

Variability in phytic acid and phytase levels and utilization of synthetic hexaploids for enhancing phytase levels in bread wheat

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Abstract

Phytic acid and phytase levels in wheat grains have greater influence on the bioavailability of micronutrients to human beings. Hence in the present investigation, two hundred fifty seven wheat genotypes including 168 synthetic hexaploids and 89 wheat cultivars were used to assess variability in phytase and phytic acid levels. The phytic acid content showed 1.5 fold variations from 1.03 to 1.58% with the mean value of 1.34% in bread wheat cultivars while higher variability (2.1 fold) was exhibited in synthetic hexaploids ranging from 0.93 to 1.98% with the mean value of 1.35%. There were 4.1 fold differences in phytase levels in wheat cultivars varying from 284 to 1155 FTU/Kg with the mean value of 516 FTU/Kg and 7.9 fold in synthetic hexaploids varying from 255 to 2025 FTU/Kg with the mean value of 751 FTU/Kg. There was very strong correlation ($R^2=0.89$) in phytase levels between duplicate samples of the wheat flours demonstrating high reproducibility of direct assay method used in this investigation. The method has additional advantage of analysing small quantity of materials available in the segregating generations of breeding programme. The selected synthetic hexaploids having high phytase levels are useful source for improving phytase levels in bread wheat cultivars.

Keywords: *Agrobacterium*, phytase, phytic acid, wheat, micronutrients, synthetic hexaploids

1. Introduction

Wheat (*Triticum aestivum* L.) is the main source of world's food energy and contains significant amount of carbohydrates, minerals, fat, vitamins and proteins essential for human health (Piironen *et al.*, 2009). However, the absorption of essential micronutrients such as Fe and Zn is strongly inhibited in the human gastrointestinal tract by the presence of anti-nutritional element known as phytic acid (Greiner and Konietzny, 2006). Phytic acid, (myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate; InsP₆) also known as phytate, contains more than 80% of the total phosphorus in the grain. The negatively charged phosphate group in phytate strongly binds to divalent cations such as Ca²⁺, Fe²⁺, K²⁺, Mg²⁺ and Zn²⁺ at physiological pH to form a mixed salt called phytin

which reduces the bio-availability of micronutrients to humans and monogastric animals (Greiner and Konietzny, 2006). This leads to Fe and Zn deficiency in human population making them prone to several diseases (Guo *et al.*, 2012). This problem is more common in infants and pregnant women who require relatively more quantity of micronutrients. In addition, some monogastric animals are not able to metabolize phytic acid due to the lack of sufficient level of phytate hydrolysing enzymes activity in their digestive tract and as a result the feed of these animals are enriched with phosphorus. Large amounts of phosphorus, micronutrients and phytic acid (PA) are excreted through faecal material and gets accumulated in the water and causes algal blooming and phosphorus

pollution (Brinch-Pedersen *et al.*, 2002). Therefore, the hydrolysis of phytic acid present in the grain is needed to solve the problem of micronutrient deficiency in human beings and monogastric animals.

The hydrolysis of phytic acid can be accomplished either by addition of isolated phytases to the production process and the use of recombinant food-grade microorganisms as carriers for phytate-degrading activity in the human gastrointestinal tract or using wheat varieties high in phytase levels. Dietary phytases are important sources of phytate breakdown in human intestine (Sandberg and Andersson, 1998). Lopez *et al.* (2003) also reported that varieties had major influence on mineral bioavailability in which improved break down of phytate was related to higher phytase levels. Phytase (myo-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8) is a subgroup of phosphatases which enables to start the sequential dephosphorylation of phytate in cereals. Development of wheat with higher phytate degrading activity may result in more extensive phytate degradation in human stomach and improved micronutrient bioavailability. However, there are limited reports on phytase variability in wheat (Kim *et al.*, 2002; Liu *et al.*, 2007; Ram *et al.*, 2010). Therefore, it is needed to see the variability in phytic acid and phytase levels occurring naturally in diverse wheat genotypes. In the present investigation, variability of phytic acid and phytase levels were evaluated for 168 synthetic hexaploids and 89 wheat varieties. The information was used in crossing of high phytase synthetic hexaploid lines (Synthetic 70) with high yielding wheat varieties (PBW343) and generating back cross populations.

2. Materials and methods

2.1 Plant material: A set of 168 synthetic hexaploids procured from CIMMYT, Mexico and 89 wheat varieties developed in India were grown and analysed at Indian Institute of Wheat and Barley Research, Karnal, India during 2014-15 and 2015-16 for phytase and phytic acid levels. Backcross population of a cross between PBW 343 and Syn70 was developed and analysed for phytase activity to enhance phytase levels in the background of PBW 343. The wheat grain samples were grounded with the Cyclotec Mill and passed through a 0.05 mm sieve and stored at 4°C for the measurement of phytase levels and phytic acid content.

2.2 Determination of phytic acid : Phytate content was determined by the modified method of Lorenz *et al.* (2007), Gao *et al.* (2007) and Ram *et al.* (2010). 80 mg of the whole meal flour was weighed and mixed with 1.6 ml of 0.65N HCl in 2 ml micro-centrifuge tubes and shaken overnight at room temperature followed by centrifugation at 10,000 g for 20 min. Wade reagent was prepared for final reaction by using 5-sulfosalicylic acid and ferric chloride as per (Lorenz *et al.*, 2007) and diluted 5 times. The absorbance was taken at 490 nm. The sodium phytate salt (Sigma) was used for preparing standard curve for phytic acid content. The phytic acid content was expressed in percentage on dry weight basis.

2.3 Phytase assay : Phytase levels were measured using modified methodologies (Kim and Lei, 2005; Okot-Kotber *et al.*, 2003 and Ram *et al.*, 2010). 100 mg of the whole meal flour was soaked overnight in 1.0 ml of 50mM sodium acetate buffer (pH 5.3) in 2.0 ml tubes at 4°C and centrifuged at 12,000 rpm for 30 min. at 4°C. 0.2 ml of the supernatant was used in enzyme assay. Two hundred microliter of 0.5% (wt/vol) sodium phytate was added in each tube and incubated at 50°C for 30 min. for enzymatic hydrolysis. The reaction was stopped by adding 0.4 ml of absolute trichloroacetic acid at room temperature. The control reaction was set by adding TCA before the enzyme to inhibit its activity. The mixture was centrifuged at 10,000 g for 30 min. and 0.2 ml of supernatant fraction was mixed with 1.8 ml of distilled water and 2 ml of fresh colour reagent and incubated at 50°C for 15 min. The colour reagent was prepared by mixing three volumes of 1M sulphuric acid, one volume of 2.5% ammonium molybdate and one volume of 10% ascorbic acid (wt/vol). Series of diluted potassium phosphate solutions were used as standards (Ullah, 1988). The absorbance of each reaction mixture was taken at 820 nm and the enzyme activity calculated. One unit of phytase is defined as the amount of enzyme required to release 1 µmol of inorganic P/min from sodium phytate at 50 °C. The phytase activity was calculated in FTU/kg on dry weight basis.

2.4 Statistical analysis : The analysis was carried out by ANOVA for both traits to test the level of significance. The ANOVA was performed from SAS PROG GLM statistical tools by using linear model. The data were expressed as mean ± SE (n=3). The reproducibility of the method was tested by regression analysis.

3. Result and discussion

3.1 Variations in phytic acid content: In order to determine genotypic differences in phytic acid levels, 257 wheat genotypes including 168 synthetic hexaploids and 89 wheat cultivars released in India were used in the present investigation. The phytic acid content showed 1.5 fold variations from 1.03 to 1.58% with the mean value of 1.34% (Fig. 1). It exhibited higher variability (2.1 fold) in synthetic hexaploids ranging from 0.93 to 1.98% with the mean value of 1.35%. Different range of phytate levels have been reported by several workers (Lopez *et al.*, 2003; Dost and Tokul, 2006; Steiner *et al.*, 2007; Liu *et al.*, 2007; Ficco *et al.*, 2009) depending on the germplasm and different assay procedures used. The analysis of variance showed significant differences among genotypes for phytic acid content and indicated potential for genetic improvement in wheat for low phytic acid (Table 1). Thus the data demonstrated that wheat germplasm with low phytic acid is available for utilization in breeding to improve high yielding varieties for micronutrient bioavailability.

Table 1. Analysis of variance (ANOVA) of phytic acid in the grains of 168 synthetic hexaploids

Source	SS	df	MS	F
Genotypes	17.43	167	0.104	58.284**
Replication	0.01	2	0.015	8.933
Error	0.299	167	0.001	
Total	17.74	336		

** Significant at 1% level of significance

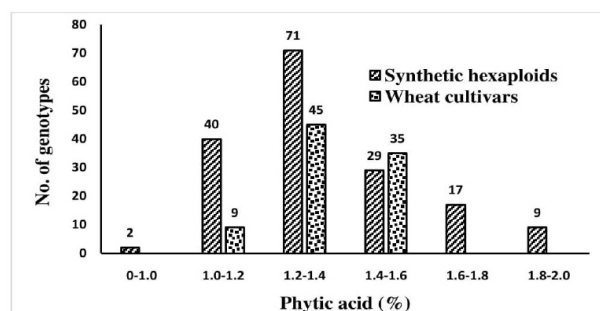


Fig. 1 Levels of phytic acid in the grains of synthetic hexaploids and wheat cultivars released in India indicating larger variability in synthetic hexaploids as compared to wheat cultivars

3.2 Variations in phytase levels: Higher phytase levels are important in increasing micronutrient bio-availability (Lopez *et al.*, 2003) by degrading phytic acid and thus releasing more micronutrients in human intestine. There were 4.1 fold differences in varieties developed in India and 7.9 fold variations in synthetic hexaploids (Fig. 2). It varied from 284 to 1155 FTU/Kg with the mean value of 516 FTU/Kg in released varieties and from 255 to 2025 FTU/Kg with the mean value of 751 FTU/Kg in synthetic hexaploids. It is interesting to note that greater variability was observed in synthetic hexaploids. Similar variations were reported by Ram *et al.*, 2010 in a set of wheat germplasm. In this investigation more numbers of synthetic hexaploids and new wheat cultivars were used for estimation of phytase levels. Large diversity in phytase levels in synthetic hexaploids may be because of the fact that synthetics were developed by crossing different species of tetraploids with different accessions of *Ae. tauschii*. Because of smaller number of hybridization events and the restricted geographic origin of these events, most of the variations were not tapped during the evolution of wheat by natural crossing between *Ae. tauschii* and tetraploid wheat. This might have led to narrow genetic diversity for bread wheat (Talbert *et al.*, 1998). The analysis of variance showed significant differences among genotypes for phytase levels and thus great possibility of genetic improvement in wheat for high phytase levels (Table 2).

Table 2. Analysis of variance (ANOVA) of phytase activity in the grains of 168 synthetic hexaploids

Source	SS	df	MS	F
Genotypes	60919574	167	167	32.91**
Replication	90731.17	2	2	8.18
Error	1851054	167	167	
Total	62861359	336	336	

** Significant at 1% level of significance

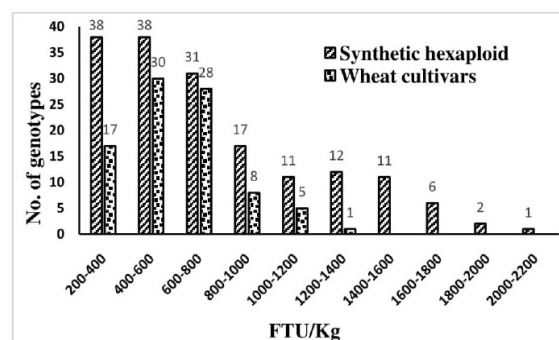


Fig. 2 Frequency distribution of phytase levels in the grains of 168 synthetic hexaploid and 89 wheat cultivars indicating larger variability in synthetic hexaploids as compared to wheat cultivars

3.3 Testing the robustness of the methodology of estimating phytase levels: Some of the earlier reports (Dost and Tokul, 2006; Steiner *et al.*, 2007; Ficco *et al.*, 2009) indicated a diverse range of phytase levels due to differences in the protocol of phytase assay. Earlier, Ram *et al.* (2010), made comparison of three different methods. In this report we used direct assay method of Ram *et al.* (2010) and validated the robustness of the assay. There was very strong correlation ($R^2=0.89$) in readings of duplicate samples of the wheat flours (Fig. 3). This demonstrated that the direct assay method is more suitable for phytase analysis as it gives both reproducibility and repeatability of results. It has additional advantage of being taking lesser time and cost in the analysis. Moreover, 100 mg of the whole meal flour sample used in the evaluation of phytase levels has the advantage of analyzing small quantity of materials available in the segregating generations of breeding programme.

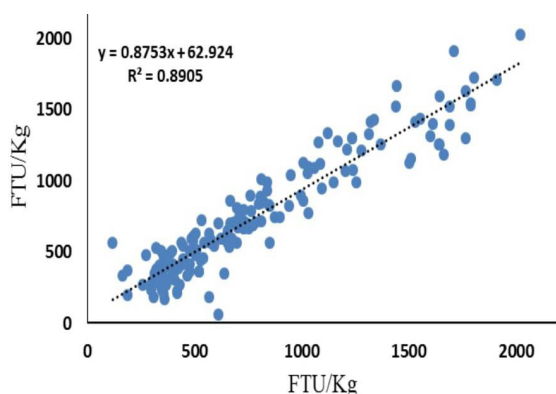


Fig. 3 Relationship between phytase levels estimated in duplicate samples using direct assay method indicating the high repeatability of the assay

3.4 Improvement of phytase levels in the background of PBW 343: One of the synthetic hexaploids (Syn70) exhibiting higher phytase levels was utilized in making crosses with PBW 343 for enhancing phytase levels in this cultivar. Phytase levels were 610 FTU/kg and 1450 FTU/kg in PBW 343 and synthetic hexaploid 70, respectively. The cross was advanced to BC_2F_4 level during 2016-17 and desirable segregants for high phytase levels were selected based on microlevel test validated in this investigation. Phytase level varied from 570 FTU/kg to 1569 FTU/kg in the backcross population (Fig. 4). Desirable segregants with high yield potential and high phytase levels will be further advanced and selected

for yield trials for identification of superior cultivar. The data clearly demonstrated that synthetic hexaploids can be used to enhance and diversify phytase levels in bread wheat for improving nutritional quality of wheat for enhanced bio-availability of micronutrients.

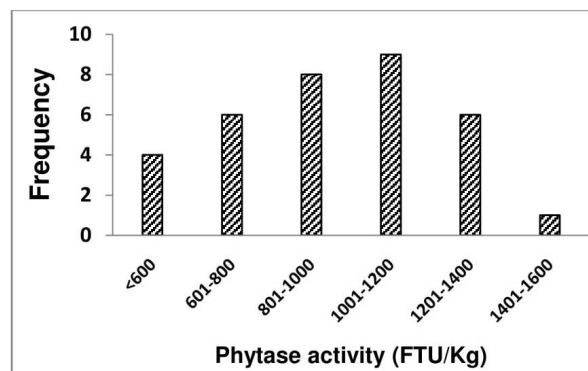


Fig. 4 Phytase levels in BC_2F_4 population of a cross between PBW 343 and Synthetic 70 indicating enhanced phytase levels in advanced lines

Large variations were observed for both phytic acid and phytase levels among synthetic hexaploids and bread wheat genotypes. The data demonstrated direct assay method of phytase activity using 100 mg of the flour has high reproducibility with the advantage of analysing large segregating generations of breeding programme. The study also demonstrated that synthetic hexaploids having high phytase levels are useful source for the improving phytase levels in bread wheat cultivars.

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