DIFFERENCE IN PHYTIC ACID CONTENT AND PHYTASE ACTIVITY BETWEEN SPRING WHEAT VARIETY AND NEW MUTANT LINES

Genetic variability in micronutrient bioavailability, which is dictated by low grain phytic acid (PA) content, is limited in wheat varieties. The PA particularly enhances such a problem as it also related to an attendant loss of indigestible complexed nutrients and charged proteins in the waste and results in reduced their bioavailability in both humans and livestock. The new spring wheat mutant lines (M, generation) developed on the genetic background of cv. Eritrospermum-35 through gamma irradiation by 100 and 200 Gy using 60Co, were used for evaluation of grain PA content, the study of time germination on phytase activity (PhyA) and its organ-specific distribution. Our investigation showed the large genotypic variation in PA content of the Eritrospermum-35 100 Gy- and 200 Gy-dosed mutant lines. The 23 mutant genotypes of which mostly 200 Gy-treated lines had significantly lower grain PA content by 1.2- to 3.5-times than that parent, with its lowest mean of 0.78 ± 0.02 mg g⁻¹ in 100 Gy-dosed lines. The variation in PhyA of cv. Eritrospermum-35 and mutant lines was dependent on time germination (3 and 6 hours) and doubling the germination time enhanced grain PhyA to 1.47-fold in cv. Eritrospermum-35, and to 1.36-3.53-fold in mutant lines, indicating that the effect of germination on PhyA was strongly time and genotypes related. Organ-specific distribution (grain, roots, and shoots) of PhyA in cv. Eritrospermum-35 and 100 Gy- and 200 Gy-dosed mutant lines different by grain PA content, in 12- and 24-hours seedlings showed a gradual decrease in grains enzyme activity while its level in roots and shoots of both hours' seedlings was the same. A mutant line numbered 35/1, had maximum PhyA in grains, roots, and shoots of 12-hour seedlings, which exceed the PhyA of cv. Eritrospermum-35 by 3.05-, 3.03- and 8.43-fold, respectively, which is valuable in developing spring wheat cultivars with high PhyA.

Key words: variability in grain phytic acid content, spring wheat, phytase activity.
С.С. Кенжебаева1*, А.А. Альнурова1, Ж.Ч. Тулендиева1, А. Абекова2, К. Миятжанова1, С.Ш. Асрандина1
1Казахский национальный университет имени аль-Фараби, Казахстан, г. Алматы
2Казахский институт земледелия и растениеводства, Казахстан, Алматинская обл., с. Алмалыбак,
*e-mail: kenzhebaevas@mail.ru, Kenzhebaevas@kaznu.kz

Различия в содержании фитиновой кислоты и активности фитазы в сорте яровой пшеницы и новых мутантных линий

Генетическая изменчивость биодоступности микронутриентов, обусловленная высоким содержанием фитиновой кислоты (ФА) в зерне, у сортов пшеницы ограничена. ФА особенно углубляет эту проблему, поскольку она связана с сопутствующей потерей не перевариваемых, связанных с ней комплексов микронутриентов и заряженных белков в отходах, и приводит к снижению их биодоступности для людей и домашнего скота. Новые мутантные линии яровой пшеницы (поколение M 7), созданные на генетическом фоне сорта Эритроспермум-35 посредством гамма-облучения в дозах 100 и 200 Гр с использованием 60Со, были взяты для оценки содержания ФА в зерне, исследования времени прорастания на активность фитазы и ее органоспецифического распределения. Выявлена существенная генотипическая изменчивость содержания ФА у Эритроспермум-35 100- и 200-Гр генерированных мутантных линий, из которых, в основном, линии, созданные 200 Гр, имели значительно более низкое содержание ФА в зернах, в 1,2–3,5 раза, чем у родителя, с его самым низким средним значением 0,78 ± 0,02 мг/г в 100 Гр линии. Вариации активности зерновой фитазы у сорта и мутантных линий обусловлены временем прорастания (3 и 6 часов); увеличение времени прорастания вдвое повышало активность фитазы в 1,47 раза у сорта Эритроспермум-35 и в 1,36-3,53 раза в мутантных линиях, что свидетельствует о зависимости активности фитазы от времени прорастания и генотипа. Органоспецифическое распределение (зерно, корни и побеги) активности фитазы у сорта и мутантных линий, различающихся по содержанию ФА, 12- и 24-часовых проростках, показало постепенное снижение зерновой активности и одинаковое увеличение ее уровня в корнях и побегах проростков. Мутантная линия под номером 35/1 имела максимум активности фитазы в зернах, корнях и побегах 12-часовых проростков, который превышал таковой сорта 35 в 3,05, 3,03 и 8,43 раза соответственно, что является ценным при разработке сортов с высокой активностью фитазы.

Ключевые слова: вариабельность содержания фитиновой кислоты в зерне, яровая пшеница, активность фитазы.

Introduction

Phytases (myo-inositol hexakisphosphate 3-, 6- and 5-phosphohydrolase, EC 3.1.3.8, EC 3.1.3.26 and EC 3.1.3.72) belong to phoshatases that can catalyze the stepwise hydrolysis of myoinositol-(1,2,3,4,5,6)-hexakisphosphate, IP6, also known as phytic acid (PA). Monogastric animals poorly digest PA, as they lack the phytase enzyme, which is responsible for the release of phosphate residues [1]. As the result of phytases catalyzed reaction, phosphorus (P), inositol phosphates, and inositol are formed required for a range of cellular activities [2]. In addition, phytases are considered as pro-nutritional enzymes that make chelated with PA nutrients, such as macro elements and microelements, bioavailable. Moreover, for many years’ phytases have practical inquiries, in this aspect researches have been centered on the actual need for improving utilization of PA-phosphorus in humans and single-stomached animal’s diets, and reduced the anti-nutritional action of non-digested PA associating micronutrients in their digestive tracts. Therefore, phytases are considered as tools for managing global phosphate resources and for alleviating human nutrients deficiencies mainly in the developing world [3].

In plants, PA is the main storage form of phosphate, typically amounting 2/3 of the total P content in the grain and seed [3]. PA has a strong...
chelating ability and stores in the grains and seeds as an insoluble salted complex with cations called phytin [4]. Phytin forms spherical crystalloid inclusions called globoids inside protein storage vacuoles [5]. The globoids are the principal site of phosphorous (P), potassium (K) and magnesium (Mg) in the mature cereal grain and they also contain calcium (Ca), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), sodium (Na), sulfur (S), and protein [6]. The anti-nutritional action of PA is particularly valid for Fe and Zn, making these two micronutrients are not bioavailable in humans and animal’s diets [7, 8]. There many human diseases that are associated with nutritional deficient and two-thirds of all children’s deaths are related to nutrients malnutrition. One of the common consequences of Fe deficiency is anemia. Currently, over three billion people have malnutrition of nutrients with the constantly rising numbers [9, 10]. Being essential bulk of nutrients in the human diet, there is a need genetic enhancement of quality grain wheat including a decrease in PA content which is one of the most cost-effective and powerful approaches for preventing global micronutrient malnutrition problem [9, 11]. Therefore, breeding for grain low phytic acid content is an actual goal to overcome all of these issues [12]. The different approaches were used wherein most of considerable efforts have been concentrated on the development of low PA (LPA) genotypes [13]. Transgenic cereals that express and accumulate microbial phytase in seeds have been developed but these materials require laborious processing steps before the product can be fed to animals [14, 15]. Consequently, more economical and sustainable solutions are necessary [15]. One of an important strategy to decrease PA content is mutagenesis [16]. The LPA mutants have been identified in several crop plants including maize (Zea mays) [17, 18], barley (Hordeum vulgare) [19], soybean (Glycine max) [20], rice (Oryza sativa) [21], and wheat (Triticum aestivum) [22, 23]. However, the progress in developing LPA wheat is relatively small compared to the achievements made for other cereal crops. Mutagenesis is a powerful tool for crops improvement and is free of the regulatory restrictions, licensing costs, and societal opposition imposed on genetically modified (GM) organisms. The aim of mutation induction is to increase mutation rate in traits or genes in a short duration that then could be readily exploited by plant breeders for developing new plant varieties without any limitations of GM approaches [16].

In our previous research, spring wheat M₄ mutant lines were developed through physical mutagenesis on background parent cultivar, cv. Zhenis, for searching a mutation resource with low grain phytic acid (PA) content, for correlations between yield, grain size, and quality parameters [24]. The Zhenis developed via 100 Gy and 200 Gy gamma irradiation treatments M₄, mutant lines were diverse in several grain quality traits, and we revealed that some lines had significantly lower by 1.23-1.63 PA content compared to parent, cv. Zhenis.

The objectives of this study were: (1) to evaluate the variability in grain PA content in M₄ spring wheat mutant lines, generated on genetic base of cv. Eritrospermum-35 using treatments of 100 and 200 Gy gamma irradiation, (2) to study the effect of time germination on grain phytase activity in cv. Eritrospermum-35 and identified 100 Gy- and 200 Gy-dosed mutant lines, differing by grain PA content and (3) to investigate on tissue-specific distribution of phytase activity dependent on time germination.

**Materials and methods**

**Plant material and application of induced mutation**

Grains of spring bread wheat variety cv. Eritrospermum-35 (T. aestivum L.) were irradiated with 100 and 200 Gy doses from a ⁶⁰Co source at the Kazakh Nuclear Centre. Grains were planted after irradiation to raise M₁ plants. The M₁ generation was grown in the experiment afield of the Kazakh Institute of Agricultural and Farming near Almaty (43°15′N, 76°54′E, and elevation 550 m above mean sea level) [12]. Grains of the spring bread wheat cv. Zhenis (Triticum aestivum L.) were irradiated with 100 Gy and 200 Gy doses from a ⁶⁰Co source at the Kazakh Nuclear Centre, Almaty. After irradiation, seeds were sown to raise M₁ plants [12]. After harvesting the M₁ plants, 15 lines was selected from the original 100 Gy radiation dose. The selection criteria for these lines was grain weight per main spike (GWS) and per plant (GWP) and it was applied in the M₁, M₂, M₃ 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 M₄ generation were GWS >1.1 g and GWP >2.2 g for mutant lines. The initial number of lines in the M₄ generation was 300 each for the 100 Gy and 200 Gy radiation doses. In the M₅ generation, 61 mutant 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 M₅-M₆
generation. After harvesting the M₇ plants, 14 lines and 24 lines from the original 100 Gy- and 200 Gy-treated germplasm, respectively, were selected. The 100 Gy-dosed mutant 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. The 200 Gy-dosed 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 gamma irradiation, were used for evaluation of variability in grain PA content and study of effect of time germination on tissue-specific distribution of phytase activity in cv. Eritrospermum-35 spring wheat generated mutant lines through 100 Gy- and 200 Gy gamma irradiation.

Phytic acid extraction and determination

The extraction of PA from milled grain samples (0.3 g) was performed as described by [25] and the 2.5 mL of supernatant was treated with 2 mL 0.2% FeCl₃, and the mixture was boiled for 30 min with further centrifugation after cooling. The residue was washed twice with deionized water. The 1.5 M NaOH was added for the residue precipitation, shaken, and the solution was centrifuged. The 3 mL of 0.5 M HCl was added to precipitate and then was 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 100%. The determination of PA was based on precipitation of ferric phytate and measurement of Fe remaining in the supernatant [25]. The grain PA content was calculated by multiplying Fe content by a factor of 4.2.

Phytase extraction and activity assay.

Phytase activity was analyzed according to a method reported previously [26]. Phytic acid sodium salhydrate (Sigma P0109 fromrice) was used as substrate. The phytase activity was expressed as units, which is defined as the amount of inorganic phosphorus released from sodium phytate at a rate of 1 μmol min⁻¹ kg⁻¹ at pH 5.5 and 37°.

Results and discussion

Cereals crops bio fortification by nutrients centered on either increasing the accumulation of these in grains or improving their availabilities to realize both goals. High nutrients bioavailability, is in particular regarded as crucial, can be achieved by the reductions of anti-nutritional agents, particularly grain PA content, considering as the most important causal solution[8, 10, 27]. For the past years, it is being promulgated that Fe and Zn malnutrition as well combat of environmental issues associated with seed PA-P content could be decreased by grain PA content.

Our results revealed that relative to the spring wheat parent, cv. Eritrospermum-35, grain PA content was significantly decreased or increased in mutant lines developed (table 1, figure 1). The ranges in PA content were 0.75-3.84 mg g⁻¹ with a mean of 2.26±0.68 and 2.20±0.48 mg g⁻¹, in 100 Gy-dosed and 200 Gy-dosed lines, respectively (n=42 and n=72) (figure 1). These ranges of values define the PA 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 grain PA content by 1.2- to 3.5-times than that the 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 (figure 1).

For the past years, it is being promulgated that Fe and Zn malnutrition as well combat of environmental issues associated with seed PA-P could be increased by decreasing PA content.

Table 1 – Comparison between grain phytic acid content means and ranges for spring wheat cv. Eritrospermum-35 (parent), 100 Gy- and 200 Gy-dosed M₇ mutant lines

| Grain quality trait | cv. Eritrospermum-35 | 100 Gy-dosed lines | 200 Gy-dosed lines |
|---------------------|---------------------|-------------------|-------------------|
|                     | Mean                | Range             | Mean              | Range             | Mean              | Range             |
| Phy content (mg/g)  | 2.61                | 2.74-2.81         | 2.26              | 0.75-3.84         | 2.20              | 1.15-3.34         |
Difference in phytic acid content and phytase activity between spring wheat variety and new mutant lines

Studies of natural variation showed the huge difference for grain phytic acid content of wheat germplasm. The reported ranges in PA content in the 93 wheat accessions were from 0.59 to 2.08% [28], in modern varieties from 1.25 to 3.42% [29], and the range from 7 to 13 mg g\(^{-1}\) depending on Zn fertilization [30]. High PA content was found for whole-wheat flour (44.91 mg g\(^{-1}\)) [31](Frontela et al., 2011), and Spain grown wheat (24.6-45.4 mg g\(^{-1}\)) [32]. One of possible explanations for these inconsistent finding could be related to the method used for PA determination. The selection of the most appropriate method for the PA analysis is critical [27].

Considering that phytases are as pro-nutritional enzymes that make chelated with PA nutrients, such as macroelements and microelements, bioavailable [3] and have practical inquiries, our research was therefore carried out for determination of the effect of germination time on phytase activity (PhyA) and investigation of organ-specific distribution of phytase depending on germination time in spring wheat cv. Eritrospermum-35 and mutant lines differing by grain PA content, and to reveal is there a difference between genotypes.

The genotypic variation in PhyA of cv. Eritrospermum-35, the parent, and M, mutant lines depending on time of germination (3 and 6 hour) is shown in figure 2. Germination time from 3 to 6 hour enhanced grain PhyA by 1.47-fold in cv. Eritrospermum-35, and by 1.36-3.53-fold in mutant lines, indicating that the effect of germination on PhyA was strongly time and genotypes dependent. There was not germination time dependence of PhyA in 35/1 numbered mutant line. Some mutant lines numbered as 147/2, 153/4, 6/1, 26/2, and 38/1 exhibit a significantly increased PhyA at 6 hour germination by 1.25-1.38 comparing cv. Eritrospermum-35 (**p ≤ 0.01). Earlier reported on rice grain husks, bran, rootlets and shoots that phytase activity of all fractions increased significantly after germination, and was highest in brown rice followed by rice bran after four days of germination [33].

### Table 2 – Grain phytic acid content in spring wheat cv. Eritrospermum-35 (parent), M, 100 Gy- and 200 Gy-dosed mutant lines selected for study of effect of germination time on phytase activity and organ-specific distribution of phytase

| Spring wheat genotypes | Grain phytic acid content, mg/g |
|------------------------|-------------------------------|
| Eritrospermum-35       | 2.74 ± 0.11                   |
| 100 Gy-dosed lines     |                               |
| 151/2                  | 2.90 ± 0.14                   |
| 147/2                  | 1.48 ± 0.17                   |
| 153/4                  | 0.77 ± 0.02                   |
| 200 Gy-dosed lines     |                               |
| 6/1                    | 2.26 ± 0.09                   |
| 26/2                   | 2.78 ± 0.12                   |
| 35/1                   | 1.30 ± 0.14                   |
| 38/1                   | 2.15 ± 0.33                   |

The PhyA of cv. Eritrospermum-35 in grain, roots and shoots of 12-hour seedlings was 495.8, 694.1 and 1381.5 U kg\(^{-1}\), respectively, and that of mutant lines ranged from 942.0 to 3093.6, from 1322.0 to 4402.4 U kg\(^{-1}\), and from 522.2 to 4402.4 U kg\(^{-1}\), respectively, indicating that there is a greater difference between parent and line for shoots than other organs (figure 3). A mutant line numbered 35/1, had maximum PhyA in grain, roots, and shoots of 12-hour seedlings, which exceed the PhyA of cv. Eritrospermum-35 by 3.05-, 3.03- and 8.43-fold, respectively, which is valuable for spring wheat breeders in developing cultivars with high PhyA. In 24-hour seedlings, decrease in grain-, roots- and shoots- PhyA of cv. Eritrospermum-35 and mutant lines were observed with the highest mean in shoots (by 4.77- and 2.6- fold, respectively).
S.S. Kenzhebayeva et al.

Phytic acid particularly enhance solution as it related to an attendant loss of indigestible complexed nutrients and charged proteins in the waste and results in reduced their bioavailability in both humans and livestock. Large genotypic variation in PA content was found in spring wheat M₇, 100 Gy- and 200 Gy-dosed mutant lines developed on background of cv. Eritrospermum-35, parent, The 23 mutant genotypes of which mostly 200 Gy-treated lines had significantly lower grain PA content by 1.2- to 3.5-times than that parent, with its lowest mean of 0.78±0.02 mg g⁻¹ in 100 Gy-dosed lines.

The variation in phytase activity of cv. Eritrospermum-35 and M₇, mutant lines was depended on time germination (3 and 6 hours) and increasing time by twice enhanced grain PhyA to 1.47-fold in cv. Eritrospermum-35, and to 1.36-3.53-fold in mutant lines, indicating that the effect of germination on PhyA was strongly time and genotypes related.

Organ-specific distribution (grain, roots and shoots) of phytase activity in cv. Eritrospermum-35 and 100 Gy- and 200 Gy-dosed M₇ mutant lines differing by grain PA content, in 12- and 24-hours seedlings showed gradual decrease in grain genotypic activity with the same its level in roots and shoots of both hours seedlings.

A mutant line numbered 35/1, had maximum PhyA in grain, roots, and shoots of 12-hour seedlings, which exceed the PhyA of cv. Eritrospermum-35 by 3.05-, 3.03- and 8.43-fold, respectively, which is valuable for spring wheat breeders in developing cultivars with high PhyA.

Conflicts of interest

The authors have no conflicts of interest.

Source of financing

The research was carried out on the basis of the scientific research institute of biology and biotechnology problems of the al-Farabi National University and was funded by the Ministry of Education and Sciences of the Republic of Kazakhstan under the Project 074/GF “The creation and study of mutant genotypes of wheat for identifying valuable breeding forms and new alleles of genes controlling key adaptive properties” and AP05131881 “Development of integrated approaches for biofortification, high bioavailability of the most important micronutrients of spring wheat and health”.

Thus, in our study, to search spring wheat new resource for high bioavailability of nutrients strongly related to low grain PA content and, spring wheat new genetically stable (M₇) 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. Using the abundant variation present in wheat mutant lines, it should be possible to improve mineral bioavailability in modern cultivars. Germination is an effective process to increase PhyA level.

Conclusions

As a result of the research carried out, the following conclusions can be drawn:
27. Gibson, R.S., Bailey, K.B., Gibbs, M., Ferguson, E.L. "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. Volume 31(2S), (2010): 134-146.

28. Zhao, F.J., Su, Y.H., Dunham, S.J., Rakszegi, M., Bedo, Z., McGrath, S.P., Shewry, P.R. "Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin". J. Cereal Sci. Volume 49, (2009): 290–295.

29. Ahmad, I.; Mohammad, F.; Zeb, A.; Noorka, R.I.; Farhatullah, Jadoon, S.A. "Determination and inheritance of phytic acid as marker in diverse genetic group of bread wheat". Am J of Mol. Biol. Volume 3, (2013): 158-164.

30. Erdal, I., Yilmaz, A., Taban, S., Eker, S., Torun, B., Cakmak, I. "Phytic acid and phosphorus concentrations in seeds of wheat cultivars grown with and without zinc fertilization". J Plant Nutr. Volume 25, (2002), 113–127.

31. Frontela, C., Ros, G., Martinez, C. "Phytic acid content and “in vitro” iron, calcium and zinc bioavailability in bakery products: The effect of processing". J Cereal Sci. Volume 54, (2011): 173-179.

32. García-Estepa, R.M., Guerra-Hernández, E., García-Villanova, B. "Phytic acid content in milled cereal products and breads". Food Res Int. Volume 32, (1999): 217-221.

33. Sangsopha, J., Moongngarm, A. "Influence of germination on mineral bioavailability and phytic acid content in rice". Food and applied bioscience J., Volume 6 (sp. issue), (2018): 69–83.