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Variation in grain proteins content and nutritionally important protein fractions concentration in spring wheat mutant lines

Abstract: Wheat is a major cereal crop for both human and animal nutrition, providing 28 % of the world's edible dry matter and up to 60 % of the daily calorie intake in developing countries. Across years, wheat breeding reduced its genetic diversity by replacing traditional cultivars with modern higher yielding varieties and this has resulted in decreased nutritional quality. Spring wheat genetically stable mutant lines (M_7 generation) produced on genetic background of cv. Eritrospermum-35 after 100 and 200 Gy gamma treatments to broaden genetic variation and search for new resources were analyzed for grain protein content and nutritionally important protein fractions (albumins, globulins and prolamins). A significant positive correlation between grain protein content and yield-associated traits, such as grain number and weight per spike, were observed in the 100- and 200 Gy-dosed mutant lines with different means, r^2=0.141, ($p<0.05$) and r^2=0.068, ($p<0.05$), respectively. Albumins ranged from 139.5 to 890.4 μ g/g, globulins – from 130.1 to 344.04 μ g/g. The 200 Gy-dosed M_7 mutant lines showed the highest globulins concentration by 1.84 fold higher, compared to cv. Eritrospermum-35. Prolamins level varied from 65.1 to 398.2 μ g/g in mutant lines. High dose of irradiation (200 Gy) generated higher level of variation, when compared to 100 Gy. ANOVA analysis revealed significant variation ($p<0.05$) in globulins and prolamins. In order to improve both quantity and quality of wheat proteins and influence selection of improved raw materials for the flour and bread-making industry a more detailed knowledge of the variability of grain proteins and protein fractions accumulation among new spring wheat mutant lines varieties could be useful. In addition, to improve whole-wheat flour application in production of functional food, rich in health-beneficial components, the study of the whole grain proteins content, their structure and quality is significant.
Key words: bread wheat, correlations, gamma irradiation, concentrations, grain protein fractions, mutation.

Introduction

Wheat (*Triticum aestivum* L.) is a major cereal crop for both human and animal nutrition. It is a major source of energy, protein, and dietary fiber in human nutrition and animal feeding. It provides 28% of the world's edible dry matter and up to 60% of the daily calorie intake of the world's population [1; 2]. Currently, about 95% of the wheat grown worldwide is hexaploid bread wheat, with most of the remaining 5% being tetraploid durum wheat. Nutritionally, wheat is important sources of dietary protein, carbohydrates, the B complex of vitamins, vitamin E, iron, trace minerals, and fiber [3].

The ability of wheat flour to be processed into different foods is largely determined by the proteins. Mature wheat grains contain 8% to 20% proteins [4]. Wheat proteins show high complexity and different interactions with each other, thus making them difficult to characterize. Usually, wheat proteins are either divided into four solubility classes, called Osborne fractions, or extracted in a series: albumins, which are water-soluble; globulins, which are soluble in salt solutions, but insoluble in water; gliadins, which are soluble in 70-90 % alcohol; and glutenins, which are insoluble in neutral aqueous solutions, saline solutions, or alcohol. The respective wheat protein fractions are also applicable to other cereals and are gen-

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erally known as albumins, globulins, prolamins, and glutenins, soluble in diluted acid or sodium hyderoxide, which make up to 10-22 % of total flour protein [5; 6]. Wheat prolamins are the major component of gluten, the properties of which determine quality of wheat flour for various technological processes, including bread making [7]. An alternative classification to that described above has been proposed based on composition and structure rather than solubility described by Shewry and Halford [8], indicating that wheat proteins consist of two classes based on storage property seed, storage proteins (gliadins/glutenins) and nonseed storage proteins (albumins/globulins) [9].

Albumins and globulins of wheat endosperm represent 20% to 25% of total grain proteins [10; 11]. Albumin is biologically active protein, which is responsible for breakdown of starch and other enzymatic reactions, e.g. amylase and proteases, and is the first to be stored in significant amounts [12]. Nutritionally, albumins and globulins (non-glutens) have a very good amino acid balance. Some proteins, which mostly belong to a family of trypsin and α-amylase inhibitors, participate in plant defense [13]. The role of α-amylase and trypsin inhibitors as wheat allergens in baker's asthma has been shown [14]. Prevailing number of the physiologically active proteins influence the processing and rheological properties of wheat flour. The benefits of the use of amylases, xylanases, lipoxygenase, pentosanase, glucoseoxidase stimulated further interest in the breadmaking industry [15; 16].

The objectives of this study were: (1) to evaluate the variability in grain protein content (GPC) and protein fractions (albumins, globulins and prolamins) concentrations, in grains of spring wheat, parental cv. Eritrospermum-35, advanced mutant lines (M_7), produced after 100- and 200 Gy-gamma dose treatments; (2) to evaluate the correlations between grain number and weight per spike, thousand grain weight (TGW) and GPC; (3) to estimate the significant variation ($p<0.05$) in albumin, globulin and prolamin storage proteins by ANOVA analysis.

Materials and methods

Plant material. Seeds of the spring bread wheat awn variety Eritrospermum-35 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 [17]. 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 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 Gy 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 [17]. 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 and GWS), and thousand grain weight (TGW) which was 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. The sequential extraction of protein fractions of grain from storage proteins of grain, albumins, globulins and prolamins was carried out using the Osborne method [5]. Extraction of albumins fraction of storage proteins was performed from 0.5 g crushed grains (flour), with a 2-step extraction of 0.05 M Tris-HCl (pH 7.0) for 2 hours with constant stirring, at room temperature.

The precipitate obtained after centrifugation at 4000 g for 10 min was used to isolate the globulin fraction by 2-fold extraction with 1 M NaCl with constant stirring, at room temperature. The fraction of prolamins was obtained by 2-step procedure extraction of the precipitate obtained after centrifuging the globulin fraction using of 55% propanol with constant stirring, at room temperature.

Protein concentration in the obtained fractions was measured by the Bradfort method using a Coomassie Blue G-250 solution an Eppendorf BioPhotometer plus spectrophotometer at 595 nm.

Statistical data analysis. The data was analyzed by one-way ANOVA (single factor) using Excel 2007 for significant F-statistics, If overall F-test was significant ($p<0.05$), a Fachers T-test was performed to discern difference between the varieties.

Results and discussion

Wheat genetic improvement requires the identification of key traits in high performing cultivars to deploy in breeding programs. To generate new sources of variation in the genetic background of modern varieties by introducing more major re-organizations within the genome. Genetic changes produced by irradiation are much larger than the subtle single-base changes introduced by chemical mutagens [18]. Mutated lines generated by irradiation can be used as potential donors for genes/alleles beneficial for wheat-breeding programs to increase yield and improve grain quality [17].

In the present study, spring wheat genetically stable M_7 mutant lines were generated from parent seed (cv. Eritrospermum-35) given two doses of radiation (100 and 200 Gy). These wheat mutant resources were evaluated on grain protein content (GPC) and protein fractions (albumins, globulins and prolamins (glutenins) and determination of correlations between GPC and grain number and weight per spike and thousands grain weight.

We have plotted the pooled GPC data to show the range of values generated by the irradiation treatments. The GPC for the pooled data showed considerable variation, from 12.5 to 16.0%, with a mean of 14.7±0.4 % (n=106). Thirty genotypes (78.9%), mainly in the 200-Gy-dosed treatment, had 7.3–12.5 % higher GPC than that of the parent (cv. Eritrospermum-35). The highest GPC measured in the irradiated mutants was 16.0%, which was 12.5% increase over that of the parent cv. Eritrospermum-35, shown in Figure 1.

Analysis of variance (ANOVA) with differences in GPC among cv. Eritrospermum-35 and spring wheat mutant lines is shown in Table 1.

These results revealed significant differences between the cv. Eritrospermum-35 and derived 100 Gy- and 200 Gy-mutant lines for this grain quality character. The radiation effect of 200 Gy was highest in GPC, indicating its increased efficiency to generate mutations in the genome associated with this grain quality trait. There is also significant difference between low (100 Gy) and high (200 Gy) level of radiation to generate variation in GPC. The association between grain nutrients characteristics and yield components is important. Our results showed that no significant correlations between the parent GPC with yield-associated traits such as grain number per spike (GNS), grain weight per spike (GWS) and thousand-grain weight (TGW). A significant positive correlation between GPC and GNS and GWS were observed in the 100- and 200 Gy-dosed mutant lines with different means, $r^2 = 0.141$ ($p<0.05$) and $r^2 = 0.068$ ($p<0.05$), respectively (Table 2). These results may suggest that higher grain number and weight per spike improve the capacity to accumulate higher grain protein content.

Albumins are water-soluble proteins in wheat, which are accounted for about 10% of total grain proteins. They perform the metabolic functions in plant growth and development. The albumins are mostly monomeric physiologically active or structural proteins and include α-amylase, β-amylase/protease inhibitors (13 and 16 kDa) as well as enzymes with different physiological functions (62 kDa serine carboxypeptidase) [19; 20]. It has been reported that three wheat albumin fractions (60, 24, and 12.5 kDa) inhibited amylases activity [21]. Gao et al. analyzed non-prolamin expression profiles during grain development of bread wheat and found that most of the proteins had masses of 14-97 kDa, which were mostly distributed in the pH 4-7 range [22]. Out of 400 protein spots, 230 proteins were identified and more than 85% of the identified pro-

teins were enzymes possessing different physiological functions. It was revealed that among the identified 89 non-prolamin proteins more than 80% were various enzymes classified into eight functional categories including carbohydrate metabolism (27%), protein metabolism (27%), stress/defense/detoxification (11%), cell metabolism (6%), transcription/translation (4%), nitrogen metabolism (4%), photosynthesis (4%) and signal transduction (1%) [23]. Some high molecular weight albumins and certain globulins are considered to have a storage function [22; 23].

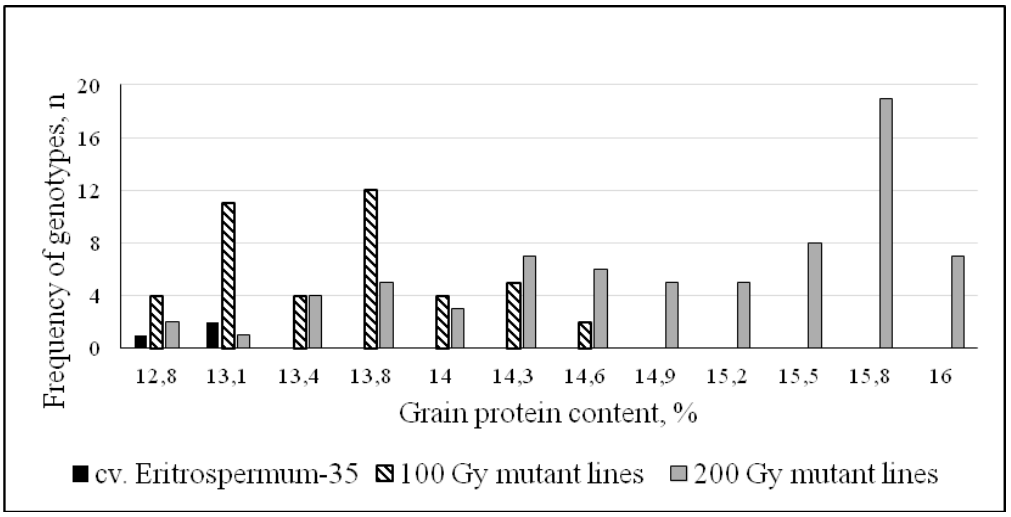

Figure 1 – Frequency of genotypes on grain protein content in the parent (cv. Eritrospermum-35), 100- and 200 Gy-dosed M_7 spring wheat mutant lines

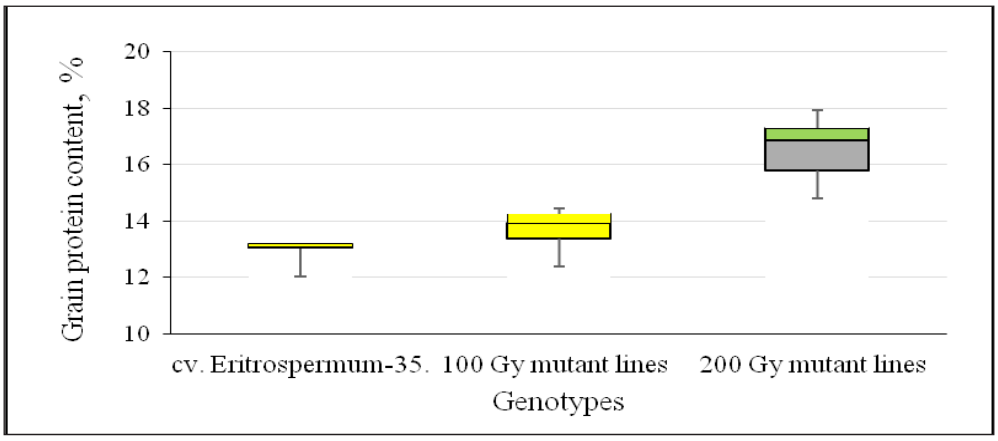

Figure 2 – Box plots showing the statistical testing
for the relationships between grain protein content for the parent.
Note: indicated are cv. Eritrospermum-35, 100 (low) and 200 (high) Gy irradiated wheat

Table 1 – Comparing grain protein content of advanced M_7 mutant lines of spring wheat developed using 100 Gy and 200 Gy and the parent cv. Eritrospermum-35 expressed as percentage of the total sum of squares from ANOVA analysis

Source of variation	Df	Grain protein content, %
cv. Eritrospermum-35 x 100 Gy- dosed mutant lines	60	37.47***
cv. Eritrospermum-35 x 200 Gy- dosed mutant lines	90	86.26***
100 Gy- x 200 Gy-dosed mutant lines	114	68.53***

Table 2 – The square R correlations correlation coefficients with p values between yield-associated traits (TWG, GNS and GWS) and grain protein content in parent (cv. Eritrospermum-35) and spring wheat M_7 100- and 200 Gy-dosed mutant lines

	GWS [g]	TGW [g]	GPC [%]
cv. Eritrospermum-35			
Grain number per spike (GNS)	0.566**	0.261	0.000
Grain weight per spike (GWS), [g]		0.118	0.250
Thousand grain weight (TGW), [g]			0.008
100 Gy-dosed M_7 mutant lines			
Grain number per spike (GNS)	0.087	0.047	0.141*
Grain weight per spike (GWS), [g]		0.087*	0.013
Thousand grain weight (TGW), [g]			0.001
200 Gy –dosed M_7 mutant lines			
Grain number per spike (GNS)	0.304***	0.001	0.014
Grain weight per spike (GWS), [g]		0.201***	0.068*
Thousand grain weight (TGW), [g]			0.030
Note: *, ** and *** denote significance at $p < 0.05$, <0.01 and <0.001 probability level, respectively			

Evaluation of new spring wheat mutant lines for grain water-soluble albumins protein has been quantified as depicted in Figure 3. Enormous variation on this grain protein fraction, ranged from 139.5 to 890.4 μ g/g is noted. It is revealed that 100-Gy-dosed M_7 mutant lines contain the highest albumins concentration.

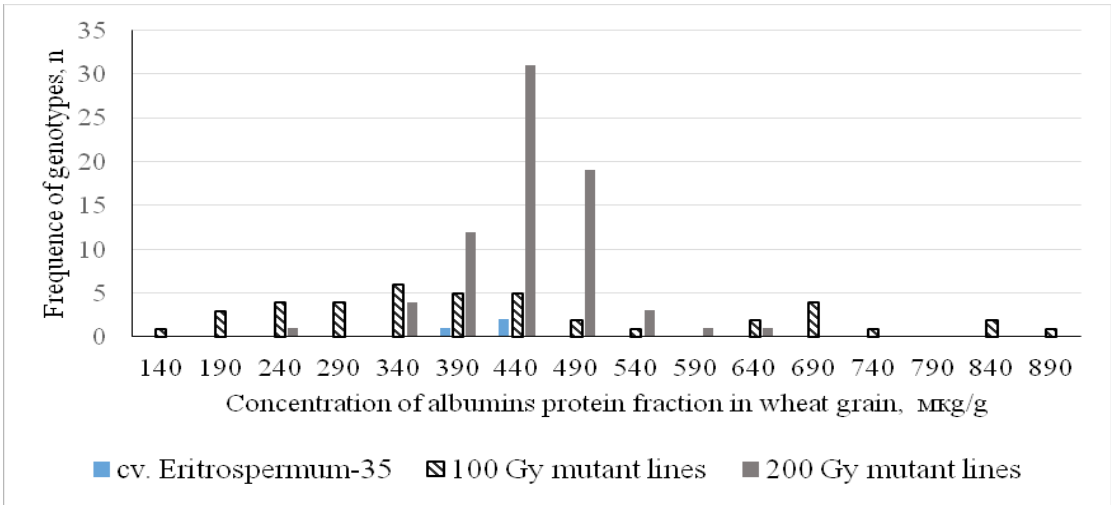

Figure 3 – Frequency of genotypes on concentration of albumins protein fraction in spring wheat grain in the parent (cv. Eritrospermum-35) and 100- and 200 Gy-dosed M_7 mutant lines

The embryo and outer aleurone layer of the endosperm contain globulins storage proteins, and those from maize embryos have been characterized in some detail [24]. These proteins are readily soluble in dilute salt solution and have sedimentation coefficients of about 7. Related proteins have been found in embryos and/or aleurone layers of wheat [25]. The 7S globulins are stored in protein bodies and appear to function solely as storage proteins. The high content of globulin storage proteins in oat grain may contribute to high nutritional value when compared with other cereals, such as barley and wheat, an important

factor in view of the widespread use of oats for livestock feed [26].

In our study, comparing the 100- and 200-Gy M_7-mutated lines of spring wheat showed that considerable variation was generated by irradiation doses for globulins storage protein fraction (Figure 4). Globulins storage protein ranged from 130.1 to 344.04 μg/g (Figure 4).

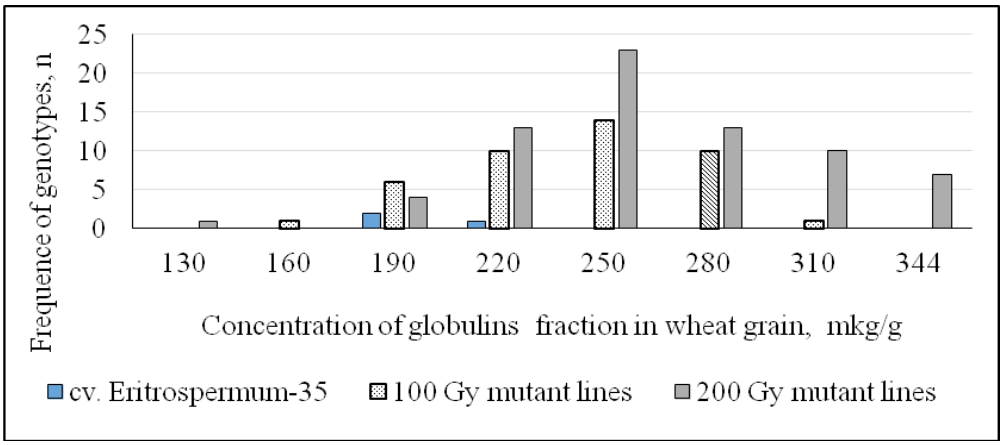

Figure 4 – Frequency of genotypes on concentration of globulins storage protein fraction in spring wheat grain in the parent (cv. Eritrospermum-35) and 100- and 200 Gy-dosed M_7 mutant lines

It was revealed that 200 Gy-dosed M_7 mutant lines showed the highest globulins storage concentration by 1.84 fold higher than that of cv. Eritrospermum-35. Similar to observed for GPC (Table 1), the radiation effect of 200 Gy was highest, indicating its increased efficiency to generate mutations in the genes associated with this grain protein fraction. It was reported the identification of three unique wheat globulin genes, *Glo-3A*, *Glo3-B* and *Glo-3C,* the genomic structure of these genes and their expression pattern in wheat seeds [27]. The *Glo-3A* gene shared 99% identity with the cDNA of WP5212 at the nucleotide and deduced amino acid level, indicating that the identified the gene(s) encoding wheat protein WP5212. In addition, southern analysis carried out in this research revealed the presence of multiple copies of *Glo-3*-like sequences in all wheat samples, including hexaploid, tetraploid and diploid species wheat seed. Importantly, the results reported indicate that a diverse group of globulins exists in wheat, some of which could be associated with the pathogenesis of type 1 diabetes (T1D) in some susceptible individuals. The identification of spring wheat mutant lines, characterized by the lowest globulins concentration offers promising donors for improving immune response in some genetically susceptible individuals, wheat proteins induce an acute mucosal inflammatory response known as celiac disease [28] or Baker's asthma [29].

Prolamins form the major endosperm storage protein fraction in all the major cereals except oats and rice [30]. The name of prolamins was originally based on fact that they are generally rich in proline and amide nitrogen derived from glutamine [30]. The combined proportions of these amino acids actually vary from about 30–70% of the total among different cereals and protein groups [30]. There also is new system of classification all of the prolamins of the Triticeae (wheat, barley and rye) which separate them to three broad groups: sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular weight (HMW) prolamins, with several subgroups within the S-rich group [30]. These groups do not correspond directly to the polymeric and monomeric fractions in wheat (glutenins and gliadins, respectively) recognized by cereal chemists, as both. The prolamins storage proteins vary greatly, from about 10 000 to almost 100 000, in their molecular masses and they are much more variable in structure than the 7S and 11u12S globulins. In wheat, the prolamins form the major components of the gluten protein fraction and commonly known as "gluten". Structurally, wheat prolamins are a complex mixture of 71–78 proteins, which constitute ~80% of the proteins in the wheat grains and form the unique viscoelastic network in doughs and is largely responsible for the ability to process wheat to form bread, pasta and many other food products.

This study quantified the concentration of prolamins storage protein fraction in spring wheat grain in the parent (cv. Eritrospermum-35) and 100- and 200 Gy-dosed M_7 mutant lines (Figure 5).

Prolamins concentration considerably varied from 65.1 to 398.2 μ g/g in mutant lines. This range of variation (6.1 times) is much higher comparing to that of globulins (Figure 4), but not albumins (Figure 3) protein fractions. Similar to globulins storage protein fraction, high dose of irradiation (200 Gy) was generated higher level of variation than 100 Gy. The lowest mean of prolamins level was revealed in 200 Gy-dosed M_7 mutant lines. This observation is seeming, importance of since prolamins are also responsible for numerous gluten-induced health disorders, such as celiac disease, gluten sensitivity and food allergies [31; 32].

Analysis of variance with differences in albumins, globulins and prolamin storage protein among cv. Eritrospermum-35 and mutant lines is presented in Table 3.

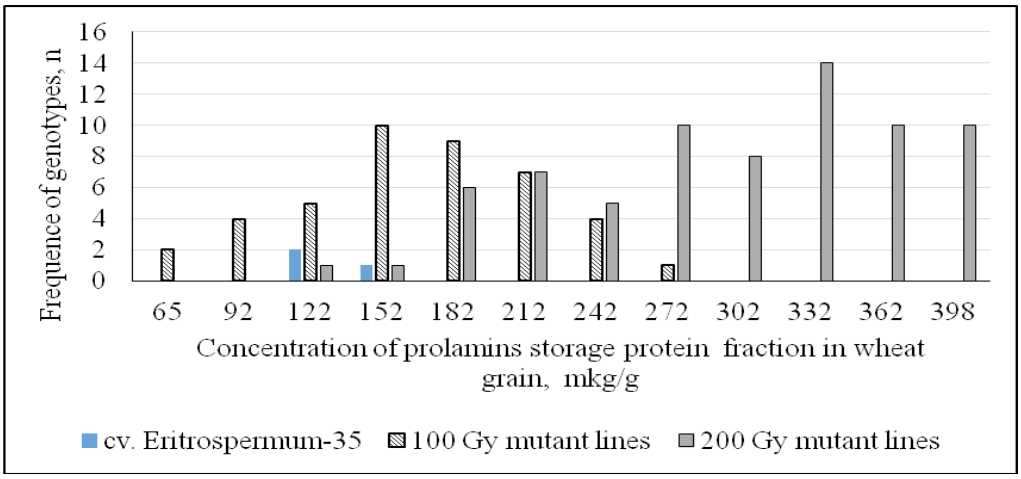

Figure 5 – Frequence of genotypes on concentration of prolamin storage protein fraction in spring wheat grain in the parent (cv. Eritrospermum-35) and 100- and 200 Gy-dosed M_7 mutant lines

Table 3 – Comparing albumin, globulin and prolamin storage proteins in the parent cv. Eritrospermum-35 and M_7 mutant lines of spring wheat developed using 100 Gy and 200 Gy and expressed as % of the total sum of squares from ANOVA analysis

Source of variation	Df	Albumins	Globulins storage proteins	Prolamins storage proteins
cv. Eritrospermum-35 x 100 Gy- dosed lines	56	0.25	36.76***	13.21**
cv. Eritrospermum-35 x 200 Gy- dosed lines	86	0.01	31.38***	101.21***
100 Gy- x 200 Gy-dosed lines	113	1.72	6.13*	94.20***

No significant differences between parent cv. Eritrospermum-35, derived 100 Gy- and 200 Gy-mutant lines for albumins protein fraction are noted, which could possibly indicate that applied doses gamma radiation were not generated mutations in genes associated with wheat predominant albumins (for instance, alpha- amylase/trypsin, serpins and purothionins) [33]. These prevailing albumins members and as well globulins serve as nutrient reserves for the germinating embryo and they also help in protecting embryo from insects and pathogens before germination [34].

Although significant differences between parent, cv. Eritrospermum-35 and derived 100 Gy- and 200 Gy-mutant lines were found for globulins protein fraction, there was not considerable residual for 100 Gy- and 200 Gy-mutant lines (Table 3).

The radiation effect of 100 Gy and of 200 Gy were lowest and highest in prolamins storage proteins, respectively, indicating its efficiency to generate mutations in the genome associated with this trait. There is also significant difference between low and high level of radiation to generate variation in

genome associated with prolamins storage proteins (Table 2). Wheat prolamins are encoded by several loci on the group one and six chromosomes [8] and study has described the relationships between allelic variability at these loci and the functional properties of dough [35].

Conclusion

Successful breeding for yield-associated traits and grain quality traits including protein composition in spring wheat grains requires genetic variation, which has to be distinguishable from environmental effects and permits breeding for genotypes based on end-use product quality and marketing parameters. Mutagenesis, a powerful tool for wheat broaden genetic variation and improvement, has been used for yield improvement, but this technique has not been as widely applied for improving nutritional quality of the grain, including the quality protein fractions to achieve desired end-use product quality. The present study reveals that some new spring wheat genetically stable mutant lines (M_7 generation) generated on genetic background of cv. Eritrospermum-35 and after 100 and 200 Gy gamma treatments have higher grain protein content by 7.3–12.5%, mainly in the 200-Gy-dosed lines, than that of parent. A significant positive correlation between grain protein content and grain number and weight per spike were observed in the 100- and 200 Gy-dosed mutant lines. These mutant lines have great nutritional potential in term of grain important proteins fractions (albumins, globulins and prolamins) characterizing by their enormous variation. High dose of irradiation (200 Gy) was generated higher level of variation in prolamins storage protein fraction as compared to 100 Gy. These new mutant resources of spring wheat can be explored for baking products and for breeding of new cultivars with high nutritional benefits for consumers. To facilitate ongoing efforts to improve both quantity and quality of wheat proteins and influence the selection of better raw materials for the flour and bread-making industry a more detailed knowledge of the variability of grain proteins and protein fractions accumulation among new spring wheat mutant lines varieties could be useful. In addition, be able to use whole wheat flour in production of functional food, rich in health-beneficial components, the study of the whole grain proteins content, their structure and quality are important.

Acknowledgments

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