means between both productivity components and grain micronutrients were observed as compared to their values from 100 Gy-treated lines (Table 1). Although, the correlation between GIC and GZnC was found for parent and 100 Gy-treated lines, these microelements were not dependent to each other in lines resulting from high irradiation dose (Table 1).

According to data obtained by us, among metals tested, high significant relation was observed between Fe and Zn in wheat RIL populations (Pu *et al.*, 2013), less positive correlation in domesticated wheat (Zhao *et al.*, 2009) and spring wheat (Velu *et al.*, 2012). This implies that the alleles of genes for Zn and Fe grain deposition co-segregate or are pleiotropic and therefore GIC and GZnC can be simultaneously improved. The near relation between metals concentrations may also suggest possible common regulatory mechanisms to transport and accumulate these micronutrients. The regulation of transporter gene expression for the steps from leaves to grain during wheat monocarpic senescence is poorly understood (Pearce *et al.*, 2014). A detailed understanding of these mechanisms will be required to engineer wheat varieties with improved nutritional quality through biofortification (Borrill *et al.*, 2014).

Wheat mutated lines generated by irradiation can be used as potential donors for genes/alleles beneficial for breeding to improve the helpful traits. The identification of novel alleles of target genes within both germplasm and mutagenized populations allow to make significant progress in functional genomics within model species and in the assessment of candidate genes for crop improvement (Parry *et al.*, 2009).

The parent and 100- and 200-dosed Gy M_7 lines, differing in GPAC from 1.29 to 2.73 mg g^{-1} were used for sequencing and gene structure of MINPP (Table 2). Among three primers *TaPhylla1, TaPhylla2, TaPhyllb* and *TaPhyllc* selected for PCR cloning of MINPP cDNAs (Supp. Table 2) (Dionisio *et al.*, 2007). The *TaPhyllc* primers amplified a focused segment of cDNA with higher levels of amplification of specific sequences comparing with others; the results are shown for 200 Gy-dosed line 35(1) (Fig. 4a). A PCR cloning with *TaPhyllc* primers were performed on all the mutant lines; some results shown on Fig. 5b revealed the presence of *TaPhyllc* MINPP gene in M_7 germplasm.

The sequence of MINPP cDNA from the parent and several M_7 mutant lines revealed them to contain a different single Open Reading Frame (ORF) (Table 2). The predicted Molecular Weights (MW) of their MINPPs ranged from 23.74 to 27.87 kDA (Table 2). Since the PA in cereal foods is major concern about the deficiency of micronutrients, various types of phytase have been isolated, purified and characterized from different varieties of cereal (Vashishth *et al.*, 2017).

The nucleotide and protein sequence similarities between parent and these M_7 lines varied from 28.2% and 30.2% to 45.7% and 56.5%, respectively (Table 2). Their predicted polypeptides differed by number of glycosylated serine, threonine and asparagine (Fig. 5).

Table 2: Molecular features of parent (cv. Eritrospermum-35) and spring wheat M_7 mutant lines *TaPhyllc* multiple inositol phosphate phosphatasesc DNA. The glycosylation site positions related to the amino acid sequences of the full-length proteins

| Genotypes | ORF/AA | MW (kDa), full length | Glycosylation site | | | Theoretical IP | Grain PA-concentration $(mg\ g^{-1})$ | Similarities of the nucleotide and protein sequence between parent and lines (%) |
			serine residue	threonine residue	asparagines residue			
cv. Eritrosper-mum-35	186/62	23.74	15	4	1	8.25	2.73±0.09	-
100 Gy-dosed line, 166(10)	798/266	26.25	14	6	0	7.25	2.63±0.20	46.9%/29.9%
100 Gy-dosed line, 171(1)	423/141	25.52	14	11	4	11.27	2.70±0.14	46.1%/28.2%
200 Gy-dosed line 7(4)	678/226	27.87	21	4	6	9.79	2.07±0.13	45.7%/30.2%
200 Gy-dosed line 35(1)	684/228	24.47	17	9	2	5.18	1.29±0.14	56.5%/30.2%

AA, amino acid; MW, molecular weight; ORF, open reading frame, IP, proteinisolectric point
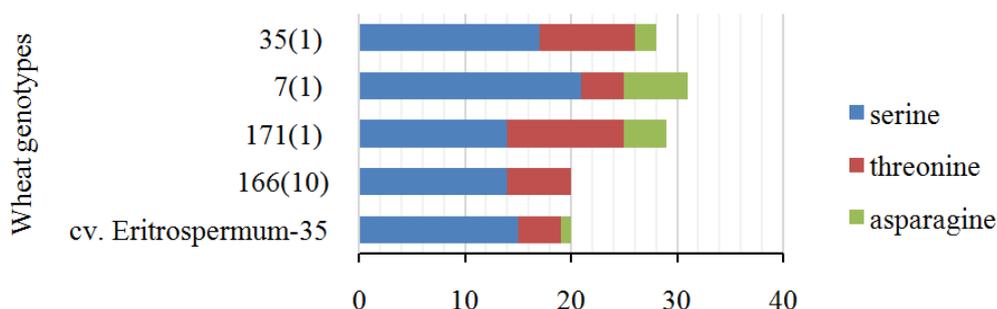

Fig. 5: Number of glycosylated serine, threonine and asparagine of the predicted polypeptides of MINPP cDNA

Relative to the parent and other lines, the polypeptides of 7(4) and 35(1) lines having low GPAC (Table 2) were characterized by a higher means of glycosylated serine residues of 17 and 21, respectively (Fig. 5). These residues in the line 35(1) were located in the beginning of the polypeptide chain with its last site at position 75. The predicted Isoelectric Points (IP) (Kozlowski, 2016) based on the AA sequence varied from 5.18 to 11.27 (Table 2), from which of 35(1) line had the lowest PIP (5.18) and only its peptide contains selenocysteine(s). This finding may be of importance in breeding directed to changing the phytase potential of wheat grain and therefore to increase genetically bio-availabilities of macro- and microelements due to PA. Previously reported for wheat and barley proteins of which contained only one putative N-linked glycosylation site (Dionisio *et al.*, 2007).

Conclusion

Spring wheat M_7 mutation resources generated to broaden genetic variation and developed on cv. Eritrospermum-35 by irradiation with 100 and 200 Gy has shown significant differences in micronutrients and PA, components of productivity between parent and mutant lines. The results clearly indicate that genetic variability for grain nutrients mutation resource is potential in wheat improving nutritional outcomes and overcome malnutrition.

Author's Contributions

Saule Kenzhebayeva: Participated in experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Alfia Abekova: Participated in all experiments, coordinated the data-analysis.

Guoping Zhang: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Dinara Zharassova: Participated in experiments, strengthened consistency of manuscript by controlling strictly the objectives and conclusion.

Fei Dai: Participated in experiments, coordinated the data-analysis.

Nargul Omirbekova: Had a significant contribution in structuring issues on the paper, controlling abstract and adjusting the paper template.

Dauren Tashenev: Participated in experiments, coordinated the data-analysis.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript.

References

Ahmad, F., A. Mohammad, I. Zeb, R. Noorka and Farhatullah *et al.*, 2013. Determination and inheritance of phytic acid as marker in diverse genetic group of bread wheat. Am. J. Mol. Biol., 3: 158-164. DOI: 10.4236/ajmb.2013.33021

Akfirat, F.S. and A.A. Uncuoglu, 2013. Genetic diversity of winter wheat (*Triticum aestivum* L.) revealed by SSR markers. Biochem. Genet., 51: 223-229. DOI: 10.1007/s10528-012-9557-6

Akhter, S., A. Saeed, M. Irfan and K.A. Malik, 2012. *In vitro* dephytinization and bioavailability of essential minerals in several wheat varieties. J. Cereal Sci., 56: 741-746. DOI: 10.1016/j.jcs.2012.08.017

Badone, F.C., M.Amelotti, E. Cassani and R. Pilu, 2012. Study of *low phytic acid1-7* (*lpa1-7*), a New ZmMRP4 mutation in maize. J. Heredity, 103: 598-605. DOI: 10.1093/jhered/ess014

Balyan, H.S., P.K. Gupta, S. Kumar, R. Dhariwal and V. Jaiswal *et al.*, 2013. Genetic improvement of grain protein content and other health-related constituents of wheat grain. Plant Breed., 132: 446-457. DOI: 10.1111/pbr.12047

Blanco, A., G. Mangini, A. Giancaspro, S. Giove and P. Colasuonno *et al.*, 2012. A relationships between grain protein content and grain yield components through quantitative trait locus analyses in a recombinant inbred line population derived from two elite durum wheat cultivars. Mol. Breed., 30: 79-92. DOI: 10.1007/s11032-011-9600-z

Borrill, P., J.M. Connorton, J. Balk, A.J. Miller and D. Sanders *et al.*, 2014. Biofortification of wheat grain with iron and zinc: Integrating novel genomic resources and knowledge from model crops. Front Plant Sci., 5: 1-8. DOI: 10.3389/fpls.2014.00053

Bouis, H.E., 2000. Special issue on improving human nutrition through agriculture. Food Nutr. Bull., 21: 351-576.

Brevis, J.C., C.F. Morris, F. Manthey and J. Dubcovsky, 2010. Effects of the chromosome region including the *Gpc-B1* locus on wheat grain and protein yield. Crop Sci., 50: 93-104. DOI: 10.2135/cropsci2009.02.0057

Cecchini, E., B.J. Mulligan, S.N. Covey and J.J. Milner, 1998. Characterization of gamma irradiation-induced deletion mutations at a selectable locus in *Arabidopsis*. Mutat. Res., 401: 199-206. DOI: 10.1016/S0027-5107(98)00009-8

Chatzav, M., Z. Peleg, L. Ozturk, A. Yazici and T. Fahima *et al.*, 2010. Genetic diversity for grain nutrients in wild emmer wheat: Potential for wheat improvement. Ann. Bot., 105: 1211-1220. DOI: 10.1093/aob/mcq024

Chhuneja, P., H.S. Dhaliwal, N.S. Bains and K. Singh, 2006. *Aegilops kotschyi* and *Aegilops tauschii* as sources for higher levels of grain Iron and Zinc. Plant Breed., 125: 529-531. DOI: 10.1111/j.1439-0523.2006.01223.x

Dai, F., J.M. Wang, S.H. Zhang, Z.Z. Xu and G.P. Zhang, 2007. Genotypic and environmental variation in phytic acid content and its relation to protein content and malt quality in barley. Food Chem., 105: 606-611. DOI: 10.1016/j.foodchem.2007.04.019

Dionisio, G., P.B. Holm and H. Brinch-Pedersen, 2007. Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) multiple inositol polyphosphate phosphatases (MINPPs) are phytases expressed during grain filling and germination. Plant Biotech. J., 5: 325-338. DOI: 10.1111/j.1467-7652.2007.00244.x

Doria, E., L. Galleschi, L. Calucci, C. Pinzino and R. Pilu *et al.*, 2009. Phytic acid prevents oxidative stress in seeds: Evidence from a maize (*Zea mays* L.) low phytic acid mutant. J. Exp. Bot., 60: 967-978. DOI: 10.1093/jxb/ern345

Eagling, T., A.A. Wawer, P.R. Shewry, F. Zhao and S.J. Fairweather-Tait, 2014. Iron bioavailability in two commercial cultivars of wheat: Comparison between wholegrain and white flour and the effects of nicotianamine and 2'-deoxymugineic acid on iron uptake into Caco-2 cells. J. Agric. Food Chem., 62: 10320-10325. DOI: 10.1021/jf5026295

Erdal, I., A. Yilmaz, S. Taban, S. Eker and B. Torun *et al.*, 2002. Phytic acid and phosphorus concentrations in seeds of wheat cultivars grown with and without zinc fertilization. J. Plant Nutr., 25: 113-127. DOI: 10.1081/PLN-100108784

FAO, 2014. FAO Document Repository.

Fredlund, K., M. Isaksson, L. Rossander-Hulthén, A. Almigren and A.S. Sandberg, 2006. Absorption of zinc and retention of calcium: Dose-dependent inhibition by phytate. J. Trace Elements Med. Biol., 20: 49-57. DOI: 10.1016/j.jtemb.2006.01.003

Frontela, C., G. Ros and C. Martínez, 2011. Phytic acid content and "*in vitro*" iron, calcium and zinc bioavailability in bakery products: The effect of processing. J. Cereal Sci., 54: 173-179. DOI: 10.1016/j.jcs.2011.02.015

Frontela, C., M.L. Scarino, S. Ferruzza, G. Ros and C. Martínez, 2009. Effect of dephytinization on bioavailability of iron, calcium and zinc from infant cereals assessed in the Caco-2 cell model. World J. Gastroenterol., 15: 1977-1984. DOI: 10.3748/wjg.15.1977

García-Estepa, R.M., E. Guerra-Hernández and B. García-Villanova, 1999. Phytic acid content in milled cereal products and breads. Food Res. Int., 32: 217-221. DOI: 10.1016/S0963-9969(99)00092-7

Gibson, R.S., 2006. Zinc: The missing link in combating micronutrient malnutrition in developing countries. Proc. Nutr. Society, 65: 51-60. DOI: 10.1079/PNS2005474

Gibson, R.S., K.B. Bailey, M. Gibbs and E.L. Ferguson, 2010. A review of phytate, iron, zinc and calcium concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. Food Nutr. Bull., 31: 134-146. DOI: 10.1177/15648265100312S206

Graham, R., D. Senadhira, S. Beebe, C. Iglesias and I. Monasterio, 1999. Breeding for micronutrient density in edible portions of staple food crops: Conventional approaches. Field Crop Res., 60: 57-80. DOI: 10.1016/S0378-4290(98)00133-6

Gupta, R.K., S.S. Gangoliya and N.K. Singh, 2015a. Screening and characterization of wheat germplasms for phytic acid and iron content. J. Agric. Sci. Tech., 17: 747-756.

Gupta, R.K., S.S. Gangoliya and N.K. Singh, 2015b. Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. J. Food Sci. Technol., 52: 676-684. DOI: 10.1007/s13197-013-0978-y

Guttieri, M.J., D. Bowen, J.A. Dorsch, V. Raboy and E. Souza, 2004. Identification and characterization of a low phytic acid wheat. Crop Sci., 44: 418-24. DOI: 10.2135/cropsci2004.4180

Guttieri, M.J., K.M. Peterson and E.J. Souza, 2006. Agronomic performance of low phytic acid wheat. Crop Sci., 46: 2623-2629. DOI: 10.2135/cropsci2006.01.0008

Hídvégi, M. and R. Lásztity, 2002. Phytic acid content of cereals and legumes and interaction with proteins. Period Polytch. Chem., 46: 59-64.

Hurrel, R. and I. Egli, 2010. Iron bioavailability and dietary reference values. Am. J. Clin. Nutr., 91: 1461S-1467S. DOI: 10.3945/ajcn.2010.28674F

Kenzhebayeva, S.S., G. Doktyrbay, N.M. Capstaff, F. Sarsu and N.Z. Omirbekova *et al.*, 2017. Searching a spring wheat mutation resource for correlations between yield, grain size and quality parameters. Crop Improv., 31: 208-228. DOI: 10.1080/15427528.2016.1276990

Kozlowski, L.P., 2016. IPC-Isoelectric Point Calculator. Biol. Direct, 11: 55-55.

Lafiandra, D., G. Riccardi and P.R. Shewry, 2014. Improving cereal grain carbohydrates for diet and health. J. Cereal Sci., 59: 312-326. DOI: 10.1016/j.jcs.2014.01.001

Liu, Q.L., X.H. Xu, X.L. Ren, H.W. Fu and D.X. Wu *et al.*, 2007. Generation and characterization of low phytic acid germplasm in rice (*Oryza sativa* L.). Theor. Applied Genet., 114: 803-814. DOI: 10.1007/s00122-006-0478-9

Liu, Z.H., H.Y. Wang, X.E. Wang, G.P. Zhang and P.D. Chen *et al.*, 2006. Genotypic and spike positional difference in grain phytase activity, phytate, inorganic phosphorus, iron and zinc contents in wheat (*Triticum aestivum* L.). J. Cereal Sci., 44: 212-219. DOI: 10.1016/j.jcs.2006.06.001

Madsen, C.K., G. Dionisio, I.B. Holme, P.B. Holm and H. Brinch-Pedersen, 2013. High mature grain phytase activity in the Triticeae has evolved by duplication followed by neofunctionalization of the Purple Acid phosphatase Phytase (*PAPhy*) gene. J. Exp. Bot., 64: 3111-3123. DOI: 10.1093/jxb/ert116

Mallick, S.A., K. Azaz, M. Gupta, V. Sharma and B.K. Sinha, 2013. Characterization of grain nutritional quality. Ind. J. Plant Physiol., 18: 183-186. DOI: 10.1007/s40502-013-0025-z

Monasterio, I. and R.D. Graham, 2000. Breeding for trace minerals in wheat. Food Nutr. Bull., 21: 392-396. DOI: 10.1177/156482650002100409

Morita, R., M. Kusaba, S. Iida, H. Yamaguchi and T. Nishio *et al.*, 2009. Molecular characterization of mutations induced by gamma irradiation in rice. Genes Genetic Syst., 84: 361-370. DOI: 10.1266/ggs.84.361

Murphy, K.M., P.G. Reeve and S.S. Jones, 2008. Relationship between yield and mineral nutrient concentrations in historical and modern spring wheat cultivars. Euphytica, 163: 381-90. DOI: 10.1007/s10681-008-9681-x

Oladosu, Y., M.Y. Rafii, N. Abdullah, G. Hussin and A. Ramli *et al.*, 2015. Principle and application of plant mutagenesis in crop improvement: A review. Biotechnol. Biotec. Eq., 30: 1-16. DOI: 10.1080/13102818.2015.1087333

Oury, F.X., F. Leenhardt, C. Remesy, E. Chanliaud and B. Duperrier *et al.*, 2006. Genetic variability and stability of grain magnesium, zinc and iron concentrations in bread wheat. Eur. J. Agron., 25: 177-185. DOI: 10.1016/j.eja.2006.04.011

Parry, M.A., P.J. Madgwick, C. Bayon, K. Tearall and A. Hernandez-Lopez *et al.*, 2009. Mutation discovery for crop improvement. J. Exp. Bot., 60: 2817-2825. DOI: 10.1093/jxb/erp189

Pearce, S., F. Tabbita, D. Cantu, V. Buffalo and R. Avni *et al.*, 2014. Regulation of Zn and Fe transporters by the *GPC1* gene during early wheat monocarpic senescence. BMC Plant Bio., 14: 368-368. DOI: 10.1186/s12870-014-0368-2

Pilu, R., D. Panzeri, G. Gavazzi, S.K. Rasmussen and G. Consonni *et al.*, 2003. Phenotypic, genetic and molecular characterization of a maize low phytic acid mutant (lpa241). Theor. Applied Genet., 107: 980-987. DOI: 10.1007/s00122-003-1316-y

Pilu, R., M. Landoni, E. Cassani, E. Doria and E. Nielsen, 2005. The maize *lpa241* mutation causes a remarkable variability of expression and some pleiotropic effects. Crop Sci., 45: 2096-2105. DOI: 10.2135/cropsci2004.0651

Pu, Z., M. Yu, Q. He, G. Chen and J. Wang *et al.*, 2013. Quantitative trait loci associated with micronutrient concentrations in two recombinant inbred wheat lines. J. Integrative Agric., 13: 2322-2329. DOI: 10.1016/S2095-3119(13)60640-1

Raboy, V., 2009. Approaches and challenges to engineering seed phytate and total phosphorus. Plant Sci., 177: 281-296. DOI: 10.1016/j.plantsci.2009.06.012

Raboy, V., K.A. Young, J.A. Dorsch and A. Cook, 2001. Genetics and breeding of seed phosphorus and phytic acid. J. Plant Physiol., 158: 489-497. DOI: 10.1078/0176-1617-00361

Raboy, V., P.F. Gerbasi, K.A. Young, S.D. Stoneberg and S.G. Pickett *et al.*, 2000. Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. Plant Physiol., 124: 355-368. DOI: 10.1104/pp.124.1.355

Rasmussen, S.K. and F. Hatzack, 1998. Identification of two low-phytate barley (*Hordeum vulgare* L.) grain mutants by TLC and genetic analysis. Hereditas, 129: 107-112. DOI: 10.1111/j.1601-5223.1998.00107.x

Rawat, N., V.K. Tiwari, N. Singh, G.S. Randhawa and K. Singh *et al.*, 2009. Evaluation and utilization of *Aegilops* and wild *Triticum* species for enhancing iron and zinc content in wheat. Genet. Resour. Crop Evol., 56: 53-64. DOI: 10.1007/s10722-008-9344-8

R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Salunke, R., N. Rawat, K. Neelam, V.K. Tiwari and G.S. Randhawa *et al.*, 2014. Effect of grain hardness on bioavailability of iron in wheat as determined using the coupled *in vitro* digestion/Caco-2 model. LWT. Food Sci. Technol., 59: 433-438. DOI: 10.1016/j.lwt.2014.04.031

Shahzad, Z., H. Rouached and A. Rakha, 2014. Combating mineral malnutrition through iron and zinc biofortification of cereals. Compr. Rev. Food Sci. Food Safety, 13: 329-346. DOI: 10.1111/1541-4337.12063

Shu, Q.E. and P.J.L. Lagoda, 2007. Mutation techniques for gene discovery and crop improvement. Mol. Plant Breed., 5: 193-195.

Tavajjoh, M., J. Yasrebi, N. Karimian and V. Olama, 2011. Phytic acid concentration and phytic acid: Zinc molar ratio in wheat cultivars and bread flours, Fars Province, Iran. J. Agric. Sci. Tech., 13: 743-755.

Vashishth, A., S. Ram and V. Beniwal, 2017. Cereal phytases and their importance in improvement of micronutrients bioavailability. Biotech, 7: 41-48. DOI: 10.1007/s13205-017-0698-5

Velu, G., I. Ortiz-Monasterio, I. Cakmak, Y. Hao and R.P. Singh, 2013. Biofortification strategies to increase grain zinc and iron concentrations in wheat. J. Cereal Sci., 59: 365-372. DOI: 10.1016/j.jcs.2013.09.001

Velu, G., I. Ortiz-Monasterio, R.P. Singh and T. Payne, 2011. Variation for grain micronutrients concentration in wheat core-collection accessions of diverse origin. Asian J. Crop Sci., 3: 43-48.

Velu, G., R.P. Singh, J. Huerta-Espino, R.J. Peña-Bautista and B. Arun *et al.*, 2012. Performance of biofortified spring wheat genotypes in target environments for grain zinc and iron concentrations. Field Crops Res., 137: 261-267. DOI: 10.1016/j.fcr.2012.07.018

Welch, R.M. and R.D. Graham, 2004. Breeding for micronutrients in staple food crops from a human nutrition perspective. J. Exp. Bot., 55: 353-364. DOI: 10.1093/jxb/erh064

Yamaguchi, H., S. Nagatomi, T. Morishita, K. Degi and A. Tanaka *et al.*, 2003. Mutation induced with ion beam irradiation in rose. Nucl. Instr. Meth. Phys. Res., 206: 561-564. DOI: 10.1016/S0168-583X(03)00825-5

Yenagi, S.N. and P.W. Basarkar, 2008. Antioxidant contents of whole grain cereals of North Karnataka. Karnataka J. Agric. Sci., 21:602-603.

Zhao, F.J., Y.H. Su, S.J. Dunham, M. Rakszegi and Z. Bedo *et al.*, 2009. Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin. J. Cereal Sci., 49: 290-295. DOI: 10.1016/j.jcs.2008.11.007

Zhao, H.J., Q.L. Liu, H.W. Fu and Q.Y. Shu, 2008. Effect of non-lethal low phytic acid mutations on grain yield and seed viability in rice. Field Crops Res., 108: 206-211. DOI: 10.1016/j.fcr.2008.05.006