Journal of Cereal Science 50 (2009) 392–397

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

Genetic variation of bioavailable iron and zinc in grain of a maize population

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A R T I C L E I N F O

Article history:
Received 24 February 2008
Received in revised form
12 May 2009
Accepted 19 June 2009

Keywords:
Bioavailability
Biofortification
Maize
Minerals

A B S T R A C T

More than one-third of the world's population is afflicted by iron (Fe) and zinc (Zn) deficiencies, since cereal grain as a staple food of the people contains low levels or low bioavailability of Fe and Zn because of phytate. In maize, 80% of grain phosphorus (P) is in the form of phytate, and P could be an indicator of phytate content. The objectives of this study were (1) to estimate genetic variation of Fe and Zn in a maize population including P/Fe and P/Zn molar ratios as quantitative traits; (2) to determine relations among yield, P, Fe, Zn, P/Fe and P/Zn molar ratios; and (3) to define the implications of those on biofortification (breeding) programmes. There were significant genetic variations and workable heritabilities for Fe, Zn, P/Fe and P/Zn estimated in 294 F4 lines of a maize population, but there were no associations among six traits according to both simple correlations and principal component analysis. Weak correlations between P and Fe and between P and Zn indicated feasibility of breeding non low-phytic acid maize genotypes with more appropriate phytate/Fe and phytate/Zn relations. Bioavailability of iron and zinc varied substantially in a maize population justifying utilisation of these unique parameters in biofortification programmes.

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1. Introduction

More than one-third of the world's population is afflicted by iron (Fe) and zinc (Zn) deficiencies, these ranking fifth and sixth among the ten most important risk causes of illness and disease in low-income countries (WHO, 2002). Among strategies for enhancing iron and zinc levels in cereal grains, plant breeding strategy (biofortification) appears to be the most sustainable and cost-effective approach (e.g. Cakmak, 2008; Graham et al., 1999; Welch and Graham, 2002). However, there are inherently low iron and zinc concentrations in cereal grains whose utilisation in the human digestive tract was additionally limited by substances that influence the bioavailability of these nutrients.

Bioavailability can be defined as the proportion of the total amount of mineral element that is potentially absorbable in a metabolically active form (House, 1999). Unrefined cereals contain high levels of phytate (myo-inositol hexaphosphate), known to be a powerful inhibitor of iron and zinc absorption in both adults (Egli et al., 2004; Mendoza et al., 1998) and children (Davidsson et al., 2004). Lönnerdal (2002) demonstrated that any reduction in dietary phytate can have a positive effect on zinc absorption. The same can be true also for iron (Mendoza et al., 1998). Thus, the negative effect of phytate on iron and zinc absorption follows a dose dependent response, and the phytate/iron or phytate/zinc molar ratio of a diet may be used to predict the proportion of absorbable dietary iron and zinc. Gibson (2006) has stated that particularly high phytate/zinc molar ratios are in diets of children from Malawi, Kenya, Mexico and Guatemala who consume only unfermented maize products.

There are low-phytate strains in maize (Raboy et al., 2000), but they express some less favourable agronomic features, e.g. reduction of seedling vigour (Bänziger and Long, 2000). Phytate also has some beneficial effects such as inhibition of different types of cancers (Somasundar et al., 2005; Vucenik and Shamsuddin, 2003). Therefore, biofortification programmes usually do not include decreasing phytate as a breeding objective beyond naturally occurring variability (Ortiz-Monasterio et al., 2007 for HarvestPlus programme).

The total phosphorus (P) in maize grain could be an indicator of phytate since 80% of the P is in the form of phytate (Raboy, 1997). Hence, identifying the relation between phosphorus and iron as well as between phosphorus and zinc concentrations in standard maize strains could be a way of increasing iron and zinc

Abbreviations: ICP-OES, inductively coupled plasma–optical emission spectrophotometry; PCA, principal component analysis; REML, restricted maximum likelihood.

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doi:10.1016/j.jcs.2009.06.014

bioavailability in maize grain. There are several published studies about genetic variation of iron and zinc in grain of maize (Ortiz-Monasterio et al., 2007 for a review), but a thorough quantitative-genetic analysis for these traits is still lacking. Furthermore, there was no attempt to include bioavailability quantitatively to biofortification studies of iron and zinc. The objectives of this study were (1) to estimate genetic variation of iron and zinc in a maize population including P/Fe and P/Zn molar ratios as quantitatively inherited traits; (2) to determine relations between yield, phosphorus, iron, zinc, P/Fe and P/Zn ratios; and (3) to define the implications of those on biofortification programmes in maize.

2. Materials and methods

2.1. Materials

As part of a biofortification project in maize at the Agricultural institute Osijek, Croatia, two temperate maize elite dent inbred lines having significantly different micronutrient concentrations according to our previous studies (Brkić et al., 2003; Brkić et al., 2004) were crossed in order to commence genetic studies. The two inbred lines (B84 and Os6-2) belong to opposite gene pools of U.S. Corn Belt germplasm: the line B84 is a well known BSSS line, while OS6-2 is related to the line C103 of Lancaster origin. Liu et al. (2003) gave detailed background of B84 and C103 and their relation. Although this breeding material is not directly utilisable for some low-income countries afflicted by mineral deficiency, temperate maize as a model system allows the opportunity for comprehensive genetic research.

Material development took place in the nursery of the Agricultural institute Osijek and in a winter nursery in Chile in 2003 and 2004. Randomly chosen F_2 plants from the cross B84 × Os6-2 were selfed to produce 300 independently derived F_4 plants. The 294 F_4 families along with six checks, which included the parents in double and the subsequent F_1 generation in double, were grown as experiments in 2005 and 2006 in Osijek, Croatia (N 45°30′, E 18°40′). Soil was eutric cambisol, a soil type of moderate fertility showing no mineral deficiency. Fertilizers were given according to usual requirements for high yielding maize, taking into account the soil characteristics and the previous cropping. No additional fertilizers with iron and zinc were applied. The experiments were conducted in two replications as a 30 ×10 alpha (0,1) design (Patterson and Williams, 1976; Piepho et al., 2006) planted at the end of April and harvested in the first ten days of October. Usual crop management practice for maize was applied in both years. The single-row plots were 6 m long with 0.75 m spacing between rows. The whole plots were manually harvested shortly after physiological maturity (black-layer formation) to obtain grain yield calculated in t/ha on a 14% grain water basis. Grain samples were taken from five hand-pollinated (selfed) ears, dried at 32 °C for a week, and then shelled by hand. Moldy kernels or kernels with insect damage were discarded.

2.2. Chemical analysis

All grain samples were ground by a Retsch ZM1 grinding mill until 97% of the sample could pass through a 1 mm screen. Phosphorus, iron and zinc concentrations were determined by inductively coupled plasma – optical emission spectrophotometry (ICP-OES) technique after microwave digestion in the laboratory of the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Science and Arts in Budapest, Hungary. Kernels were digested in 65% nitric acid (HNO_3) + 30% hydrogen-peroxide (H_2O_2) by Milestone MLS 1200 microwave (Zarcinas et al., 1987). The analyses were made by a Jobin-Yvon Ultrace 238 ICP-OES spectrometer. After verification of instrument performance (drift; interferences, background correction), concentrations of phosphorus, iron and zinc in the samples were determined by a linear regression method using blank, standard solutions and internal standards. Mineral concentrations are expressed on a dry matter basis. Bioavailable iron and zinc have been estimated as molar ratios of P/Fe and P/Zn respectively.

2.3. Statistical analysis

Data for P/Fe and P/Zn molar ratios fitted a normal distribution according to the W-test (Shapiro and Wilk, 1965), characterising the ratios as quantitative traits. Estimates of variance components: genotype variance, genotype × environment variance and error variance of 294 F4 lines and their standard errors for the five compositional traits were calculated by restricted maximum likelihood (REML) using MIXED procedure in SAS (SAS Institute, 2004). Effects of environment, replication, block, genotype, genotype × environment interaction and error were considered as random. They were estimated on adjusted entry mean values from the individual trial analyses. REML analysis adjusted the observations for the estimates of the fixed effects obtained by analysis of variance and warranted only positive variance components setting negative estimates equal to zero and re-estimation of the other variance components (Searle et al., 1992).

Heritability on a sample (plot) basis was estimated as $h^2 = s_G^2/s_{GE}^2 + s_e^2 + s_G^2$ where s_G^2 is the estimate of genotypic variance, s_{GE}^2 is the estimate of genotype × environment interaction variance and s_e^2 the estimate of error variance. Heritability on a genotype (entry) mean basis (Hallauer and Miranda Fo, 1988) was estimated as $h^2 = s_G^2/(s_{GE}^2/e + s_e^2/re + s_G^2)$, where r is the number of replications per environment and e is the number of environments.

Response to selection (R) was calculated for iron, P/Fe, zinc and P/Zn in order to demonstrate expected increase of a compositional trait made by a biofortification programme within one generation; i.e. change for a given trait produced by selection. When heritability and genotype variance were calculated, then $R = ihs_G$, where i is intensity of selection; h is square root of heritability on genotype basis and s_G is the square root of genotype variance (standard deviation). Description and discussion about properties of response to selection have been given by Falconer and Mackay (1996) in detail.

To obtain more reliable information about relationships among the six traits, data were evaluated by principal component analysis (PCA), as a multivariate data analysis technique. The first principal component (PC1) accounts for the maximum of the total variance, and the second (PC2) is uncorrelated with the first one and accounts for the maximum of the residual variance, and so on, until the total variance is accounted for. For a practical problem, it is to retain only a few components, accounting for a large percentage of the total variance. PCA will show which traits are close to each other, i.e., which carry comparable information, and which ones are unique. The algorithm of PCA can be found in Otto (1999). PCA was made by the STATISTICA programme package (StatSoft, 2000).

3. Results

There were significant differences between the means at two environments for all traits except iron (Table 1). Generally, there were higher yields, phosphorus and iron concentrations, as well as P/Fe and P/Zn molar ratios in 2006. Only zinc concentrations were significantly lower for 4.86 mg/kg in 2006 compared to 2005. Means of individual F_4 lines varied significantly for all six traits, differing in 6.24 t/ha for yield, 1011 mg/kg for phosphorus,

Table 1
Means with ± standard error, minimum and maximum values for yield, phosphorus, iron, zinc and bioavailable iron and zinc (P/Fe and P/Zn) in grain of 294 F_4 maize lines and two parent lines at two environments.

	Yield (t/ha)	Phosphorus (mg/kg)	Iron (mg/kg)	Zinc (mg/kg)	P/Fe (molar ratio)	P/Zn (molar ratio)
2005						
Mean	4.83±0.07	3190±12	24.13±0.17	24.10±0.16	241.3±1.8	282.6±1.9
2006						
Mean	5.79±0.08	3373±13	24.18±0.23	19.27±0.13	257.1±2.3	374.0±2.6
Combined						
Mean	5.32±0.06	3285±10	24.20±0.16	21.71±0.12	249.4±1.6	328.2±1.8
Minimum	2.20	2838	16.62	16.35	175.8	256.8
Maximum	8.44	3849	33.57	28.56	332.5	425.8
Parent1	3.80±0.71	3199±141	20.77±2.29	20.23±2.78	294.7±21.1	323.5±50.7
Parent2	4.63±0.72	3243±141	26.11±2.30	24.11±2.78	223.8±21.1	275.0±50.7

16.95 mg/kg for iron, 12.21 mg/kg for zinc, 156.7 points for P/Fe and 169.0 for P/Zn. Total mean of the F_4 population for yield and phosphorus transgressed the means of both parents, while for iron, zinc, P/Fe and P/Zn, it was within the range of the two parental lines. Means of parent lines differed significantly for yield, iron and P/Fe, but not for phosphorus, zinc and P/Zn ratio.

Estimates of genotypic variance among F_4 lines and error variance were highly significant ($P < 0.01$) for all compositional traits, whereas estimates of genotype × environment interaction variance were highly significant for phosphorus, iron and P/Fe, significant ($P < 0.05$) for P/Zn and not significant for zinc (Table 2). Genotype × environment interaction variances were smaller than respective genotypic variances for all traits, but iron. Heritability estimates on a sample basis was notably lower than heritability estimates on genotype mean basis, which ranged from 0.46 to 0.63.

Combining genotypic standard deviation with square root of heritability on a genotype mean basis and given selection intensity, expected response to selection was calculated for four traits of biofortification interest (Fig. 1). When response to selection is expressed as a percentage of respective total mean (Table 1), the values of traits are directly comparable. Thus, due to larger estimates of both genotypic variance and heritability, expected response to selection was higher for P/Fe than for iron alone, yielding 2.2% more response if selection intensity was strong (5% selection fraction). However, our results showed that greater response for P/Zn compared to zinc alone is not to be expected.

Correlation between phosphorus and iron for the means of F_4 lines over two environments was very weak ($r = 0.17$), while between phosphorus and zinc, it was weak ($r = 0.32$). Correlation coefficients of $r = 0.11$ between iron and zinc and $r = 0.14$ between P/Fe and P/Zn showed very weak associations between compositional traits. Grain yield was not associated with any of the compositional traits: correlation coefficients were from −0.02 to 0.02. No substantial associations among traits can be seen also according to PCA (Fig. 2). PCA yielded three principal components (PC1, PC2, PC3), explaining 82.9% of total variance in data, showing that all five compositional traits are equally distanced from each other. Grain yield was close to phosphorus concentration according to two first principal components, but the third loading (PC3) reveal that these two traits are actually distinct, carrying no comparable information.

4. Discussion

Quantities of minerals in grain are influenced by numerous complex factors including genotype, soil properties, environmental conditions and nutrient interactions (House, 1999). Our study demonstrated that mineral concentrations of grain were affected by environmental conditions and year to some extent. However, if there were no adverse soil chemical properties (Cakmak, 2008 for review) or extreme weather conditions like in our study, differences among genotypes in absorbing sufficient amounts of iron and zinc from the soil and in accumulating in the grain to achieve nutritional benefit could be clearly expressed. The range in iron concentrations in our study was somewhat wider than those obtained in evaluations that included a large number of landraces, varieties and breeding germplasm, but zinc had a smaller range than that in previous studies (Bänziger and Long, 2000; Ortiz-Monasterio et al., 2007). Generally smaller genotype × environment interaction variances for zinc, P/Fe and P/Zn in our study than respective genotypic variance, demonstrate that environmental conditions have a smaller influence, especially on zinc status in maize grain. Relatively small, though partly significant genotype × environment interactions for iron and zinc grain concentrations were also detected in rice and wheat as reviewed by Welch and Graham (2002). However, Oikeh et al. (2004) found highly significant genotype × environment interactions for iron and zinc concentrations in grain of 20 tropical maize genotypes, and Oikeh et al. (2003a) reported significant genotype × environment interactions for kernel-iron in 49 tropical maize varieties grown at six environments in Africa. There were no heritability estimates presented in those studies.

In our study, there is a constant difference between the two types of heritability estimates, indicating that genotypes should be

Table 2
Restricted maximum likelihood (REML) estimates of variance components and heritability on a sample basis and on a genotype mean basis with corresponding standard errors (±SE) for phosphorus, iron, zinc and bioavailable iron and zinc (P/Fe and P/Zn molar ratios) in 294 F_4 lines of a maize population.

Estimate	Phosphorus	Iron	Zinc	P/Fe	P/Zn
Genotype	20014.00±2814**	3.06±0.65**	2.42±0.35**	402.47±70.15**	484.37±84.02**
Genotype×environment	6923.61±2201**	3.31±0.67**	0.00	350.87±63.01**	150.54±83.39*
Error	32293.00±1946**	8.07±0.49**	6.59±0.32**	705.84±42.70**	1400.59±83.84**
Heritability (sample)	0.34±0.04	0.21±0.04	0.27±0.03	0.28±0.04	0.24±0.04
Heritability (genotype)	0.63±0.04	0.46±0.07	0.59±0.04	0.53±0.06	0.53±0.06

*, ** Variance component significant at 0.05 and 0.01 probability level, respectively, according to Wald test.

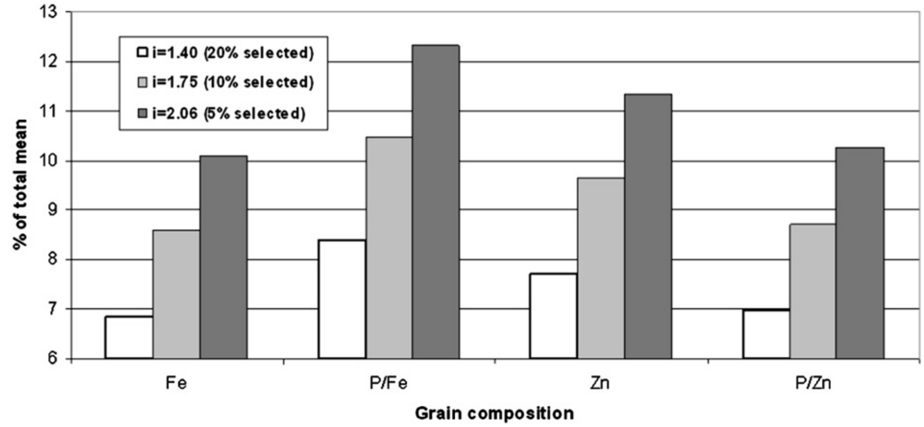

Fig. 1. Response to selection (R) expressed as percentage of total mean in relation to change of intensity of selection (i) for four grain compositional traits in maize.

evaluated over a number of environments to obtain accurate estimates for traits investigated, as implied by Oikeh et al. (2003a) and Oikeh et al. (2004). Generally, we can conclude, according to the large sample size of 294 investigated genotypes, that heritabilities on a genotype basis were not low as suggested by Ortiz-Monasterio et al. (2007), so our results support the conclusions of Graham et al. (1999) that there exists significant and sufficient genetic variation and workable heritabilities to improve iron and zinc in maize. The estimates are even larger for iron if bioavailability of this element, defined as P/Fe molar ratio, is considered.

Mean values of P/Fe and P/Zn molar ratios might not be interpreted in absolute terms, rather as indications of differences in nutrient bioavailability among genotypes. For a biofortification programme it can be important, since our study showed that bioavailability of iron and zinc (when phosphorus and/or phytate are to be considered) varied substantially in the maize population. Statistical normality of P/Fe and P/Zn ratios and their significant genetic variations justify utilising these parameters in biofortification programmes. We supposed that there was no

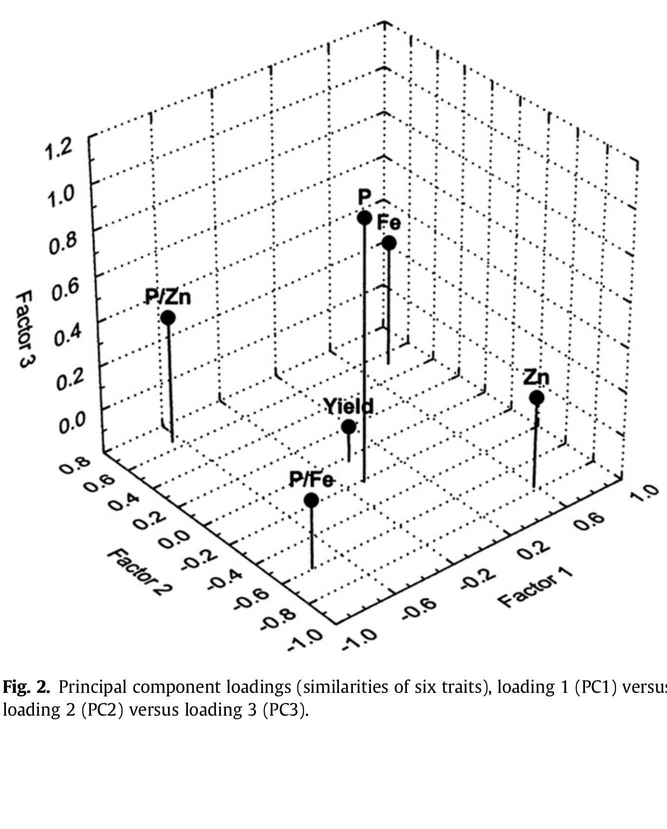

Fig. 2. Principal component loadings (similarities of six traits), loading 1 (PC1) versus loading 2 (PC2) versus loading 3 (PC3).

genotypic variation of the ratio phytate/total P among maize genotypes assuming the ratio to be constant at 80%. Calculation of P/Fe and P/Zn molar ratios in cereal grain is just a preliminary screening method for predicting Fe and Zn bioavailability. It is cost-effective since only one compositional analysis (ICP) is necessary to determine approximate bioavailability levels. For precise bioavailability measurements in vitro/in vivo models are required (e.g. in vitro digestion/Caco-2 cell model). Additionally, different forms of iron should be considered (Wortley et al., 2005), as well as other inhibitor or promoter substances which interfere with iron and zinc availability (Glahn et al., 2002; White and Broadley, 2009).

Response to selection can demonstrate the power of long-term selection (conventional plant breeding) in altering the expression of complex traits, especially in maize. For example, the Illinois Long-Term Selection Experiment for grain protein and oil concentration clearly shows that newly developed strains spanned the known phenotypic extremes from almost 0% to approximately 30% for protein (normally 8–12%) and from 0% to more than 20% oil (normally 4–6% oil). Realized selection responses of both protein and oil are greater than 20 standard deviations from the original population mean in the positive direction and four standard deviations in the negative direction (Moose et al., 2004). In our case, a considerable increase of iron and zinc concentrations as well as a decrease of P/Fe and P/Zn ratios seems to be achievable after a number of generations of selection. It could probably take fewer generations than improvement for protein and oil concentrations due to lesser number of genes involved in control of iron and zinc accumulation. Brkić et al. (2003) reported about simple genetic control of iron and zinc accumulation, suggesting possible more rapid developments in biofortification. Stein et al. (2007) demonstrated that conventional plant breeding can be very cost-effective to control zinc deficiency in India, while Wardyn and Russell (2004) reported about good prospects to develop maize genotypes with low phosphorus to obtain more favourable P/Fe and P/Zn ratios in grain.

Big phenotypic change in concentrations of iron and zinc by themselves is not to be expected through faster approaches such as mutagenesis or genetic modifications. However, transgenic approaches to increase bioavailability of iron and zinc in food focused on reducing the concentrations of other inhibitors and increasing the concentrations of promoter substances and iron-binding proteins, can be successful (Drakakaki et al., 2005; White and Broadley, 2009). Eventually, the combination of conventional long-term selection for higher mineral concentrations together with transgenic approaches of manipulating concentrations of inhibitor and/or promoter substances could solve mineral deficiency in the long run.

Perhaps a greater problem for biofortification programmes appears to be lack of associations among the traits. Some previous studies in maize demonstrated that grain yield was generally negatively correlated with grain iron and zinc concentrations (Bänziger and Long, 2000; Brkić et al., 2003) as a result of increased carbohydrate content in high-yielding genotypes, which dilutes a given concentration of iron and zinc. However, Bänziger and Long (2000) reported on correlation coefficients between yield and iron, ranging from -0.60 to 0.16 across 12 environments, suggesting that the relation could be affected by specific environmental conditions. Furthermore, in the study by Brkić et al. (2004), the estimated correlation coefficient between yield and iron was $r = -0.09$ in a set of 121 maize genotypes. It indicates that the relation is also dependent on particular genetic material under investigation. Murphy et al. (2008) have recently shown that iron and zinc were not negatively correlated with yield in a set of 63 wheat cultivars, suggesting feasibility of simultaneous increase of iron and zinc along with yield in the presence of positive selection pressure.

There were no associations among five compositional traits according to both simple correlations and PCA analysis either, suggesting a need to measure and evaluate all traits: phosphorus, iron and zinc concentrations but also iron and zinc bioavailability ratios. PCA showed that there were no two traits carrying comparable information, indicating uniqueness of all five compositional traits. Welch and Graham (2002) reviewed, however, tight correlation between iron and zinc concentrations in wheat. Shi et al. (2008) reported on a significant and positive relation between phosphorus and zinc concentrations in a wheat population, while a recent report of Morgounov et al. (2007) demonstrated a negative correlation between these traits, suggesting that the relation could be affected by specific environmental conditions. In maize, Menkir (2008) found non-significant correlations of iron and zinc with phosphorus, but positive and significant correlations between iron and zinc in a vast set of tropical-adapted inbred lines, while Oikeh et al. (2003b) detected a significant negative relationship between kernel-P concentration and bioavailable iron.

The population investigated in our study cannot be considered as an ideal breeding source for biofortification programmes since we found that iron:phytic acid ratios theoretically would be greater than 1:10, indicating maximal inhibition of iron uptake by phytic acid (Glahn et al., 2002). Other maize germplasm should be evaluated in order to find more favourable iron:phytic acid ratios. Further work should be done to elucidate underlying physiological mechanisms of the relation among phosphorus, iron and zinc, particularly in cases when the relations are not obscured by deficiency of any element. Nonetheless, weak correlations between phosphorus and iron and between phosphorus and zinc indicated feasibility of breeding non low-phytic acid maize genotypes with more appropriate phytate/iron and phytate/zinc relations.

Acknowledgments

The authors thank I. Kadar and J. Koncz at the Laboratory of the Research Institute for Soil Science and Agricultural Chemistry of Hungarian Academy of Science and Arts in Budapest, Hungary for ICP-OES analyses.

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