Selecting Wheat Seeds of Moderate Phytate Using Colorimetric Method

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Abstract

A simple and effective method of determining the phytate content of a single wheat seed was considered necessary for selecting the materials of moderate phytate during early generations breeding. 23 wheat genotypes (16 winter wheat cultivars and 7 spring wheat cultivars) were firstly used as determining samples of phytate content (PC) and inorganic phosphorus content (IPC) of the whole meal, secondarily they were planted in two locations for two times (2004 and 2005). The all single plants were harvested by a plot from those sites, then 6 individual plants were randomly selected to save their seeds for each plot, and the seeds of other plants were grinded into whole meal to assay their PC and IPC. Subsequently, every seed from per individual plant was cut into two semi-grain seeds: the semi-grain seed without embryo (SGSWOE) and the semi-grain seed with embryo (SGSWE); the SGSWOE was carried out to assay colorimetric scoring values by inorganic phosphorus content (CSVIPC), while according to the CSVIPC index, the satisfied SGSWEs were saved to plant at next generation or the dissatisfied SGSWEs were eliminated. The results showed that there was significantly negative correlation between PC and IPC of the wheat meal from either the first samples or the materials harvested at those sites, and for the latter there was significantly negative correlation between PC and CSVIPC. There was no significant difference of CSVIPC among the SGSWEOs and SGSWE of the spikes and individual plants from the same plot or the same genotype at any sites. In conclusion the CSVIPC of the SGSWOEs can indirectly express PC level of the wheat meal: the higher the former was, the lower the latter was. So the CSVIPC of SGSWOE can be applied to assaying the level of corresponding SGSWE according to their index, and screening the individual plant of moderation phytate. Moreover, this method is characterized by high efficiency during breeding processing.

Keywords: Phytate content, Inorganic phosphorus content, Individual plant selecting, Wheat (*Triticum aestivum* L.), Colorimetric method

1. Introduction

With development of the wheat meal food and the feed wheat in many countries including China, many people have concerned with the wheat meal nutrition, especially the effect of many anti-nutritive factors (Guttieri M, et al., 2004; Robay V, 2001; Dominguez BM, et al., 2002) on human and animals bioavailability on mineral elements, such as Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, $Cd^{2+}$and Fe^{2+}(Lopez HW, et al., 2002; Lopez HW,et al., 2004; Rodrigues-Filho, et al., 2005) and proteins (Gilani GS, et al., 2005) in the food and the feed, respectively. Phytate has been deemed as an anti-nutrition factor, which can make non-ruminant animals including human restrain absorbing nutrition. Moreover, the phosphorus of phytate is excreted from body into environment to lead to inorganic phosphorus waste and global phosphorus pollution (Robay V, 2001; Oatway L et al., 2001; Turner BL, et al., 2002).

Phytate mainly accumulates in protein storage vacuoles, and it locates in the aleurone layers of wheat, barley and rice or in the embryo of maize (Lisbeth Bohn, *et al*; 2008). Selecting moderate phytate crop cultivars is considered as more important means than adding exogenetic phytase or sourdough (Leenhardt, Fanny, et al, 2005; Haros M, et al., 2001; Lopez HW, et al., 2003) into the food or the feed from wheat materials, or adjusting pH of dough (Leenhardt, Fanny, et al., 2005) to reduce phytate content. Whether hybridization, bioengineering or mutation breeding, it is necessary that the breeders can screen individual plant of low or moderate phytate content effectively at early generations of breeding procedure (Henrik B.P, et al., 2002; Vicky Roslinsky, et al., 2007; Holm Preben B, et al., 2002). Presently, there are many methods on determining phytate content such as HPLC or HPIC (Prachuab Kwanyuen and Joe W. Burton, 2005; Skoghund E, et al., 1998; Chen QC, 2004). Although those means are effective and exact on assaying the phytate contents of food, feed and excretion, they are trivial and low effective on selecting individual plant at early generations because of single grain seed disposal and gigantic samples analyzed. Moreover, many plant breeding organizations in developing countries have not those costly apparatus such as chromatogram and microtest plate reader. The aim of this paper is to explore a simple method on screening semi-grain seeds of low or moderate phytate effectively so that it can provide basic means on selecting moderate phytate wheat cultivars on methodology.

2. Materials and methods

2.1 Commercial materials from different origin areas

Sixteen winter wheat and seven spring wheat genotypes(Table 1) were obtained from main areas of planting wheat in China for determining samples and planting seeds respectively, and according to wheat genotype, the former(using the sample of 1,000g each genotype) was milled into wheat meal in order to assay phytate content (PC) and inorganic phosphorus content (IPC) respectively.

2.2 Crop establishment and agronomy

Those 23 wheat genotypes were planted at Anhui(the South in China, one of the main area of wheat, 31.4°N and 117.5°E at 240 m above sea level) and Tianjin(the North in China, one of the main area of wheat, 41.1°N and 117.3°E at 130 m above sea level) in 2004 and 2005, respectively. Experimental layout was a randomized complete blocks design with three replications in each environment. Sowing was done by an experimental drill in 1.0m×2.0m plots, consisting of six rows with 20cm between rows. Seeding rate was 300 seeds m^{-2} for each location. Fertilizer application was 90 kg N ha^{-1} and 60 kg P_2O_5 ha^{-1} at planting. Annually, field materials after mature phase were divided into two parts, one part was harvested by individual plant to be air-dried and used to assay six seeds CSVIPC (only using the semi-grain seed without embryo) per spike, containing three spikes of five random individual-plant samples per wheat genotype, and another was collected by plot to be milled into wheat meal (filtered by 60 screen mesh) in order to assay those PC and IPC.

2.3 Chemical

Purified Milli-Q water (Millipore Co., Bedford, MA) was used as the preparation for all reagents and buffers. All were filtered through 0.2-μm membranes. Criterion phytate (HPLC grade) was purchased from Sigma, and other chemical (the inorganic chemical was analytical grade, and the organic chemical was HPLC grade) were obtained from Tianjin Chemical Reagent Co., Tianjin, China.

2.4 Harvested samples preparation

Wheat seeds were ground in a centrifugal grinding mill equipped with 1.0-mm stainless-steel ring sieve, by which a uniform particle (the size of less than 0.3mm) was produced. For convenience, extraction was done for 1h in a 50-mL vial with 0.5N HCl in a ratio of 1:20 (W/V.) while stirring. 0.10g of sample and 2mL of 0.5N HCl were used through those experiments. Approximately 0.4mL of crude extract from each sample was centrifuged at 18,000*g* for 10min. 0.2mL of supernatant containing phytate was then filtered through 0.45-μm syringe filter. Filtered samples could be stored at 4°C for several days prior to HPLC analysis.

2.5 HPLC analysis of the samples

Phytate was determined by using the method of Prachuab Kwanyuen and Joe W. Burton (2005). The HPLC system used for eluting phytate consisted of a binary pump, a vacuum micro-degasser for buffers, and an autosampler. An isocratic pump was used for the delivery of Wade's color reagent in a postcolumn reaction with phytate. The absorbance was monitored at 500nm, and the detector signals and/or phytate peaks were processed and integrated by the chromatographic data acquisition system.

2.6 Assay of inorganic phosphate

Wheat meal was suspended in 1% glacial acetic acid. After incubation at 42℃ for 30min, the samples were

centrifuged at 3,000g for 10min, and the supernatant was assayed at OD_{820} as described by Ames (1996) and Kai Xiao et al.(2005).

2.7 CSVIPC acquirement of semi-grain seeds

Six seeds were collected from the same individual plant sample, and the each seed was split into two parts by a shaver: a semi-grain seed with embryo (SGSWE) and a semi-grain seed without embryo (SGSWOE), respectively (Figure 1, I). the SGSWEs were reserved, while their SGSWOE were crushed into powder by a glass, weighed and placed into a cell of microtest plate (containing 96 cells, Figure 1, II) from the same seed. To each sample analysed, 10μL HCl (0.4 M) mg^{-1} sample (dry weight) was added into the cell, and then the micro-test plate was placed in icebox at 4℃ for about 24 h. Next day, 10μL sample solution of the cell, 90μL distilled water and 100μL display reagent(the composition was explained at next paragraph) were extracted to a cell of another micro-test plate in turn, mixed, then it was saved at room temperature for one day to be used to compare with criterion phosphorus solutions.

The display reagent (5mL) contained 1mL of 3 M H_2SO_4, 1mL of 2.5% $(NH_4)_6MoO_{24}$ (W/V), 1mL of 10% L-Ascorbic Acid (W/V, stored at 4℃) and 2mL of distilled water, which were instantly confected as they were used.

Standard phosphorus was used as criterion solution. Five 100-μL standard phosphorus solutions with 0.0μg, 0.54μg, 1.08μg, 1.62μg and 2.70μg of KH_2PO_4 per 100μL distilled water, were put into five cells of a micro-test plate. After this, 100μL of the display reagent was added to the cells again. The next day, chroma (it was blue by originally colorized figure) of the cells was directly observed, moreover, they turned thicker and thicker with accretion of criterion phosphorus contents (Fig. 1, standard phosphorus). We named colorimetric values of the standard phosphorus solutions by 1, 2, 3, 4 and 5 in turn. Compared with the chroma of the standard phosphorus solutions, all samples also might be scored by 1 to 5 based on identical or adjacent principle. Colorimetric value 1, 2, 3, 4 and 5 contained 0.0mg, 0.54mg, 1.08mg, 1.62mg and 2.70mg of inorganic phosphorus per 1.0g sample (dry weight), respectively.

2.8 Statistical analysis

The total number of the samples analysed was lined on table 5. Data were subjected to ANOVA analysis, the means comparisons to the Newman Keuls test ($p < 0.05$), and Pearson correlations according to the Statistic software package version 5.0.

3. Results

3.1 Correlation analysis between PC and IPC of wheat meal

The PC and IPC are determined from the samples of the wheat meal, and the ranges of PC and IPC are 0.41 to 1.85 mg g^{-1}, 0.11 to 2.98 mg g^{-1} in all materials tested, respectively,(Table 2 and 3). The tables show that, i. there are not differences between the mean of PC or IPC in whether the different planted places or the different years; ii. there is significantly negative correlation between PC and IPC of wheat meal from both different planting areas and the same planting condition. As a consequence, the ranges of PC and IPC are wide from the materials tested, and all of F values reach significant difference level too. It suggested that the wheat cultivars tested might meet this trial. According to the CC between PC and IPC, because there is reverse relation at significant level (p=0.01) from any origins, the relationship between PC and IPC is tight, namely, the higher IPC is from the same wheat meal, the lower PC is.

3.2 Consistency analysis of the CSVIPC between SGSWE and SGSWOE from the same grain seed

The same grain seed is cut into a SGSWE and a SGSWOE (Figure 1), in like manner, 42 seeds are randomly sampled and analyzed per plot. The results indicate that there is high consistency in CSVIPC between SGSWE and SGSWOE from the same grain seed (Table 4), so the CSVIPC of SGSWOE may exactly express the CSVIPC of SGSWE from the same grain seed, namely, if one SGSWOE is proved moderate on PC, the corresponding SGSWE will be used as a seed of next generation planting.

3.3 Distributing relationship between IPC of wheat meal and CSVIPC of semi-grain seeds at the level of individual plant

According to Figure 2, there is not significant difference on genotype numbers among 4 sites based on the range of IPC of wheat meal, and the IPC of the same wheat cultivar assayed has not significant difference among 4 times trials too (not shown). At the same range of IPC, the individual plant numbers of corresponding CSVIPC with the IPC are the highest of all five scores, and it contains over 50% of all. If individual plant numbers of other one or two corresponding CSVIPC in the vicinity of the range assayed are counted into total amount, its

value is over 90%, mainly. So it suggests that there be basic consistency between the CSVIPC and the IPC.

3.4 Range analysis between the CSVIPC of semi-grain seeds and PC and IPC of wheat meal

Among 6 random seeds from one individual plant, when 4 or more seeds are appraised by the same score, the individual plant is sampled to assay its IPC of wheat meal. Hundreds of individual plants scored by 1 to 5 are analyzed from all wheat genotypes of 4 sites (Table 5). The results show that, the mean of PC is lower 1.0mg g^{-1} dry wheat meal when CSVIPC of SGSWOE is score 3 or more, while the mean of PC is about 0.5mg g^{-1} dry wheat meal when the CSVIPC of SGSWOE is score 5. If 0.5 to 1.0 mg g^{-1} of PC is moderate, and score 3 or 4 on the SCVIPC of SGSWOE is regarded as screening criterion of the SGSWE of "moderate" PC.

4. Discussion

The fact that an increase in Pi was inversely related to phytic acid (Vicky Roslinsky, et al., 2007; Raboy V et al., 2000) is provided by this paper using wheat, so phytate levels of the seeds will be indirectly determined by using a colorimetric assay for inorganic phosphorus (Vicky Roslinsky, et al., 2007; Chen PS, et al., 1956). Raboy et al (2000; 2001) used uniform of inorganic phosphorus for all six seeds as classified criterion of "low" or "normal" phytic acid on the lines of a segregating population. There are two different points between the former and this paper: on this paper, first, inorganic phosphorus is scored by intuitional means of the colorimetric method based on criterion phosphorus in microtest plate, so it needn't any apparatus and there are more classified types (score 1 to 5); second, only have the SGSWOE been assayed, the SGSWE can be saved to be used easily as next generation seeds.

This method is high efficiency too. If 18 grain seeds from 3 spikes per individual plant are assayed, 5 individual plants from one microtest plate may be performed by us. According to this efficiency, one people can assay 4 plates one day, so she or he will screen 180,000 seeds from 2,000 individual plants for 100 day before next breeding phase. At early generation selection, the efficiency of screening individual plant can well meet the need of selecting low phytate cultivars on wheat.

During wheat seeds germination, phytate is hydrolysed by endogenous phytase(s) and other phosphatases to release phosphate, inositol and micronutrients to support the emerging seedling. Phytate and its derivatives are also implicated in RNA export, DNA repair, signalling, endocytosis and cell vesicular trafficking (Lisbeth Bohn, et al; 2008). Moreover, we also have noted that, phytic acid may have beneficial effects on human nutrition, reducing the risk of colon and breast cancer through action either as an anti-oxidant (Evers, et al., 1999; Graf. and Eaton, 1993), or through control by the amount of free iron present within the body (Thompson, et al., 1991), and phytate content of crops is too low to increase their yield (Vicky Roslinsky, et al., 2007), as a consequence, very low phytate of wheat seeds will go against plant breeding. Based on barley phytate content range (Vicky et al., 2007) and wheat phytate content ranges (Xinglin Li, 2007), so we think, if 0.5 - 1.0 mg phytate per g of crop seeds is moderate content (Tbale 2 and Table 3), the colorimetric value 2 to 4 of semi-grain seeds is fit for breeding aim (Figure 1: III, IV and V).

Abbreviations: CC, correlation coefficient; CSVIPC, colorimetric scoring value of inorganic phosphorus content; HPIC, high-performance ion chromatography; HPLC, high-performance liquid chromatography; IPC, inorganic phosphorus content; PC, phytate content; SD, standard deviations; SGSWE, semi-grain seeds with embryo; SGSWOE, semi-grain seeds without embryo.

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Table 1. The wheat types and their cultivars names

Wheat types	Cultivars Names
Spring wheat	Annong No 2; Annong 8455; Annong 8729-10. Shanyou 225; Liken No 2; Xiaoying No 6; Youxuan 14; Zhongzuo 8131-1; PH82-2-2; Lu884187; Luzhi79-1; Yannong 15; Zhenzhou 8603; Yumai 14; Taiyuan 136; Yunmai 33
Winter wheat	Zhangchun 11; Liaochun No10; Liaochun13; Mengyou No 1; Longchun 17; Longchun 22; Wumai No 6

Table 2. The contents of both the phytate content and inorganic phosphorus of wheat meal (dry weight) and the correlation coefficient between the phytate content and inorganic phosphorus content (n=23)

	Winter wheat			Spring wheat		
	Range	Mean±SD	F value	Range	Mean±SD	F value
PC(mg g^{-1})	0.60-1.85	1.08±0.24	30.48[a]	0.73-1.66	1.07±0.32	28.56[a]
IPC(mg g^{-1})	0.15-2.98	0.71±0.13	26.12[a]	0.26-2.25	0..57±0.22	20.91[a]
γ	-0.5767[b]					

Note: Mark γ represents CC between PC and IPC; Mark [a] and [b] are at significant level (p=0.01). CC, correlation coefficient; IPC, inorganic phosphorus content; PC, phytate content; SD, standard deviations.

Table 3. PC and IPC of wheat meal (dry weight) from the same planting condition and CC between PC and IPC (n=23, only using the mean of triplicate trials used)

		Anhui			Tianjin		
		Range	Mean±SD	F value	Range	Mean±SD	F value
2004	PC (mg g^{-1})	0.41-1.52	0.97±0.23	22.41[a]	0.54-1.71	1.04±0.22	39.24[a]
	IPC (mg g^{-1})	0.11-2.46	0.88±0.16	27.86[a]	0.14-2.33	0.79±0.07	23.73[a]
	γ	-0.9002[b]			-0.9125[b]		
2005	PC (mg g^{-1})	0.47-1.43	1.02±0.27	25.42[a]	0.57-1.46	0.95±0.15	30.22[a]
	IPC (mg g^{-1})	0.13-1.87	0.65±0.12	24.27[a]	0.23-1.92	0.76±0.18	25.83[a]
	γ	-0.8414[b]			-0.8943[b]		

Note: Mark γ represents CC between PC and IPC; M ark [a] and [b] are at significant level (p=0.01). CC, correlation coefficient; IPC, inorganic phosphorus content; PC, phytate content; SD, standard deviations

Table 4. Homology ratio (%) of the CSVIPC between SGSWE and SGSWOE from the same grain seed (the number of seeds tested: 3×12×6=216)

year	CSVIPC from SGSWOE	Location	
		Anhui	Tianjin
2004	1	97.7	96.6
	2	98.3	96.4
	3	94.7	99.0
	4	98.3	98.1
	5	99.6	95.6
2005	1	98.3	99.4
	2	94.7	95.5
	3	96.3	97.7
	4	99.4	97.2
	5	98.8	96.7
Mean±SD		97.4±4.3	97.9±3.5

Notes: CSVIPC, colorimetric scoring value of inorganic phosphorus content; SD, standard deviations; SGSWE, semi-grain seeds with embryo; SGSWOE, semi-grain seeds without embryo.

Table 5. PC and IPC of wheat meal according to CSVIPC of SGSWOE (23 wheat genotypes)

CSVIPC of SGSWOE	Total numbers of individual plant	IPC of wheat meal (mg g^{-1})		PC of wheat meal(mg g^{-1})	
		Range	Mean±SD	Range	Mean±SD
1	112	0.10-0.31	0.15±0.05	1.27-1.92	1.45±0.38
2	134	0.26-0.53	0.38±0.10	0.89-1.66	1.13±0.22
3	66	0.47-1.18	0.84±0.32	0.70-1.16	0.82±0.26
4	61	0.95-1.67	1.25±0.33	0.56-0.84	0.68±0.14
5	38	1.33-2.62	2.05±0.47	0.37-0.77	0.53±0.06

Notes: CSVIPC, colorimetric scoring value of inorganic phosphorus content; IPC, inorganic phosphorus content; PC, phytate content; SD, standard deviations; SGSWOE, semi-grain seeds without embryo.

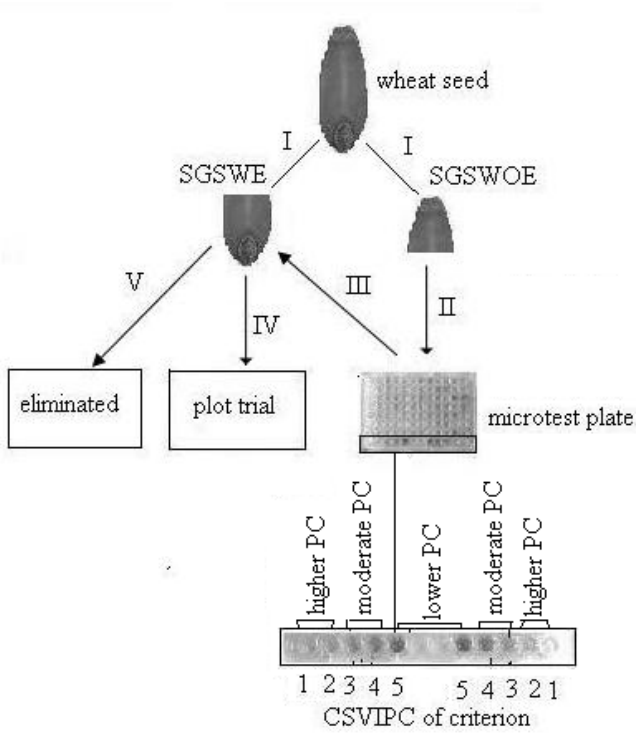

Figure 1. Screening route for low PC using SCVIPC of SGSWOE during early generation breeding

Mark I indicates that one seed is cut into SGSWE and SGSWOE by knife; Mark II indicates that SGSWOE is placed into cell of microtest plate and its CSVIPC is assayed based on criterion phosphorus; Mark III indicates that SGSWE is classified as "moderate" PC sort or "lower or higher" PC sort by CSVIPC of SGSWOE; Mark IV and V indicates that SGSWE of "moderate" PC sort will be planted in field, while SGSWEs of "lower" and "higher" PC sorts will be eliminated.

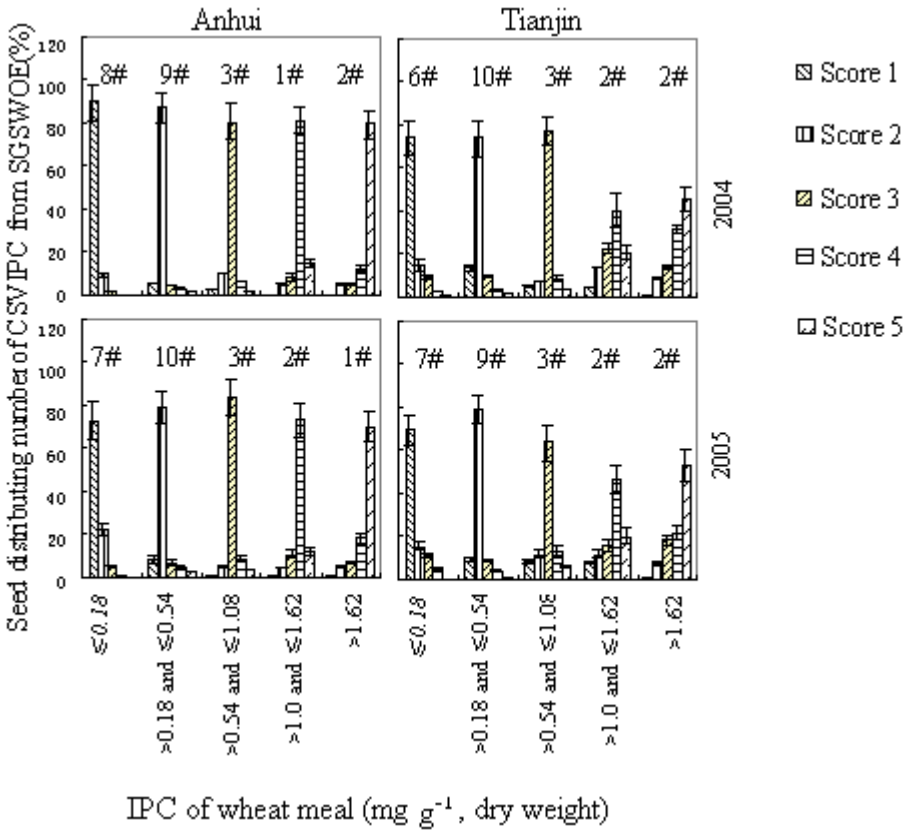

Figure 2. IPC of wheat meal and their CSVIPC from semi-grain seeds (23 wheat genotypes, 4 sites, in triplicate). Mark # represents wheat genotype number by IPC ranges

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